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Characterization of Cell Signaling Events in Human Cardiomyocytes

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Introduction

Cardiotoxicity is a significant concern for anticancer therapeutics. "On-target" toxicity may occur if a drug target that regulates cancer growth also plays an important role in regulating normal cardiac function. We are characterizing iCell® human cardiomyocytes (derived from induced pluripotent stem cells) for their utility in elucidating mechanisms of cardiac toxicity associated with anticancer agents. Our primary goal is to use *in vitro* systems to inform potential risk for cardiac toxicity in early discovery and preclinical development. Several cell signaling pathways and contractile proteins (e.g. ErbB-mediated pathways, troponins) are known to mediate critical cardiac functions *in vivo*. We measured expression and activity of two major cellular pathways involved in cardiac myocyte signaling and myocyte response to injury. Our data support the use of iCell® human cardiomyocytes to explore toxicological mechanisms, but more detailed characterization of cellular function and pathway activity is required. This *in vitro* model presents an opportunity to explore certain mechanisms of cardiotoxicity. Endpoints qualified in this system may be added to a battery of preclinical tests used to define risk of cardiac liabilities.

Objectives

- Determine expression of ErbB and endothelin-1 receptors, Erk1/2, AKT, and cardiac troponins (cTn-I and cTn-T)
- Confirm activity of ErbB and endothelin receptors by measuring ligand-induced phosphorylation of Erk1/2 and AKT cell signaling pathways
- Examine the effect of tyrosine kinase inhibitors and anthracyclines on iPS cell-derived cardiomyocytes cell death, mitochondrial membrane potential and troponin release

Cell System and Materials

Cell Culture

iPS cell-derived human cardiomyocytes (Cellular Dynamics International) were thawed, plated and maintained per the manufacturer's instructions; viable cells were plated at the following per well densities: 6 x 10^5 cells, 2.4 x 10^5 cells, 1.2 x 10^5 cells or 2 x 10^4 cells in 0.1% gelatin-coated 6-, 12-, 24- or 96-well plates, respectively, for 7 – 8 days in culture when cells were beating synchronously.

Reagents

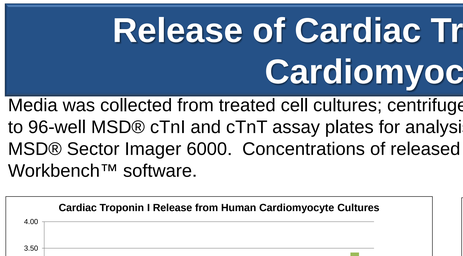
- Primary antibodies were purchased from Cell Signaling or abcam. Infrared dye conjugated secondary antibodies (LI-COR) against the appropriate species were used for detection in western blots.
- JC-10 assay for mitochondrial membrane potential (AAT Bioquest) – **3 µM** for 30 minutes
- Sytox Green Nucleic Acid stain for cell death (Invitrogen Life Technologies) – 0.1 µM co-incubated with JC-10 dye and Hoechst 33258 nuclear dye (Enzo Life Sciences) at **4 µg/ml**
- Human cardiac troponin I and T assay kits were purchased from Meso Scale Discovery®

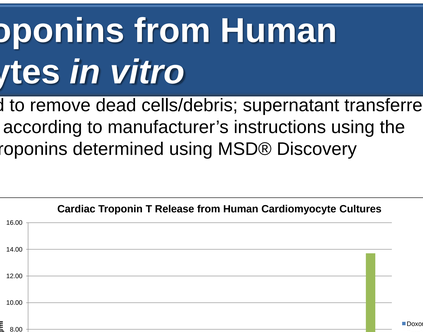
Cell Treatments

- CCCP (Tocris Bioscience; used to abolish mitochondrial membrane potential) – **1 µM** for 30 minutes (added with JC-10 indicator dye)
- Endothelin (Sigma; used to bind endothelin receptors and activate signaling pathways) – **100, 250, or 500 nM** for 10, 20, or 30 minutes
- Neuregulin (R&D Systems; used to bind ErbB receptors and activate Erk and Akt signaling pathways) – **20 or 50 ng/ml** for 10, 20, or 30 minutes
- Doxorubicin (Enzo Life Sciences), Staurosporine (Sigma), Sorafenib (LC Laboratories), Lapatinib (NCI Repository) Sunitinib (NCI Repository) – 24 hours at **0.05, 0.1, 0.5. 1.0, 2.0, 5.0, or 10 µM**

ErbB and Endothelin, AKT, Erk1/2 and Troponin Protein Expression in Human Cardiomyocytes

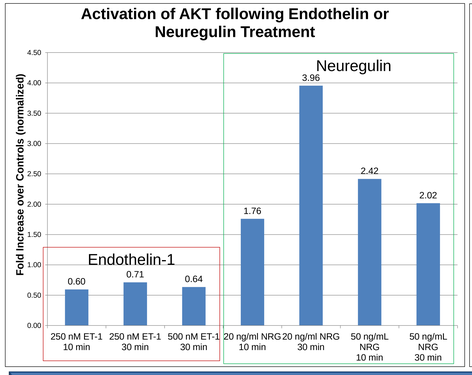
Cells were lysed directly in plates using either RIPA buffer supplemented with 0.5% SDS, phosphatase and protease inhibitors, or M-PER lysis buffer (Pierce) supplemented with phosphatase and protease inhibitors. Following electrophoresis, protein was transferred to a nitrocellulose membrane. Membranes were imaged using the Odyssey Infrared Imaging System (LI-COR). Relative intensities of protein bands were quantified using the Image Studio 2 software program (LI-COR). Signal intensities were normalized to GAPDH.

