

# Identifying Cardiotoxic Drugs Using a Two Cell Model Combined with Multiparametric Analysis: Applications for *In Vitro* Toxicity Screening

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## ABSTRACT

*In vitro* cell-based models able to predict toxicity specific to the heart would be of considerable value in early drug development. Predicting organ-specific toxicity requires cells from different tissues and a test set of drugs with known organ-specific toxicity. Drugs considered primarily toxic to the heart (anthracyclines) were compared to those considered primarily toxic to the liver (camptothecin and rotenone). Rat cardiomyocytes isolated from 1-2 day old neonates and a rat liver hepatoma (H4IIE) cell line were used as the cell models. Both cell systems were exposed to 1, 5, 10, 20, 50, 100, and 300  $\mu$ M of the test drugs for 1, 3, 6, or 24 hr. Cytotoxicity was assessed using markers for general toxicity, oxidative stress, apoptosis, and hypertrophy. Mitochondria were the most sensitive targets in both cell models. However, the TC50 values were 2- to 3-fold lower in the liver cells when compared to the heart cells. The average TC50 value for adriamycin in cardiomyocytes at 24 hr was 5  $\mu$ M versus 18  $\mu$ M in H4IIE cells. In addition, a reduction in reduced glutathione was observed in cardiomyocytes with a concomitant dose- and time-related increase in ROS as measured by DCFDA. Cardiac hypertrophy markers ANP and BNP were also measured. At 3 hr, adriamycin showed a 2-fold increase in ANP and a 4-fold increase in BNP. Endothelin-1 at 0.1  $\mu$ M showed a 2-fold increase in ANP and a 4-fold increase in BNP. Camptothecin and rotenone were more toxic in the H4IIE cells. A decrease in cell proliferation and reduction in mitochondrial function were observed in H4IIE cells that was much greater than in the cardiomyocytes. The average TC50 value for camptothecin in H4IIE cells at 24 hr was 77  $\mu$ M versus 298  $\mu$ M in cardiomyocytes. The average rotenone TC50 was 0.3  $\mu$ M in H4IIE cells versus 32  $\mu$ M in cardiomyocytes. These data suggest that a two cell model may allow *in vitro* screens to predict cardiac versus non-cardiac toxicity. Thus, the incorporation of a two cell model for identifying organ specific toxicity appears to be a useful tool.

## INTRODUCTION

Rat cardiomyocytes were tested against a rat liver cell (H4IIE) using test compounds considered primarily cardiotoxic versus those that were considered primarily hepatotoxic. The main objective of this study was to develop a cell-based model for distinguishing cardiotoxicity from systemic toxicity. Using a robust set of biochemical markers and gene expression tools, differences in cell sensitivity was measured by comparing the effects observed in several cardiotoxins to those observed in several hepatotoxins. By comparing the effects of new test compounds to these known cardiotoxins and hepatotoxins, some perspective regarding the predicted *in vivo* toxicity of the new test compounds can be achieved.

## METHODS

### Cell Culture Conditions

For H4IIE cells, flat bottom 96-well plates were seeded with cells in 100  $\mu$ L of media 48 hr prior to dosing. Cells were cultured at 37°C, 5% CO<sub>2</sub> in Modified Eagle Media supplemented with 20% serum. On the third day after seeding, test compounds prepared in medium were added to the plates. For rat cardiomyocytes, cells were harvested from 1-2 day old rat neonates and seeded in coated 96-well plates in 100  $\mu$ L of medium. After serum-free medium exchange the following day, cells were allowed to acclimate overnight. Cells were dosed with test compound the following day in medium supplemented with 20% serum. Cells were exposed to 1, 3, 6, or 24 hrs and then analyzed using various biochemical and gene expression markers.

### Test Compound

The test compound stock was used to prepare dosing solutions of 1, 5, 10, 20, 50, 100, and 300  $\mu$ M in cell culture medium containing 20% serum.

