

# A NEW APPROACH TO *IN VITRO* TOXICITY SCREENING BASED ON MULTI-ENDPOINT ANALYSIS PROVIDES INFORMATION ON MECHANISM AND PREDICTS *IN VIVO* TOXICITY

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## ABSTRACT

Toxicity data provided at a time when new drug candidates are being optimized can be used to reduce toxicity while maintaining efficacy. The present study describes a new paradigm for toxicity screening based on monitoring changes in several key biochemical processes including mitochondrial function (MTT, ATP), cell number (CyQUANT®), and cell death (alpha-glutathione S-transferase, GST). When performed in parallel these assays provide information on mechanism, toxicity relative to other compounds, and assist in predicting *in vivo* toxicity. Rat hepatoma (H4IIE) cells were seeded into 96-well plates. After 48 hr, the cells were treated with rotenone (0, 0.1, 1, 5, 10, 50, 100 μM). Following a 6-hr exposure GST was unchanged while cell numbers were reduced in a concentration-related manner (35% relative to controls, 100 μM). The reduction in cell numbers, in the absence of GST data, could have been due to cell death or reduced proliferation. By combining GST and cell number, it was possible to deduce that the reduction in cell numbers was the result of reduced proliferation. There was a significant reduction in ATP (50% at 0.3 μM) and MTT (50% at 8 μM) relative to controls. Without supporting data from the other assays, these reductions could have been due to cell death, reduced proliferation, or a direct effect on biochemical pathways. By combining data from all assays, it was possible to determine that rotenone caused a reduction in cell number that was not due to cell death, but rather to a decrease in cellular ATP, resulting in reduced proliferation. In conclusion, these data indicate that an *in vitro* cluster approach to toxicity screening can provide comprehensive toxicity data early in the drug discovery process.

## INTRODUCTION

### Problem

One-third of compounds fail late in development due to toxicity

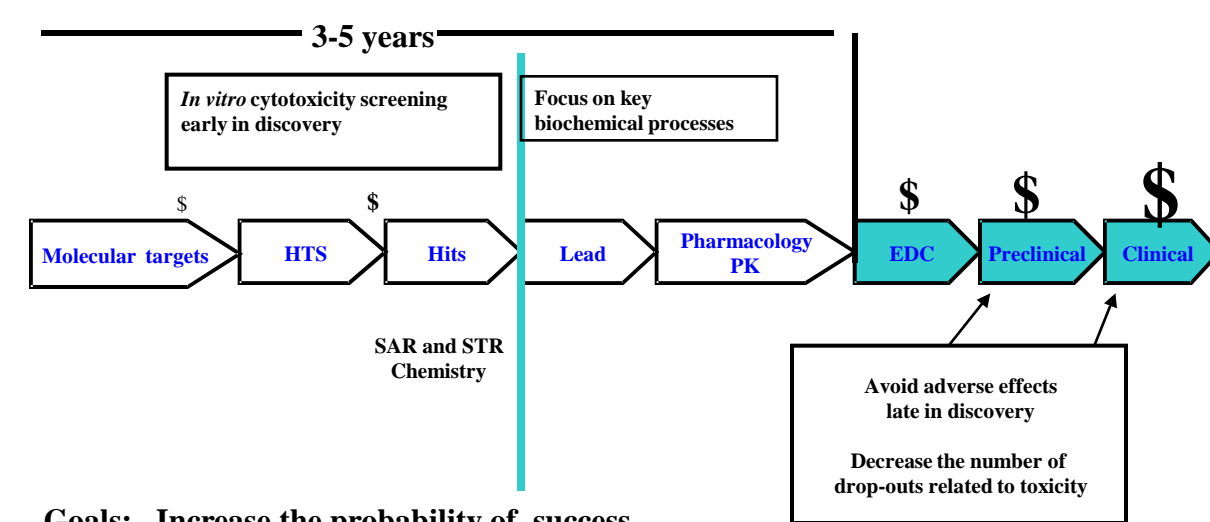
### Objective

Reduce toxic liability of compounds by identifying potential issues early in discovery

