A Multi-Assay Approach to the Study of Cellular Toxicity

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Goals

- Use more than one technique to gain a measure of cell toxicity
- Define methodologies that are applicable across multiple cell types and experimental conditions
- Keep the method as simple and inexpensive as possible
- Ultimately, define a toxicity score for each of our compounds prior to screening
Rationale

- As a Core Laboratory, we have the equipment, knowledge and resources to run a multifactorial study that is somewhat unique.

- Compound screens can have many end-point measures, some use cell death.

- Positive results are only useful if specific to the biology, not frank toxicity.

- By knowing the toxicological potential for each compound in our collection, we can avoid false positives (negatives).

- Known toxic compounds can be used as positive controls for the assay but need to be excluded from the ‘hit’ list.
The Tools

- xCelligence Real-Time Cell Monitoring (RT-CES)

1- cell growth with the xCelligence instrument can either confirm growth as expected or show growth patterns that were completely unexpected

2- alterations in cell growth patterns are changes in impedance that can foretell future cell physiology

3- can use the cells grown in the E-plates for most assays but not suitable for microscopy and high content analysis
The Tools

- WST-1 - cell viability end point analysis

  1- As with any end-point assay, only gives a snapshot in time

  2- There are large compound screens that depend upon this or similar reagents (e.g., MTT)

  3- WST-1 uses absorbance as a readout

  4- Assay is additive and easy to perform
The Tools

- Real-Time qPCR
- 1- The ability to run the reverse transcriptase reaction from cell lysates without purifying RNA is essential when dealing with large numbers of samples (e.g., multi-well plates)
- 2- Lysates provide unbiased content compared to RNA or DNA isolation
- 3- Challenge is finding the correct transcript cohort for the assay conditions
- 4- The method is rapid, relatively inexpensive with high quality data.
The Tools

- High Content Screening

  1- Gives a direct look at the cells at any given time during an experiment

  2- Rather than gathering a single data point/well, every cell that is imaged in the well becomes an N=1

  3- Multiple mechanisms for readouts: eGFP or other genes; cell dyes, Ab-based detection, transcription sites, etc.

  4- Data analysis is multifactorial and complex with many features of each cell recorded: nuclear size and shape, cell size and shape, fluorescence localization, intensity, etc.
Initial Study
PPARγ Agonists
PPARγ Compounds

- Used 4-PPARγ agonists with known toxicity profiles in HepG2 Cells as a model system - thiazolidinediones

- Ciglitazone - early anti-diabetic drug, liver tox, toxic in cells @ 30 - 100 µM

- Troglitazone - anti-diabetic, drug-induced hepatitis & liver tox, toxic in cells @ 30 - 100 µM

- Rosiglitazone - anti-diabetic, increased risk of myocardial ischemia, nontoxic in cells @ 30 - 100 µM, Avandia; w/ metformin - Avandemet; w/glimepiride - Avandaryl

- Pioglitazone - hypoglycemic action, nontoxic in cells @ 30 - 100 µM, Actos (US), Glustin (Europe)
A 2006 study by Guo, et al. identified 34 genes associated with cell death that were changed between rosiglitazone/pioglitazone versus ciglitazone/troglitazone in primary rat hepatocytes.

The microarray results were not confirmed by real-time qPCR.

We used a subset of these transcripts to test in HepG2 cells.

Concentration Effects of PPARγ Agonists using the MTT Assay - 16 H

Guo, et al.

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**A: Cytotoxicity to rat primary hepatocytes**

- Troglitazone
- Ciglitazone
- Rosiglitazone
- Pioglitazone

- JTT-501

Viability (% of control) vs. Concentration (µM)

**B: Cytotoxicity to human HepG2 cell**

- Troglitazone
- Ciglitazone
- Rosiglitazone
- Pioglitazone

- JTT-501

Viability (% of control) vs. Concentration (µM)
RT-CES

- Instruments

&

- E-plates

Figures courtesy of Roche
Roche xCelligence Instrument - Principles

- Impedance increase: Function of cell attachment
- Impedance increase: Function of cell number
- Impedance increase: Function of cell spreading

