Development of a Live-Cell Based Reactive Oxygen Species (ROS) Assay for use in High-Content Screening of Drug Candidates Using The BioTek Synergy Mx Microplate Reader

Thomas A. Lyons[1], Xavier F. Amouretti[2], Paul G. Held[2] P, and John J. Naleway[1]

[1]Marker Gene Technologies, Inc., University of Oregon Riverfront Research Park, Eugene, OR, USA • [2]BioTek Instruments, Inc., Winooski, VT, USA; *presenting author.

Introduction

Abstract: In healthy aerobic cells, reactive oxygen species (ROS) generation typically occurs at a controlled rate. But under stress conditions or by application of various drug candidates, ROS production can be greatly increased. The resulting changes in many cell components including DNA, proteins and lipids can influence cell viability, metabolism and growth. We have developed a live cell, high-throughput, microplate-based ROS assay that utilizes the cell permeable substrate, 2',7'-dichlorofluorescin diacetate, a reliable fluorogenic marker for ROS detection. Upon enzyme activity, the highly fluorescent dye, 2',7'-dichlorofluorescein is produced, with EX: 495 nm and EM: 530 nm. By using such a diacetate derivative, the assay can be efficiently adapted for intracellular applications, since the acetates help with effective loading into live cells. The acetates are then cleaved by endogenous intracellular esterases inside the cell, releasing the intact substrate. Application of this assay to the kinetic and end-point measurement of ROS activity in both adherent and non-adherent cell lines, upon treatment with the common chemotherapeutic drugs doxorubicin and idarubicin is presented. Dose response data is also compared with negative and positive controls (no drug or t-butyl hydroperoxide (TBHP) treatment). Methods for extrapolation of this assay for high-content screening applications are presented.

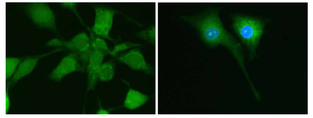

Figure 1 – NIH3T3 murine embryonic fibroblast adherent cells stained with 2',7' dichlorofluorescin diacetate (**M0807**) (left panel) and counterstained with the nuclear stain DAPI (right panel). Cells were treated with TBHP for one hour to induce ROS production and were subsequently stained with **M0807** for 30 minutes. Green fluorescence indicates ROS activity. Captured using a Zeiss AxioObserver A1 microscope equipped with a Chroma Technology FITC filterset. 40X magnification, exposure 40 ms.

Methods

Assays were run in the BioTek monochromator-based Synergy™ Mx Multi-mode Microplate Reader. Adherent cells were plated in 96-well tissue culture plates at a density of 25,000 cells/well and allowed to grow for 24 hours before the assays. Non-adherent cells were grown in culture flasks to a density of 6 x 10^5 cells/mL and plated out at 100,000 cells/well in 96-well plates for use in the assays. Cells were loaded with **M0807** (2',7' dichlorofluorescin diacetate) at 20 µM final concentration for 30 minutes before treating with inducing agents for 4 hours. Non-adherent cells were centrifuged and resuspended in HBSS buffer containing 10% fetal calf serum after dye loading and then treated with ROS inducing agents. Adherent cells were loaded with **M0807** for 30 min., washed and put back under HBSS with 10% serum before treatment with ROS inducing drugs doxorubicin, idarubicin, or TBHP (as a positive control) at concentrations between 25 -100 µM for up to 4 hours. Both end point and area-scan readings were taken using the Synergy Mx Plate Reader after 4 hours, and kinetic readings were taken at 30 minute intervals. Plates were read at 485 nm with emission at 528 nm using a 20 nm bandwidth.

BioTek Instrumentation

The Synergy™ Mx Multi-Mode Microplate Reader's quadruple monochromator system employs Ultra Fine-Tuned™ technology to select wavelengths with a repeatability of ±0.2 nm.

Turnover of Non-Fluorescent 2,7 Dichlorofluorescin Diacetate

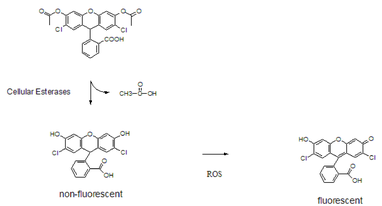

Figure 2 – Conversion of **M0807** 2',7' dichlorofluorescin diacetate to 2',7' dichlorofluorescein by cellular esterases and subsequent oxidation by reactive oxygen species to produce quantifiable fluorescence.

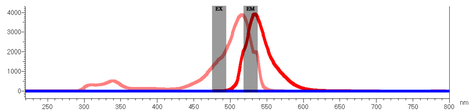

Figure 3 – Excitation and emission spectra of 2',7'-dichlorofluorescein product and 2',7'-dichlorofluorescin diacetate (**M0807**, substrate). The pink line is the absorption spectra for 2',7' dichlorofluorescein, and the red line is the emission spectrum. The blue line gives the emission spectra for **M0807** at the same excitation (lines overlapping on base line). Dashed lines show the assay excitation and emission wavelength settings (with 20 nm slit widths) used with the Synergy Mx plate reader.