### Cell Number

Cell number was determined in a separate plate using a specific nucleic acid binding dye that fluoresces upon intercalation with DNA and RNA. There is a direct correlation between intracellular RNA/DNA levels and cell number. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

### Membrane Leakage

Cell death was determined by monitoring membrane leakage from cells using either ELISA (Biotin GST kit) or activity assays. The marker enzymes were specific for the tissue. Calculations were expressed as percent change relative to cell death as determined by complete cell lysis. Treatment values were divided by cell death values and subtracted from 100 to determine percent live cells.

### MTT Assay (Tetrazolium Dye Reduction)

Cells were evaluated for their ability to reduce soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan MTT (purple). Viable cells have the greatest amount of MTT reduction and hence the highest absorbance readings. Reduction of MTT has been linked to mitochondrial respiration and extramitochondrial reductase activity. Percent change relative to controls was calculated by dividing the treatment value by the control values and multiplying by 100.

### Adenosine Triphosphate (ATP)

Cellular Adenosine triphosphate (ATP) was determined using a luciferase-based assay. The percent change relative to controls was calculated by dividing the treatment cell number by the control cell number and multiplying by 100.

### ANP and BNP Gene Expression

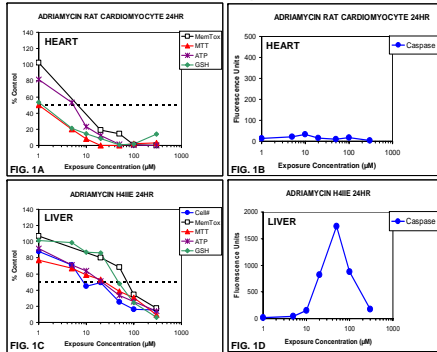
Expression of ANP and BNP mRNA levels was determined by bDNA analysis of mRNA. Induction of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) gene expression were used as markers of cardiac hypertrophy. Endothelin-1 was used as a positive control and results were compared to untreated controls.

### DCFDA Analysis

DCFDA-loaded cells were exposed to test compound and analyzed for reactive oxygen species (ROS) over time. Results were compared to untreated controls.

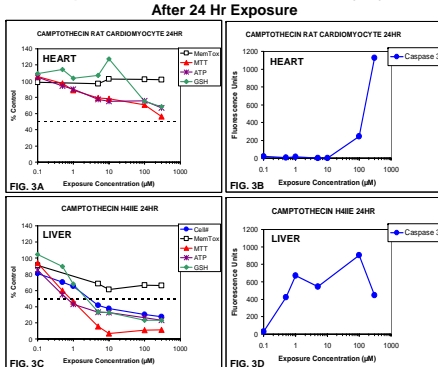
## RESULTS

**FIGURE 1:**  
Adriamycin in Rat Cardiomyocytes vs. H4IIE Cells  
After 24 Hr Exposure



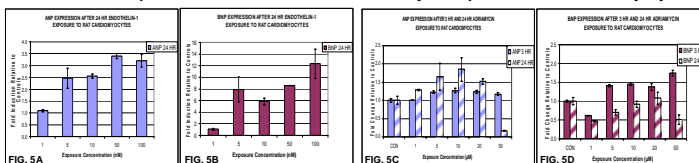
Following a 24 hr exposure of rat cardiomyocytes to adriamycin (Fig. 1A-1B), toxicity was observed beyond 1  $\mu$ M as shown by decreases in MTT, ATP, and GSH. Cell death was observed between 1-20  $\mu$ M as determined by membrane leakage (MemTox). In H4IIE cells (Fig. 1C-1D), cell death was observed between 100-300  $\mu$ M. An increase in caspase 3 activity (a committed step in apoptosis) was observed in the H4IIE liver cell line. This was not detected in cardiomyocytes. Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