Figures courtesy of Roche
Experiment - 1

- 1 96-well E-plate
- HepG2 human liver cells plated at 25,000 cells/well
- Grow cells overnight, treat with 4 drugs over a large range of concentrations 100 nM; 300 nM; 1µM; 3 µM, 10 µM; 30 µM; 100 µM
- Measure the effect of compounds on cells using the ACEA RT-CES reader for 45 H
PPARγ Effects Measured by RT-CES

Troglitazone Concentrations with HepG2 Cells

Ciglitazone Concentrations with HepG2 Cells

Pioglitazone Concentrations with HepG2 Cells

Rosiglitazone Concentrations with HepG2 Cells
Experiment 2 - Complete Analysis

- E-Plate with 4-drugs at 3 concentrations plus DMSO & cells alone controls - all in 2-triplicate sets
- 1 optical plate with a single set of drugs at the same concentrations plus controls
- Seed plates at 25,000 HepG2 cells/well
- After 9 H growth, add drugs and monitor for 18H
- Perform WST-1 cell viability assay on wells in one half of the E-plate cells, make cell lysates for real-time qPCR with the other half of the plate
- High content cell analysis using automated scanning microscope, IC-100 using an optical plate
RT-CES Results
Toxic Compounds

Effect of Ciglitazone on HepG2 Cells

Effect of Troglitazone on HepG2 Cells

- Drug Added

Normalized Cell Index

Time (H)
RT-CES Results
Non-toxic Compounds

Effect of Rosiglitazone on HepG2 Cells

Effect of Pioglitazone on HepG2 Cells

Normalized Cell Index

Time (H)

Drug Added

Normalized Cell Index

Time (H)
WST-1 Results
Cell Viability Assay

WST-1 Assay Data (18 H)
Real-Time qPCR - 18 H Post Drug

- 50 µl “Lysate Reagent” + Protector RNase inhibitor added per well, mix
- Cell lysates harvested into PCR plate for storage at 4°C
- 50 µl RT reaction, 12.5 µl cell lysate, Transcriptor
- 2 µl cDNA into 20 µl PCR, Roche PCR MM
- LC480 detection - 96 well block
Real-Time qPCR vs Microarray Results

- Real-time data from means of triplicate wells; 1-RT, 1-PCR
- Gene values normalized to β-Actin value from same well
- UPL assays used for all but β-Actin assay
- ΔΔCt value from DMSO controls
- Real-time data compares favorably to that from the microarray study

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Real-Time PCR Amplification Curves
High Content Cell Analysis

- Optical plate with cells treated with drugs in tandem with E-plate
- Stain cells with Mitotracker red dye, 2 hours, wash, fix and stain with DAPI and Cell Mask Far Red dyes
- Mitotracker dye concentrated in viable mitochondria, stained false green in the following figures
- DAPI stain, blue, specific for the nuclei
- Cell Mask Far Red, stains the cytoplasm at low concentrations
DMSO-Treated HepG2 Cells - 18 H

8 x 8 Montage, 40X high N.A. lens

Zoomed in view
Ciglitizone-Treated HepG2 Cells - 18 H

8 x 8 Montage, 40X high N.A. lens
Summary

- Can detect the real-time effect of a compound on living cells without internal probes using the RT-CES system.
- Our RT-CES results mirror those of the WST-1 end-point assay - others have shown the same.
- The new cell lysis reagent works as a means for producing cDNA without RNA isolation - no losses.
- Our initial real-time qPCR data is consistent with a published report using microarray.
- High content imaging data provides an in depth look at individual cells and their metabolic state.
Expanded Study - 2009
Experimental Setup & Conditions

- **3 Cell lines**
  - 1- HeLa - human cervical cancer line
  - 2- 624- human melanoma cell line
  - 3- CHO- Chinese Hamster Ovary cell line

- **Cell-based Assays**
  - Real-Time Cell Monitoring - xCelligence 6-position reader
  - End point imagining- Caspase 3 & Mitotraker Red

- **Biochemical Assays**
  - WST-1
  - Real-Time qPCR (final assay set not determined)
Current Work Flow