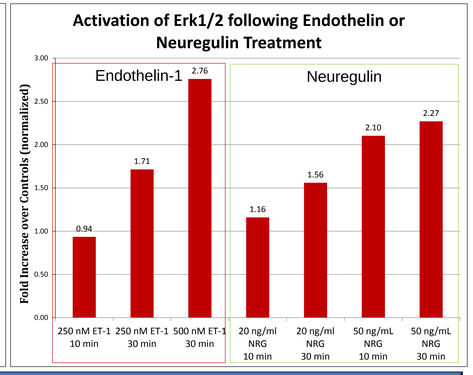




Induction of AKT and Erk1/2 Phosphorylation in Human Cardiomyocytes

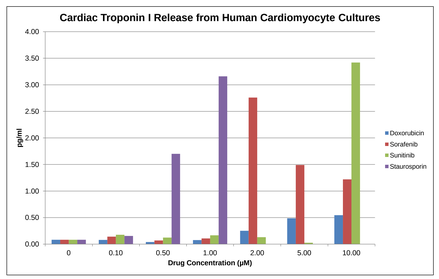
Cells were treated as outlined in the "Cell Treatment Section" and cell lysates prepared as described above. Signal intensities were normalized to total AKT or Erk1/2 and GAPDH.

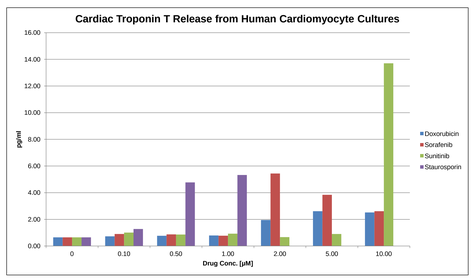




Release of Cardiac Troponins from Human Cardiomyocytes *in vitro*

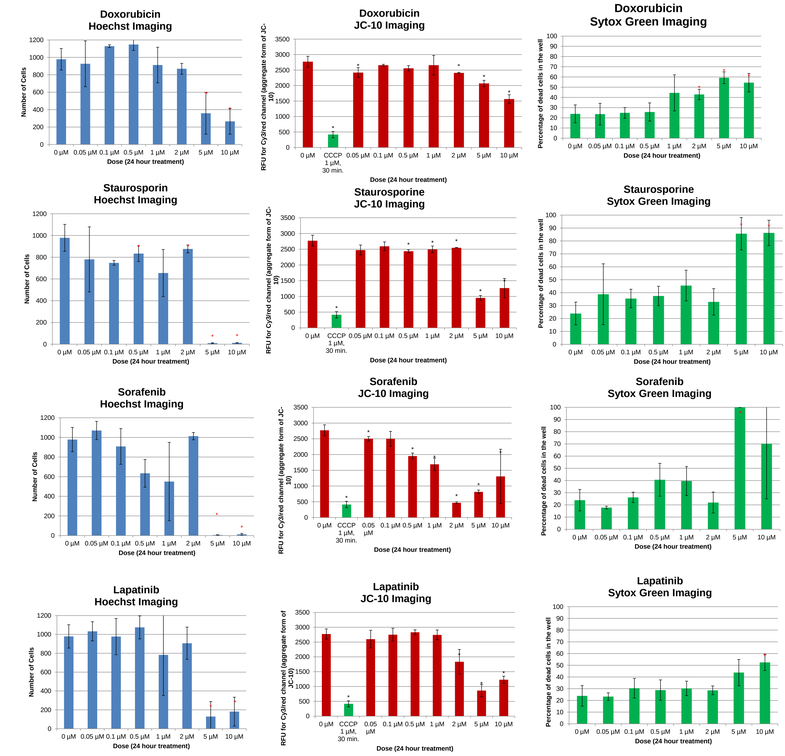
Media was collected from treated cell cultures; centrifuged to remove dead cells/debris; supernatant transferred to 96-well MSD® cTnI and cTnT assay plates for analysis according to manufacturer's instructions using the MSD® Sector Imager 6000. Concentrations of released troponins determined using MSD® Discovery Workbench™ software.





Multiplexed Cytotoxicity Assay

Cells were treated in 96-well plates for 24 hours, then stained for mitochondrial membrane potential (JC-10), cell death (Sytox Green) and total cell number (Hoechst) by co-incubating dyes for 30 minutes and imaged using GE IN Cell Analyzer 2000. Images were analyzed using the Multi-Target Analysis Module from IN Cell Investigator v2.0 (GE Healthcare).



Conclusions

- Treatment with ErbB or endothelin receptor ligands activates Erk1/2 and AKT signaling pathways in this cell model.
- Selected anticancer agents induce cell death that is associated with disruption of mitochondrial membrane potential and cardiac troponin release.
- This model may represent an opportunity to explore clinical mechanisms of cardiotoxicity using iCell® human cardiomyocytes *in vitro* and to investigate risk of cardiotoxicity associated with anticancer therapeutic agents.

Acknowledgments

This project is funded by NCI Contract Number HHSN261200800001E