**FIGURE 3:**  
Camptothecin in H4IIE Cells vs. Rat Cardiomyocytes  
After 24 Hr Exposure



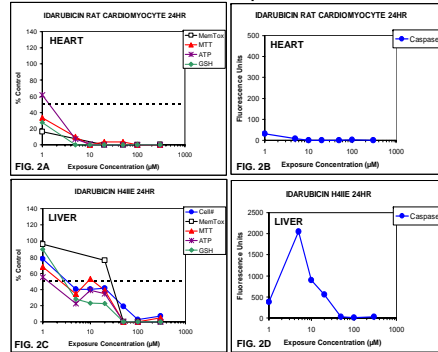
Following a 24 hr exposure of rat cardiomyocytes to camptothecin (Fig. 3A-3B), a decrease in the mitochondrial markers MTT and ATP were observed between 1-300  $\mu$ M that occurred in the absence of cell death (membrane leakage). H4IIE cells showed a large increase in the apoptotic marker caspase 3 prior to cell death. A decrease in the mitochondrial markers and a reduction in cell proliferation were observed (Fig. 3C-3D). Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

**FIGURE 5:**  
ANP and BNP Expression After Endothelin-1 and Adriamycin Exposures in Rat Cardiomyocytes



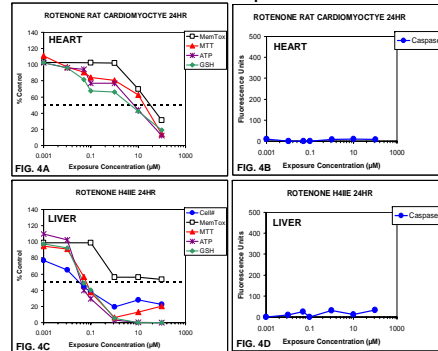
Following 24 hr Endothelin-1 exposure to rat cardiomyocytes (Fig. 5A-5B), a 3.5-fold increase in ANP and 12-fold increase in BNP were observed over 1-100 nM exposure range. Adriamycin (Fig. 5C-5D) showed almost 2-fold increase in ANP at 10  $\mu$ M (24 hr) and almost 2-fold increase in BNP at 50  $\mu$ M (3 hr).

**FIGURE 2:**  
Idarubicin in Rat Cardiomyocytes vs. H4IIE Cells  
After 24 Hr Exposure



Following a 24 hr exposure of rat cardiomyocytes to idarubicin (Fig. 2A-2B), toxicity was observed at <1  $\mu$ M. The effect of idarubicin on H4IIE cells (Fig. 2C-2D) showed no cell death (membrane leakage) until 20  $\mu$ M. Effects on the mitochondrial markers (MTT and ATP), loss of cells, and decrease in total glutathione (GSH) were observed. An increase in caspase 3 activity was observed in the H4IIE liver cell line. This was not detected in cardiomyocytes. Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

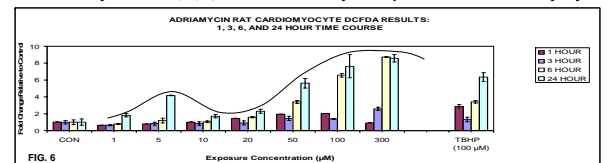
**FIGURE 4:**  
Rotenone in H4IIE Cells vs. Rat Cardiomyocytes  
After 24 Hr Exposure



Following a 24 hr exposure of rat cardiomyocytes to rotenone (Fig. 4A-4B), a decrease in viability was tracked by effects on mitochondria between 1-100  $\mu$ M. Cell death was observed between 10-100  $\mu$ M. H4IIE cells showed cell death as early as 1  $\mu$ M as demonstrated by apoptotic marker caspase 3 prior to cell death. A decrease in the mitochondrial markers and a reduction in cell proliferation were observed (Fig. 4C-4D). Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

**FIGURE 6:**

**ADRIAMYCIN RAT CARDIOMYOCYTE DCFDA RESULTS:**  
1, 3, 6, AND 24 HOUR TIME COURSE



A dose-related increase in DCFDA was observed after adriamycin exposure in rat cardiomyocytes (Fig. 6). The bimodal response at 24 hr may represent an initial stress at 10-20  $\mu$ M, followed by compensatory response at 10-20  $\mu$ M, and lassy toxicity at 10-300  $\mu$ M.