### Conditions

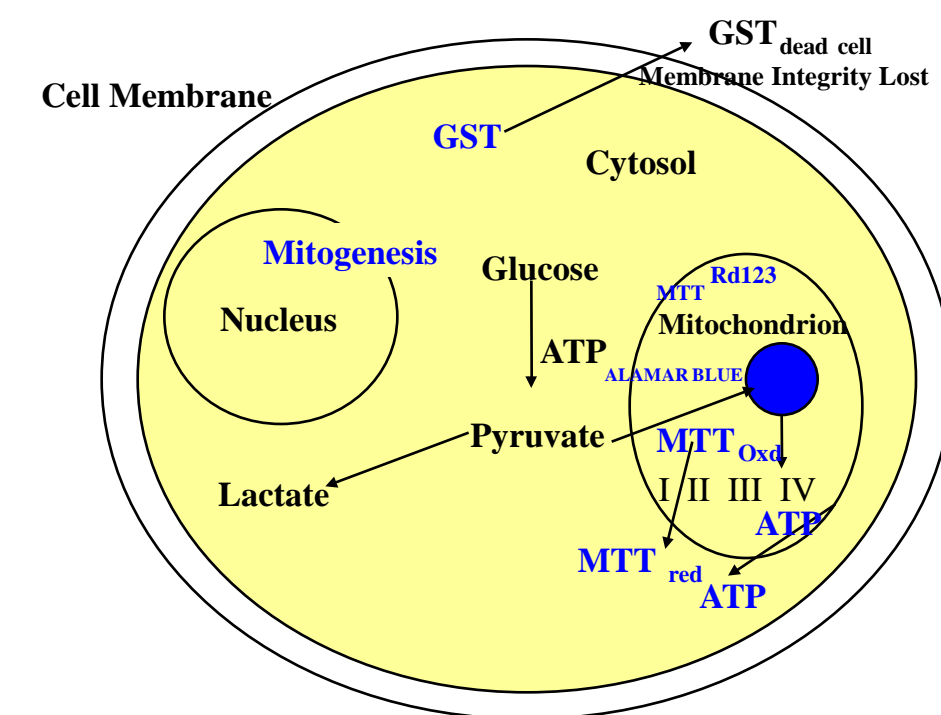
Establish a system without delay and make it useful to all discovery areas  
High throughput capacity and rapid turnaround  
Should only require a small amount (5-10 mg) of test material

## A Solution: Change the Paradigm of Drug Discovery



Goals: Increase the probability of success  
Decrease cost, cycle time, and time to market,  
competitive advantage

## Rationale for Tox-Cluster Analysis: Monitor Changes in Key Cellular Processes

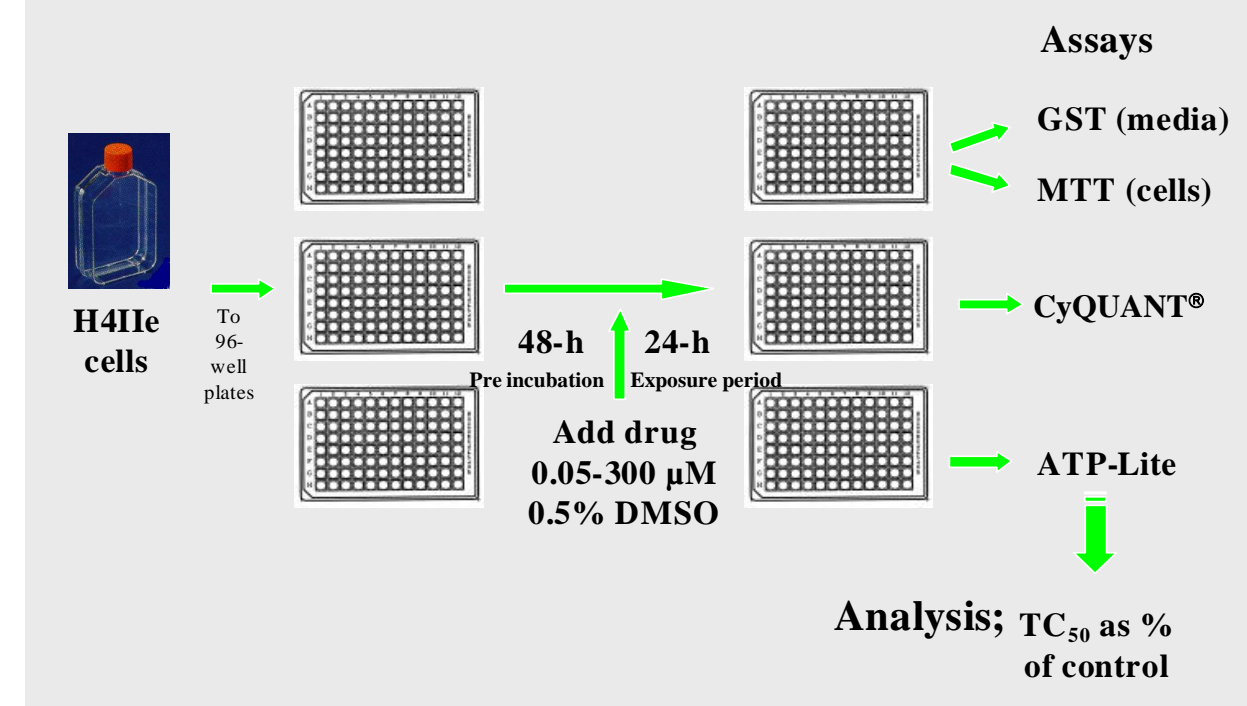


Information on the mechanism of toxicity, relative toxicity, and *in vivo* toxicity can only be achieved by monitoring several key biochemical endpoints over a broad range of exposure concentrations.

