

Utilization of a Rat Cardiomyocyte Model to Identify Heart-Specific Toxicity Using Multiple Acute Biochemical Endpoints and Gene Expression Markers

Paul C. Wilga, Diane K. Petrella, Ariel J. Swanson, Jeffrey F. Pregenzer, Sean Thompson, Karen Rutherford-Root, and James M. McKim, Jr.
CeeTox Inc., 4717 Campus Drive, Kalamazoo, MI 49008

ABSTRACT

The present study was designed to evaluate a rat cardiomyocyte (CM) model using biochemical assays and gene expression markers to help identify heart-specific toxicity and differentiate risk between compounds. Understanding risk profiles for drugs with idiosyncratic toxicity are also important. Relative sensitivity of heart over liver was assessed by using rat cardiomyocytes isolated from 1-2 day old neonates and a rat hepatoma (H4IIE) cell line. The anti-diabetic drugs troglitazone (TRO), rosiglitazone (ROSI), and pioglitazone (PIO) were tested in both cell systems at exposure concentrations of 1, 5, 10, 20, 50, 100, and 300 µM for 24 or 72 hr. Cytotoxicity was determined using markers for acute toxicity, oxidative stress, and apoptosis. Gene expression markers were assessed in CM cells at 24 hr for regulation of apoptosis (BAX/BCL2), glucose transports (GLUT1/GLUT4), and hypertrophy (atrial natriuretic peptide, ANP). In the H4IIE assays, all glitazones showed significant loss of total glutathione and inhibition of cell proliferation with a potency order of TRO>ROSI>PIO. In rat CM cells, cell death was observed at 300 µM TRO. No acute cytotoxicity was observed with ROSI or PIO, but there was a time-dependent decrease in total glutathione for all glitazones at 24 and 72 hr. An increase in the pro-apoptotic marker BAX was also observed in rat glitazones with an concomitant suppression of the anti-apoptotic marker Bcl2. ROSI showed an increase in membrane lipid peroxidation at 24 and 72 hr. An increase in the hypertrophy marker ANP was also determined for ROSI at 24 hr that was not observed with TRO or PIO. In addition, ROSI showed a dose-dependent increase in GLUT1 and corresponding decrease in GLUT4. The GLUT1/GLUT4 reversal is characteristic of compounds known to cause cardiac hypertrophy. These results suggest that the rat CM model is a useful tool for identifying differences between compounds relating to cardiotoxicity and can be used to prioritize or rank-order compounds.

INTRODUCTION

The thiazolidinedione class of drugs (glitazones) have demonstrated effectiveness for treating type II diabetes. They do so by stimulating PPARγ to modulate transcription of insulin-sensitive genes that control glucose metabolism, thus resulting in reduced glucose in the bloodstream. While Pioglitazone has also been shown to reduce risk of heart attack and stroke, Rosiglitazone has been associated with an increased rate of heart failure. It would be highly beneficial to identify these issues early in the discovery process. Thus, the main objective of this study was to develop a cell-based model for identifying heart-specific toxicity to assess risk among compounds within a given drug class. Rat cardiomyocytes were tested against a liver cell line (H4IIE) against anti-diabetic glitazone class of drugs. Using a robust set of biochemical markers and gene expression tools, differences in cell sensitivity was measured to assess potential risk related to compounds within a given class. By comparing the effects of new test compounds to those of known cardiotoxic and hepatotoxic, some perspective regarding the predicted *in vivo* toxicity of new test compounds can be achieved.

METHODS

Cell Culture Conditions

Rat cardiomyocytes harvested from 1-2 old rat neonates or H4IIE cells were seeded in 96-well plates in 100 µL of medium. After 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 for 24 and 72 hr and then analyzed using various biochemical and gene expression markers.

Test Compound

Test compound stocks were used to prepare dosing solutions of 1, 5, 10, 20, 50, 100, and 300 µM in cell culture medium containing 20% serum.

Cell Mass

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

Membrane Leakage

Cell death was determined by monitoring membrane leakage from cells using ELISA assays for Troponin (heart) or α-GST (liver). 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.

MTT Assay (Tetrazolium Dye Reduction)

Cells were evaluated for their ability to reduce soluble-MTT (yellow), (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.

Total Glutathione (GSH)

Total glutathione was measured via a modification of the enzymatic recycling method described by O. W. Griffith (Analytical Biochemistry, 1980). The rate of color production using 5,5'-dithiobis(2-nitrobenzoic acid) reductase is directly proportional to the concentration of GSH in the sample.

8-Isoprostane (8-ISO)

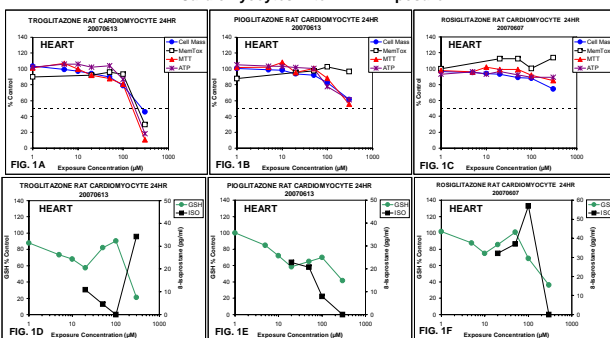
The presence of 8-Isoprostane in the culture media was analyzed with an EIA kit. 8-Isoprostane is produced by reaction of membrane phospholipids with oxygen radicals.