## SUMMARY

The objective of the present study was to determine if utilization of *in vitro* cell-based models, coupled with a robust set of biochemical and gene expression markers, could be used to distinguish cardiotoxicity from hepatotoxicity.

Use of *in vitro* cell-based models able to predict toxicity specific to the heart would be of considerable value in the early drug development.

Adriamycin and the anthracycline group were selected as reference compounds because they are well known for causing cardiac toxicity.

Cardiac toxicity observed following anthracycline treatment has been linked to production of reactive oxygen species resulting in depletion of reduced glutathione (GSH), resulting in damage to cellular macromolecules including mitochondria.

Expression of ANP and BNP mRNA levels can be used as markers of hypertrophy, especially when response is measured in the absence of toxicity. Gene expression was determined by bDNA analysis of mRNA. Endothelin-1 (ET-1) was included as a positive control.

Rotenone and camptothecin were included as known hepatotoxins.

## CONCLUSIONS

Utilization of a two cell model (rat cardiomyocyte vs. H4IIE, rat liver hepatoma) showed distinct differences in cell sensitivity consistent with reported organ specific toxicity.

For those compounds considered to be primarily cardiotoxic (anthracyclines), the mean TC50 values in the heart cell model (rat cardiomyocyte) were substantially lower compared to the liver cell model (H4IIE):

- The mean anthracycline TC50 values were 2-10 times lower in the heart model vs. the liver model. Adriamycin TC50 was about 4 times lower in the heart vs. the liver, and idarubicin TC50 was about 10 times lower in heart vs. liver.
- Thus, the anthracyclines tested showed more toxicity in the heart cell model compared to the liver cell model.

Conversely, for those compounds considered to be primarily hepatotoxic (camptothecin and rotenone), the mean TC50 values in the liver cell model (H4IIE) were substantially lower compared to the heart cell model (rat cardiomyocyte):

- The mean camptothecin TC50 value was about 4 times lower in the liver model vs. the heart model. The mean rotenone TC50 value was about 100 times lower in the liver model vs. the heart model.
- Thus, camptothecin and rotenone showed more toxicity in the liver cell model compared to the heart cell model.

Factors to keep in mind include severity of toxicity and therapeutic range:

- Compounds that are acutely cytotoxic may provide a more accurate prediction of cardiotoxicity, especially if the toxicity is close to the therapeutic window.
- Compounds that are not acutely cytotoxic and have toxicity well beyond the therapeutic window may show effects in the secondary markers such as hypertrophy (ANP/BNP) or production of reactive oxygen species (ROS by DCFDA).
- The mitochondria and loss of reduced glutathione (GSH) represent the most sensitive targets in both cell models.
- The heart cells appears to be more sensitive to the effects of glutathione depletion compared to the liver.

ANP and BNP are considered biomarkers for hypertrophy in the heart. Expression of these markers in rat cardiomyocytes can be used as markers to identify compounds that may cause cardiac hypertrophy, especially when the response is observed in the absence of toxicity. This is especially useful for compounds that are not acutely cytotoxic.

- Changes in ANP/BNP expression in the absence of acute cytotoxicity are consistent with cardiac hypertrophy.
- Changes in ANP/BNP expression for acutely cytotoxic compounds may represent a stress response and not a hypertrophic response.

These data indicate that a two cell model can provide *in vitro* data to help predict cardiac versus non-cardiac toxicity. Thus, the incorporation of a two cell model for identifying organ specific toxicity is a useful tool.

## REFERENCES

References available upon request.