## METHODS

### Cell Culture, Experimental Design, and Cytotoxicity Assays

#### Tier I Cytotoxicity Assays: Procedures



### General Cell Health Assays (Tox-Cluster)

#### Cell Number (CyQUANT®)

Cell number in each well was determined in a separate plate with the CyQUANT® Cell Proliferation Assay from Molecular Probes.

#### Glutathione S-transferase (GST) Leakage

The presence of GST in the culture media was analyzed with an ELISA kit obtained from Biotrin Inter. LLC α-GST is a specific liver protein that provides information on cell death that is more reliable than the release of lactate dehydrogenase (LDH).

#### MTT

Cells remaining in each well were evaluated for their ability to reduce soluble 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT). The reduction of MTT has been linked to mitochondrial respiration and extramitochondrial reductase activity.

#### Alamar Blue (AB) Reduction

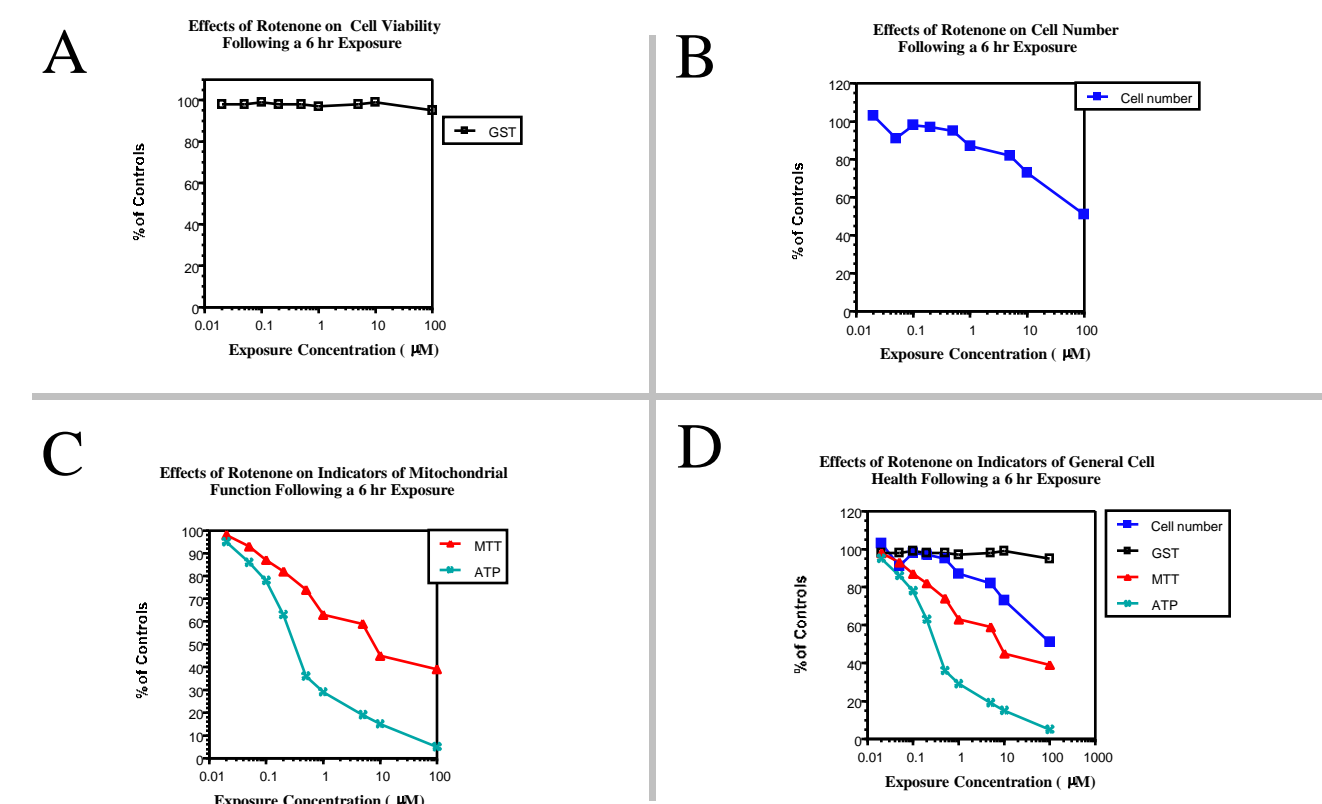
Alamar Blue™ (Accumed Int.) is a tetrazolium dye that becomes fluorescent after chemical reduction. Like MTT, it has a mitochondrial reduction component, but it has a different interaction with the electron transport chain.