Gene Expression

Expression of mRNA levels was determined by bDNA analysis. Induction of BAX/Bcl2, ANP/BNP, and GLUT1/GLUT4 gene expression were used as markers of cardiac apoptosis, hypertrophy, and glucose transport, respectively.

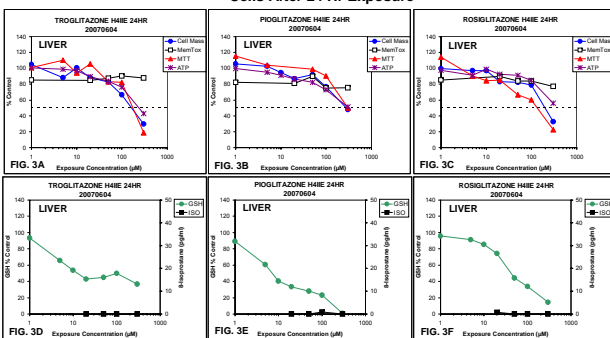
RESULTS

FIGURE 1:
Troglitazone, Pioglitazone, and Rosiglitazone in Rat Cardiomyocytes After 24 Hr Exposure



Following 24 hr exposure of rat cardiomyocytes to glitazones (Fig. 1A-1F), potency order was determined to be TRO>PIO>ROSI. Troglitazone toxicity was observed at 300 µM as shown by decreases in Cell Mass, MTT, ATP, and GSH. Pioglitazone showed decrease in Cell Mass, MTT, and ATP. Little toxicity was observed with Rosiglitazone. Total glutathione (GSH) was most affected with Rosiglitazone vs. Troglitazone and Pioglitazone. Membrane lipid peroxidation (8-ISO) was also highly increased. Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

FIGURE 2:
Troglitazone, Pioglitazone, and Rosiglitazone in H4IIE Cells After 24 Hr Exposure



Following 24 hr exposure of H4IIE cells to glitazones (Fig. 2A-2F), potency order was determined to be TRO>ROSI>PIO. Decreases in Cell Mass, MTT, ATP were observed in the absence of cell death. Decreases in total GSH were observed prior to effects on the other markers. Membrane lipid peroxidation was not observed. Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

FIGURE 5 and FIGURE 6:
BAX/BCL2 and ANP/BNP Expression After 24 Hr Glitazone Exposure to Rat Cardiomyocytes

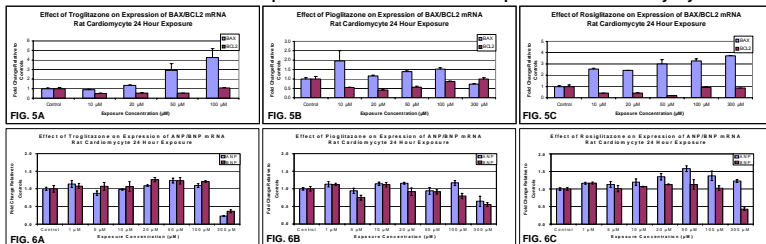
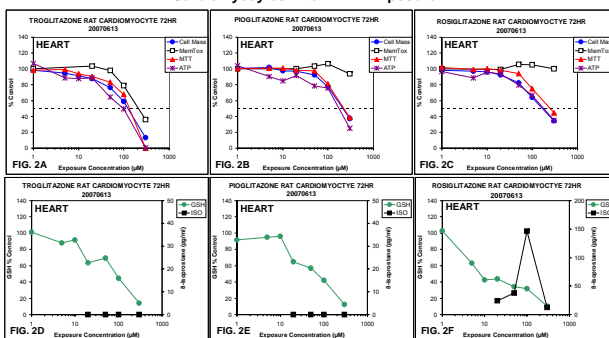
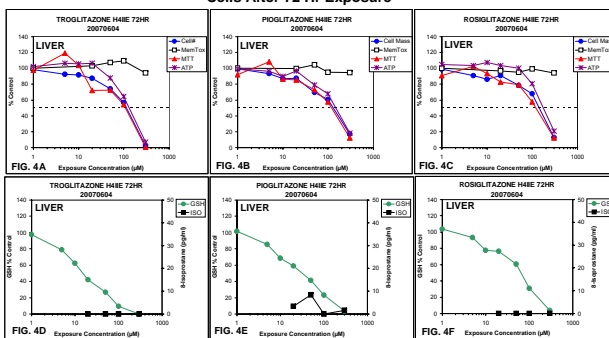


FIGURE 2:
Troglitazone, Pioglitazone, and Rosiglitazone in Rat Cardiomyocytes After 72 Hr Exposure