X-Celligence Plate Workflow

Blank Baseline → Cell Baseline → Add Compounds → Wash → Add WST-1 → Record → Wash → Add Lysis Reagent → RT rxn → Real-Time qPCR

Cell Imaging Plate Workflow

Grow cells as above → Add Compounds → Add Fluorescent Reagents → Incubate → Wash & Fix DAPI stain → Image

Both plates are processed in tandem
Compounds

- 5 Compounds used, a variety of targets
- Rosiglitizone and Ciglitazone - PPARγ agonists
- Lovastatin - USDA approved drug - lowers cholesterol
  - Inhibits HMG CoA Reductase
- Nocodazole - anti-neoplastic drug, used to synchronize cells in a laboratory setting
  - Inhibits microtubule polymerization
- Staurosoporine - potential antibiotic - very toxic
  - blocks kinase activity, ATP binding
X-Celligence Cell Monitoring

Red = Cells alone
Green = 1% DMSO
Blue = 1 µM Lovastatin

Result: No effect on cell growth over DMSO
624 Cells show a novel growth pattern
X-Celligence Cell Monitoring

Red = Cells alone  
Green = 1% DMSO  
Blue = 1 µM Nocodazole

Result: Growth inhibition for 624, CHO and HeLa
X-Celligence Cell Monitoring

Red = Cells alone
Green = 1% DMSO
Blue = 30 µM Ciglitazone
Purple = 30 µM Rosiglitazone

Result: Ciglitazone- little effect on CHO; dramatic effect on HeLa and intermediate effect on 624 cells
Rosiglitazone- no effect on CHO or HeLa but long term effect on growth for the 624 cells
X-Celligence Cell Monitoring

Red = Cells alone
Green = 1% DMSO
Blue = 1 µM Staurosporine

Result: Growth inhibition for 624, CHO and HeLa

HeLa Staurosporine
CHO Staurosporine
624 Staurosporine
Real-Time qPCR & WST-1

HeLa Cells, 16H Treatment, 1E4 Cells/Well
8 Transcripts Assayed

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# Real-Time qPCR & WST-1

624 Cells, 16H Treatment, 1E4 Cells/Well
8 Transcripts Assayed

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**Graphs**

- **Nocodazole**
- **Staurosporine**
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Following 6H treatment with 1 µM Staurosporine nearly 100% of the HeLa cells have entered apoptosis.
Mitotracker Red should only stain viable mitochondria but here we see the mitochondria stained even in cells we know have been induced to enter apoptosis - we had the wrong version of the dye.
Following 16H treatment with 30 µM Ciglitazone the cell number is greatly reduced with positive staining with the Caspase 3 reagent.
Following 16H treatment with 30 µM Rosiglitazone, the cell number is not significantly affected, no staining with the Caspase 3 reagent.
Following 16H treatment with 1 µM Lovastatin the cell number is near control cell levels with no significant staining with the Caspase 3 reagent
Following 16H treatment with 1 μM Nocodazole, the cell number is reduced with significant staining with the Caspase 3 reagent.
Summary

* Each technique has to be optimized—still a work in progress

* The best marriage of techniques is the real-time cell analysis with high content microscopy

* Real-Time qPCR can give valuable information on the physiological state of the cell at assay

* Biochemical assays such as WST-1 & MTT have served as viability end-point assays for a long time

* Whether more informative but also more sophisticated and expensive techniques will provide enough added content to supplant the WST-1, et al., remains to be seen
Future Directions

- Finish assay optimization for HCS and real-time qPCR
- Will run 24 384-well Tox plates from Bar Harbor Biotechnologies to determine the best transcript set for multiple cells lines = Tox panel
- Use Pocket Tips for dispensing drugs to lower the amount of DMSO to 0.1 % in the experiment
- Screen a plate of known toxic compounds in conjunction with a natural products compound library
- Strive to determine a quantitative toxic index measure for each compound based on one or a combination of the techniques in use
Acknowledgements

- UTHSC-Houston
  Cliff Stephan
- Mary Sobieski
- Ying Wang
- Nancy Shipley
- Roche
  Jochen Renzing
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