#### Adenosine Triphosphate (ATP)

Cellular Adenosine triphosphate (ATP) was determined with the ATP-Lite kit from Packard Instruments according to the manufacturer's instructions. This assay is based on a reaction between ATP + D-luciferin + oxygen catalyzed by luciferase to yield Oxyluciferin + AMP + PPI + CO<sub>2</sub> + light. The emitted light is proportional to the amount of ATP present.

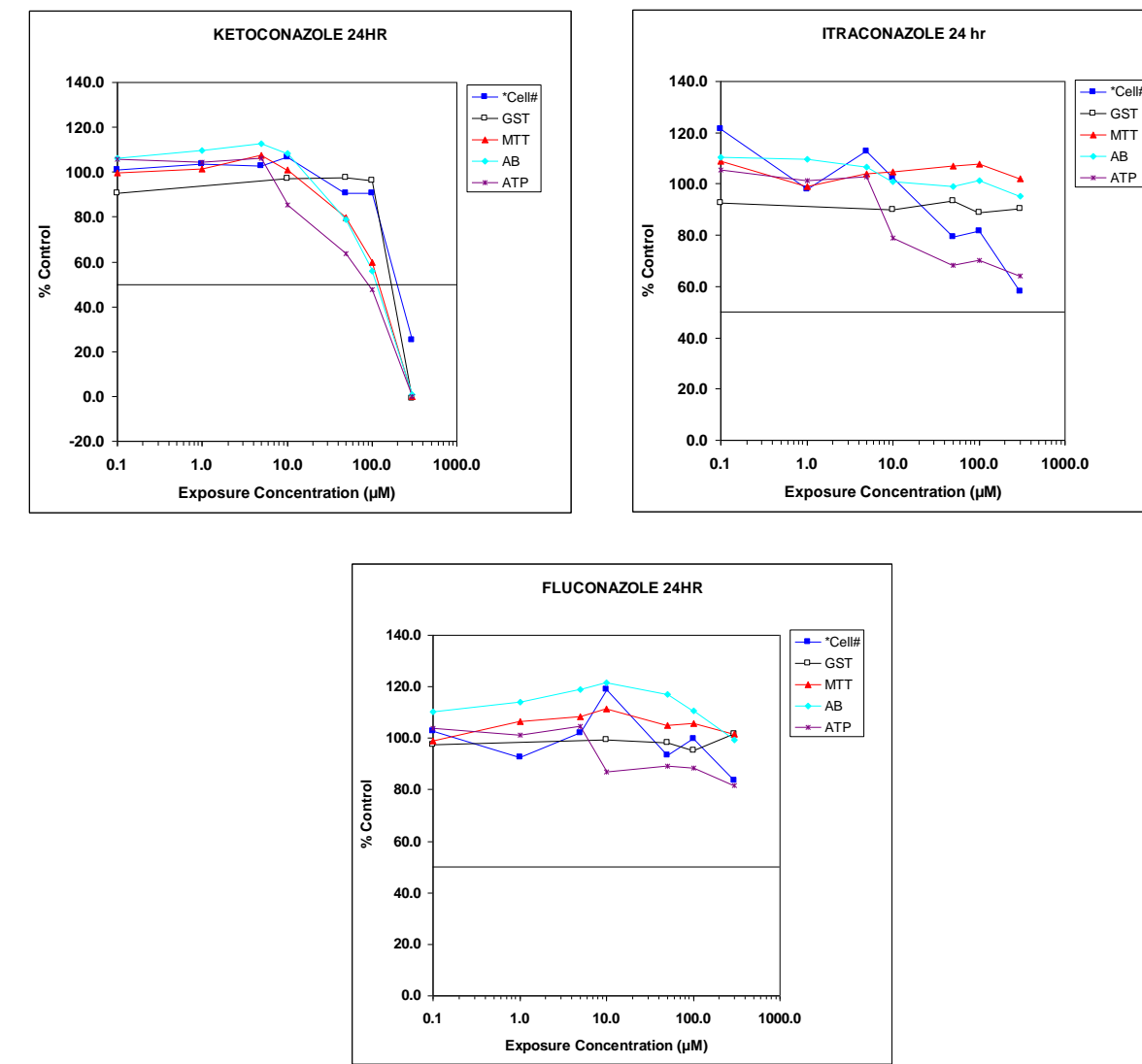
## RESULTS

### Figure 1. Multi-Endpoint Analysis (Tox-Cluster) To Assess The Effects of Rotenone on General Cell Health



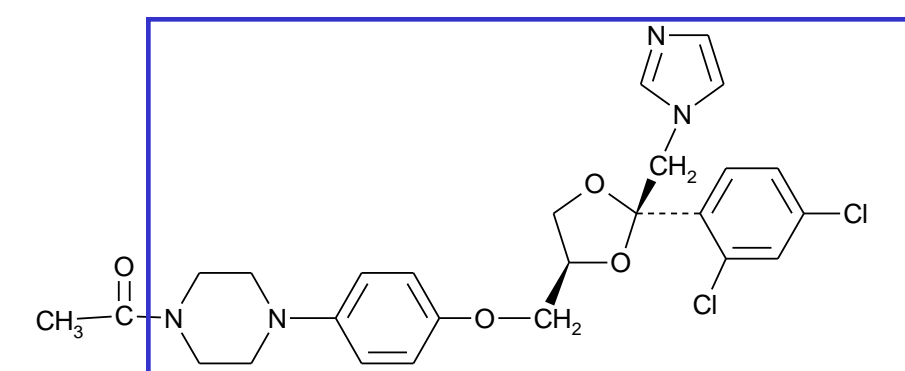
The mechanism of rotenone cytotoxicity is inhibition of oxidative phosphorylation. After 6 hr of exposure, the marker for membrane integrity (α-GST) was unchanged, indicating no toxicity (A). If a marker for cell number had been used (B) a small concentration dependent decrease would have been detected. However, the reason for the reduction would not be clear. The reduction in cells could have been due to cell death or a reduction in the rate of proliferation. When panels A and B are viewed together it is clear that the reduction in cell number was due to a reduced rate of cell proliferation. If only MTT or ATP had been used to assess toxicity the results would have shown a dramatic concentration dependent reduction in both markers (C). Without additional information, these reductions could have been produced by cell