Following 72 hr exposure of rat cardiomyocytes to glitazones (Fig. 2A-2F), potency order was determined to be TRO>PIO>ROSI. Troglitazone toxicity was observed between 50-300 µM as shown by decreases in Cell Mass, MTT, ATP, and GSH. Pioglitazone and Rosiglitazone showed decrease in Cell Mass, MTT, and ATP in the absence of cell death. Decreases in total GSH were observed prior to other effects and was especially pronounced with Rosiglitazone. Rosiglitazone also showed a large increase in membrane lipid peroxidation (8-ISO). Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

FIGURE 4:
Troglitazone, Pioglitazone, and Rosiglitazone in H4IIE Cells After 72 Hr Exposure



Following 72 hr exposure of H4IIE cells to glitazones (Fig. 3A-3F), potency order was determined to be TRO>ROSI>PIO. Decreases in Cell Mass, MTT, ATP were observed in the absence of cell death. Decreases in total GSH were observed prior to effects on the other markers. No significant membrane lipid peroxidation was observed. Each value represents the mean of 3-7 replicates. Std error not shown for clarity (CV<20%).

FIGURE 7:
GLUT1/GLUT4 Expression After 24 Hr Glitazone Exposure to Rat Cardiomyocytes

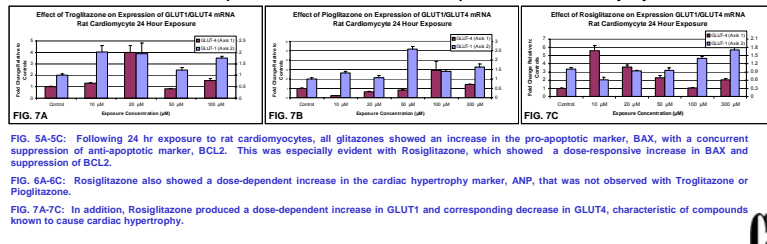


Fig. 5A-5C: Following 24 hr exposure to rat cardiomyocytes, all glitazones showed an increase in the pro-apoptotic marker, BAX, with a concurrent suppression of anti-apoptotic marker, BCL2. This was especially evident with Rosiglitazone, which showed a dose-responsive increase in BAX and suppression of BCL2.

Fig. 6A-6C: Rosiglitazone also showed a dose-dependent increase in the cardiac hypertrophy marker, ANP, that was not observed with Troglitazone or Pioglitazone.

Fig. 7A-7C: In addition, Rosiglitazone produced a dose-dependent increase in GLUT1 and corresponding decrease in GLUT4, characteristic of compounds known to cause cardiac hypertrophy.

SUMMARY

The objective of the present study was to utilize a rat cardiomyocyte model to help identify heart-specific toxicity using multiple biochemical endpoints and gene expression markers.

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

Multiple biochemical markers can be used to rank-order compounds from greatest to least toxicity.

Secondary toxicity markers for assessment of total glutathione (GSH) and membrane lipid peroxidation (8-ISO) may be valuable for identifying idiosyncratic toxicity.

Expression of key gene expression markers are important for investigating more heart-specific toxicity and idiosyncratic effects.

CONCLUSIONS

Utilization of a two cell model (rat cardiomyocyte vs. H4IIE, rat liver hepatoma) showed differences in cell sensitivity after exposure to glitazones, with rat cardiomyocytes being more sensitive than the liver cell line.

In H4IIE cells, all glitazones showed significant loss of total glutathione and inhibition of cell proliferation. The potency order based on the TC50 values from the 72 hr chronic analysis was TRO>ROSI>PIO.

Rat cardiomyocytes also showed significant loss of total glutathione and cell mass. The potency order based on the TC50 values from the 72 hr chronic analysis was TRO>ROSI>PIO.

Rosiglitazone had the greatest loss of total GSH of the three glitazones tested and this occurred prior to effects on the other markers.

Rosiglitazone also showed a large increase in the membrane lipid peroxidation marker, 8-isoprostane, at both 24 and 72 hr. This was tracked by the decrease in total GSH.

Membrane leakage (cell death) was observed in Troglitazone at the highest exposure concentration at both 24 and 72 hr.

All glitazones showed an increase in the pro-apoptotic marker, BAX, with a concurrent suppression of the anti-apoptotic marker, BCL2. This was especially evident with Rosiglitazone, which showed a dose-responsive increase in BAX.

Rosiglitazone also showed dose-dependent increase in the cardiac hypertrophy marker, ANP, that was not observed with Troglitazone or Pioglitazone.

Rosiglitazone also produced a dose-dependent increase in GLUT1 and corresponding decrease in GLUT4, very characteristic of compounds known to cause cardiac hypertrophy.

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 when 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), apoptosis (BAX/BCL2), or glucose transport (GLUT1/GLUT4).

These data suggest incorporation of a two cell model is a useful tool for prioritizing or rank-ordering compounds. The results also suggest that the rat cardiomyocyte model is a useful tool for identifying differences between compounds relating to cardiotoxicity.

REFERENCES

*References available upon request.