Results

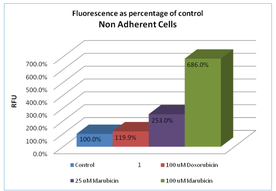

Figure 4 – Staining of non-adherent HL60 human promyelocytic leukemia cells with **M0807** at 20 µM for 30 min., followed by four hour incubation with 25-100 µM idarubicin or 100 µM doxorubicin for ROS induction and reading at EX485/EM528 nm.

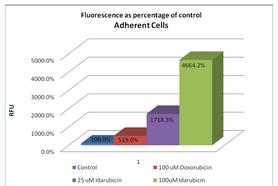

Figure 5 – Staining of NIH3T3 murine embryonic fibroblast adherent cells with **M0807** at 20 µM for 30 min., followed by four hour incubation with 25-100 µM idarubicin or 100 µM doxorubicin for ROS induction and reading at EX485/EM528 nm.

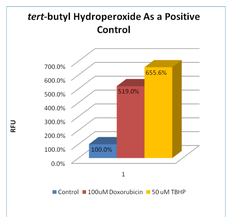

Figure 6 – Comparison of staining of NIH3T3 murine embryonic fibroblast adherent cells with **M0807** at 20 µM for 30 min., followed by ROS Induction using either doxorubicin (100 µM) or tert-Butyl Hydroperoxide (TBHP, 25 µM). Activity is shown as a percentage of uninduced control.

Structures

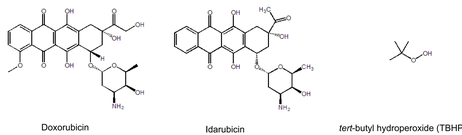

Figure 7 – Compounds used for ROS induction; idarubicin and doxorubicin are both anthracycline chemotherapeutic drugs; and tert-butyl hydroperoxide (TBHP) is a common inducer of ROS production used as a positive control.

Kinetic Data

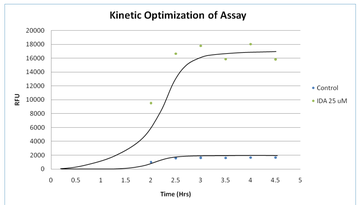

Figure 8 – Kinetic assay for NIH3T3 murine embryonic fibroblast adherent cells with **M0807** at 20 µM for 30 min. Readings were carried out starting at 2 hours after addition of ROS inducing agents every 30 minutes.

Discussion and Conclusions

- The use of 2',7' dichlorofluorescin diacetate as a fluorogenic marker for intracellular detection of ROS activity in a microplate assay format has been successfully validated using BioTek's Synergy Mx Microplate Reader.
- For a 4 hour incubation, fluorescent signal was from 6 and 46 times control for cells treated with the anthracycline drugs idarubicin and doxorubicin respectively as test ROS inducing agents and 6.5 times control with TBHP.
- When using non-adherent (HL60) cells, the fluorescent signal was lower, likely due to loss of dye through multiple differential centrifugation and wash steps required after loading the non-adherent cells with the substrate.
- In trials where cells were first treated with drugs and then stained, the signal was found to be only 2-3X controls.
- Kinetic data indicate that the assay may be optimized for even shorter time courses.
- The assay is amenable to high throughput format assays for monitoring increases in reactive oxygen species production upon application of potential drug candidates.
- The authors would like to acknowledge MitoSciences, Inc., for their assistance in assay development and BioTek Instruments, Inc., for help with instrumentation setup and collaborative efforts.

References

1. Ciapetti G, Granchi D, Verri E, Savarino L, Cenni E, Savoli F, Pizzoferrato A, (1998) Fluorescent microplate assay for respiratory burst of PMNs challenged in vitro with orthopedic metals. *J. Biomedical Materials Res.* 41(3):455-460.
2. Eder A, Arriaga E, (2006) Capillary Electrophoresis Monitors Enhancement in Subcellular Reactive Oxygen Species Production upon Treatment with Doxorubicin. *Chem. Res. in Toxicology* 19(9): 1151-1159.
3. Karbowski M, Kurono C, Wozniak M, Ostrowski M, Teranishi M, Nishizawa Y, Usukura J, Soji T, Wakabayashi T, (1998) Free radical-induced megamitochondria formation and apoptosis. *Free Radical Biology & Medicine* 26(3/4): 396-409.
4. Teranishi M, Karbowski M, Kurono C, Nishizawa Y, Usukura J, Soji T, Wakabayashi T, (1999) Effects of Coenzyme Q10 on Changes in the Membrane Potential and Rate of Generation of Reactive Oxygen Species in Hydrazine-and Chloramphenicol-Treated Rat Liver Mitochondria. *Archives of Biochem. and Biophy.* 366(1): 157-167.
5. Wang G, Klein J B, Kang Y J, (2001) Metallothionein inhibits doxorubicin-induced mitochondrial cytochrome c release and caspase-3 activation in cardiomyocytes. *J. of Pharm. and Exp. Ther.* 298(2): 461-468.
6. Cardaci S, Filomeni G, Rotilio G, Ciriolo M R, (2008) Reactive oxygen species mediate p53 activation and apoptosis induced by sodium nitroprusside in SH-SY5Y cells. *Molecular Pharmacology* 74(5): 1234-1245.