death, a reduced rate of proliferation, or direct inhibition of the enzymes responsible for MTT reduction or ATP synthesis. When all biochemical parameters were combined (D) and the concentration profiles analyzed, it was clear that rotenone inhibited mitochondrial function and cellular ATP levels which led to a decrease in the rate of cell proliferation. Values represent the mean of N=8 wells. Error bars not shown for clarity. CV ≤ 10%.

### Figure 2. Effects of Three Antifungal Agents on Cell Health: An Evaluation of Structure Toxicity Relationships

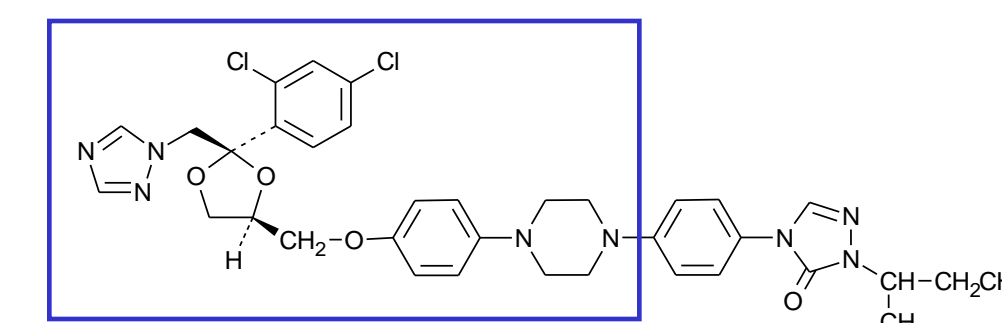


Ketoconazole and itraconazole are structurally similar, while fluconazole possesses a much different chemical structure. Multi-endpoint analysis provided information on relative toxicity, potential mechanisms of toxicity, and structure toxicity relationships. Based on the results of the cytotoxicity assays it was possible to rank the three azoles according to their toxicity as ketoconazole > itraconazole >>> fluconazole. Both ketoconazole and itraconazole inhibit State-3 respiration in isolated rat liver mitochondria. In comparison, fluconazole had no effect on State-3 respiration. In the rat hepatoma cell line ketoconazole and itraconazole showed initial effects on mitochondrial function as determined by ATP, MTT, and cell number while fluconazole had no detectable impact on any of the general health parameters (Fig 2).

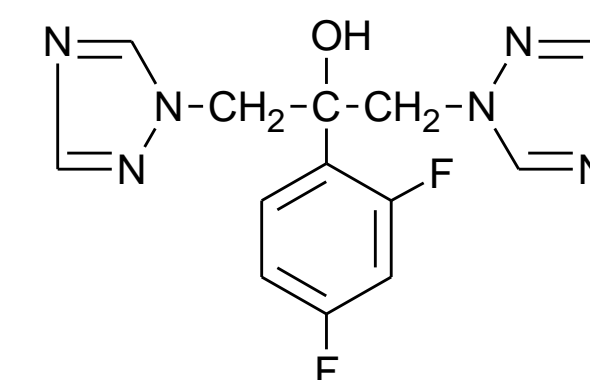
### Ketoconazole



### Itraconazole



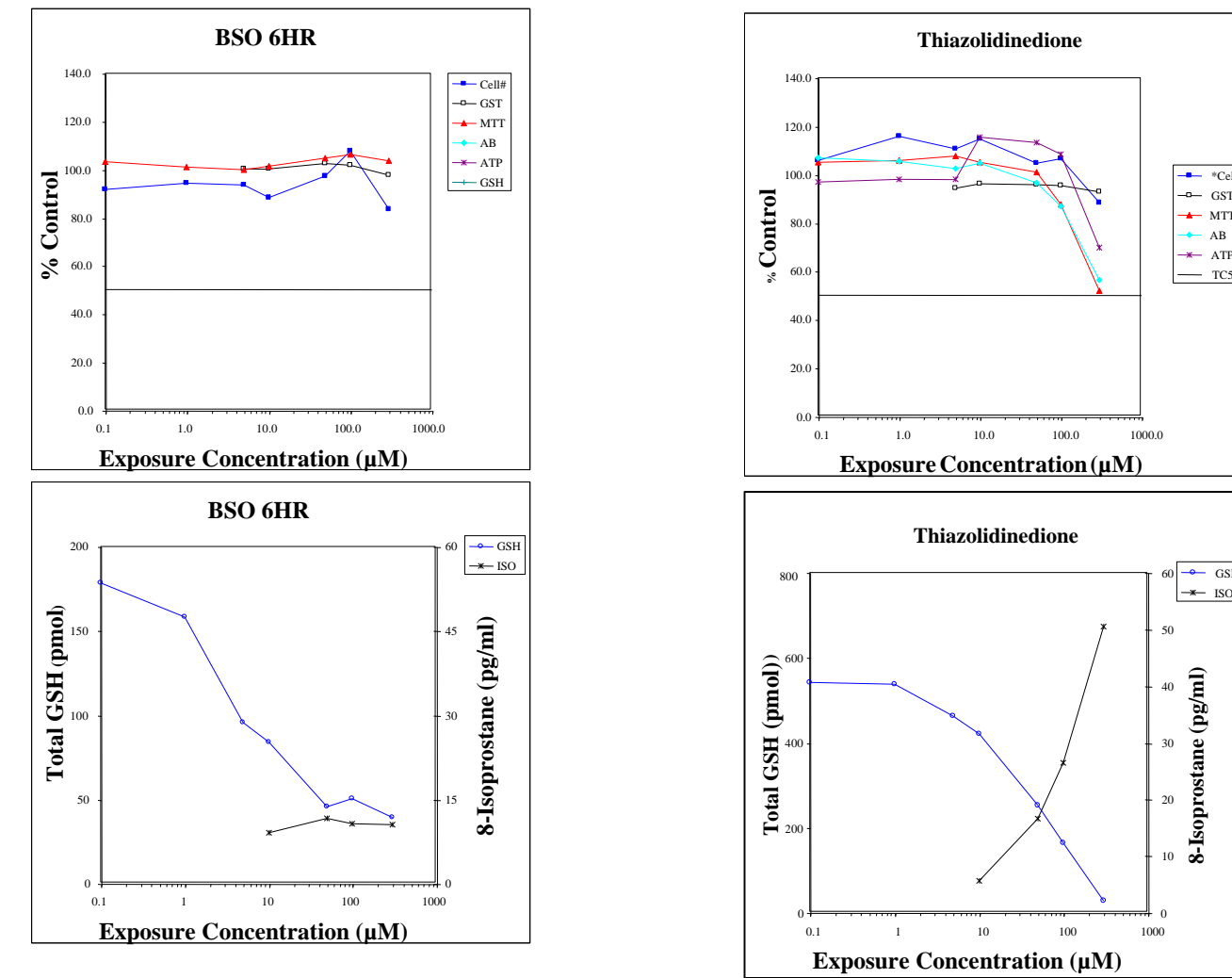
### Fluconazole



The boxes highlight the similarities between ketoconazole and itraconazole.

## RESULTS

### Figure 3. Markers of Oxidative Stress Provide Valuable Information

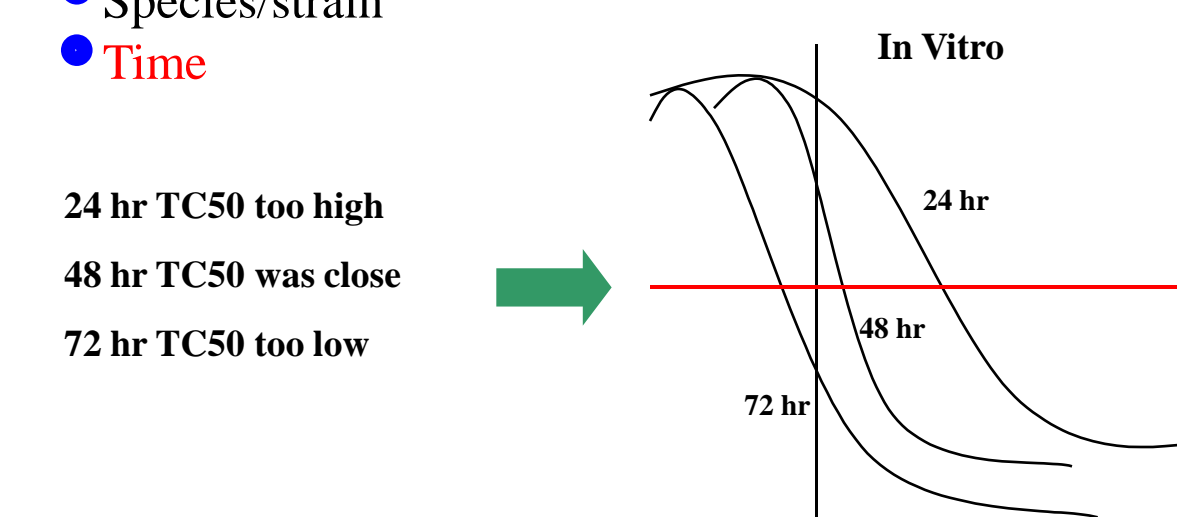


The addition of two oxidative stress markers, reduced glutathione (GSH) and 8-isoprostane (ISO), provided information on early events that may lead to toxicity upon prolonged exposure. In Fig 3 above, the test compounds had little effect on the markers of general cell health, while significant changes in GSH and ISO were observed. L-buthionine sulfoximine (BSO) inhibits GSH synthesis. Thiabendazole is a member of the glitazone antidiabetic drugs. Values represent the mean of N=8 wells. Error bars were not shown for clarity. CV ≤ 10%.

## Factors That Influence *In Vivo* Toxicity

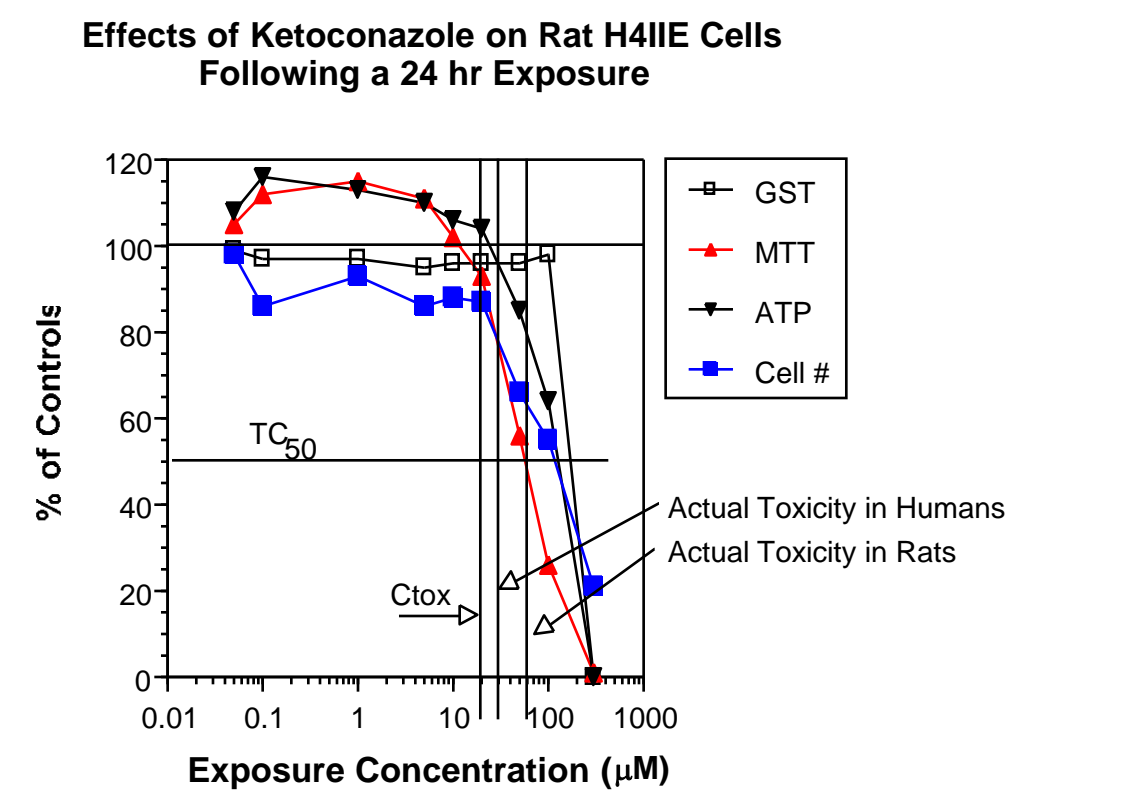
- Dose
- Route
- ADME
- Gender
- Species/strain
- Time

### Plasma Concentration Target Organ Dose



The value used to relate *in vitro* data to the *in vivo* situation was C<sub>max</sub>, which is expressed in μM. Retrospective analysis of compounds tested in 14-day rat studies revealed that the concentration that produced a half maximal response (TC<sub>50</sub>) *in vitro*, did not provide an acceptable estimate of *in vivo* toxicity. C<sub>max</sub> values equal to or greater than the *in vitro* TC<sub>50</sub> were toxic, however toxicity was also observed at lower C<sub>max</sub> values. Thus, the *in vitro* estimate using the TC<sub>50</sub> at 24 hr was too conservative. Dose-response curves generated at three time points produced curves similar to those depicted above. It was determined from these data that the NOAEL at 24 hr provided an estimate closer to the TC<sub>50</sub> observed at 48 and 72 hr. Therefore, the *in vitro* NOAEL at 24 hr became the estimated plasma concentration *in vivo* where toxicity would first be expected to occur. This value was defined as C<sub>tox</sub>. Validation with more than a dozen compounds has shown that C<sub>tox</sub> generated *in vitro* provides an accurate estimate of the plasma concentration *in vivo* where toxicity would be expected to occur.

### Figure 4. Multi-Endpoint Analysis (Tox-Cluster) Provides a Good Estimate of *In Vivo* Toxicity



The data shown in Figure 4 above illustrate how the C<sub>tox</sub> value determined from the *in vitro* experiments can provide a good estimate of the plasma concentration where toxicity *in vivo* would be expected to occur. *In vitro* experiments with ketoconazole yielded a C<sub>tox</sub> of approximately 20 μM. The actual plasma concentration in rats and humans where toxicity was observed was 60 μM and 30 μM respectively. These values were similar to the predicted C<sub>tox</sub> value of 20 μM.

## SUMMARY

The rat hepatoma cell line (H4IIE) provides an excellent *in vitro* model for toxicity screening.

Assays that monitor key biochemical processes such as membrane integrity, mitochondrial function, and mitogenesis are key to *in vitro* toxicity screening.

A single endpoint measured at one or two concentrations cannot provide reliable data for toxicity screening.

The utilization of multiple endpoints reduces false positive and false negative results.

The test system and the assays described allow a high "er" throughput system, which significantly decreases turnaround time.

Careful evaluation of the test system including its strengths and weaknesses combined with retrospective validation of *in vitro* screening results has resulted in a means of predicting *in vivo* toxicity based on *in vitro* data.

## CONCLUSIONS

Successful *in vitro* toxicity screening requires an *in vitro* cell system that has been well characterized in terms of its biochemical properties. The success of the simple approach described here is the inclusion of assays that monitor several key biochemical processes over a wide exposure range. These data provide strong evidence to support the idea that screening for toxic liability early in the drug discovery process can provide important information on toxicity relative to other compounds in the same chemical class, potential mechanisms of toxicity and subcellular targets, the existence of structure toxicity relationships, and an estimate of *in vivo* toxicity.