

# Pharmaceutical DISCOVERY

The Industry's Global, Peer-Reviewed Resource

## A Biochemical Approach to *In Vitro* Toxicity Testing

The authors describe a new approach to *in vitro* toxicity testing that is based upon cellular biochemical processes that are essential for survival. The platform combines cell-based assays, multiple endpoint analysis, concentration – response data and a database of non-proprietary drugs and chemicals that ensures a high degree of *in vivo* predictability. The information generated enables researchers to rank order compounds based upon whole-cell efficacy (IC<sub>50</sub>) and whole-cell toxicity (TC<sub>50</sub>) data, understand subcellular targets and perform structure–toxicity relationship analyses – all of which can help identify safer drugs with higher probabilities of success earlier in the development process.

James M. McKim, Jr., Paul C. Wilga, Jeffrey F. Pregenzer and Diane K. Petrella

---

**T**he development of a new drug from concept to market requires from 12 to 15 years of research and development, at an estimated cost of nearly \$1 billion (1, 2). The development costs increase significantly during preclinical animal testing for safety and in clinical trials. The loss of a new drug candidate in these late-stage tests due to unanticipated toxicity represents a major problem for the pharmaceutical industry. Even worse, a substantial number of promising drugs that survive the intense scrutiny of pharmaceutical and regulatory scientists are withdrawn from the market due to significant and sometimes lethal toxicity in humans. The late-stage attrition of new drugs pre- or post-market is costly for the pharmaceutical company and frustrating to clinicians and patients who look to the pharmaceutical community for new treatments.

Most scientists in the pharmaceutical industry agree that the only way to reduce late-stage attrition of new drug candidates is to front-load the drug discovery process with new testing paradigms that provide information on the potential liabilities associated with a new chemical entity early in the development process. Systems that provide reliable data validated against *in vivo* reality could provide discovery chemists with the ability to design out toxicity while maintaining efficacy.

The application of *in vitro* assays to help design safer drugs is not new. Companies have long used the Ames mutagenicity assay as an industry standard for assessing the carcinogenic potential of new

drugs. It is well recognized that a large portion of late-stage attrition is due to poor absorption, distribution, metabolism and elimination (ADME) properties. As a result, nearly every pharmaceutical company has implemented assays to evaluate these properties and is using these data to improve the drug development process (3, 4). More recently, it has been recognized that some compounds can alter cardiac function by causing QT interval prolongation, which has been linked to torsade de pointes in humans (5, 6, 7). This information has resulted in the implementation of new assays designed to identify new chemical entities that produce this effect.

Although *in vitro* cytotoxicity assays have been used by scientists for many years, these data have not been applied in a consistent manner to the decision-making process in early drug development. The primary reason for this is that *in vitro* toxicity data has not proven itself to be a reliable predictor of *in vivo* toxicity.

Most scientists within large pharmaceutical companies acknowledge that any system that could increase the probability of success during mandatory safety testing in animals would improve the drug development process greatly and would result in annual savings estimated to be in the hundreds of millions of dollars. Many new technologies and approaches have been suggested during the past 5–6 years; however, these have not provided the predictive power required for the data to be used to make “go/no-go” decisions on new drug candidates early in development.

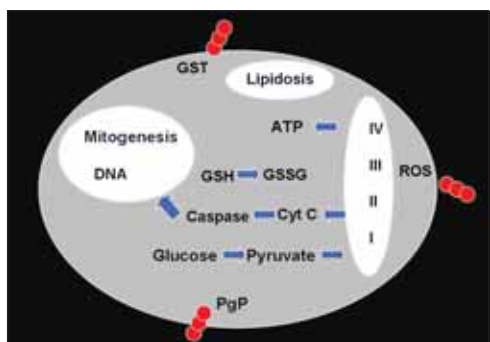


## In Vitro Toxicity Testing

This article describes an approach to *in vitro* toxicity screening that is based upon cellular biochemical processes that are essential for survival. The idea is to combine monitoring of multiple parameters with detailed concentration–response data, a novel algorithm for analysis of the toxicity response profiles and a database to provide a high level of *in vivo* predictivity. This approach has bridged the gap successfully between cells and whole-animal effects because it focuses on key functional endpoints that are not organ- or species-specific, and it has been validated in animal studies. The platform has been developed by CeeTox Inc. (Kalamazoo, Michigan, USA), which also maintains a database comprising a variety of non-proprietary drugs and chemicals; this allows clients to evaluate the relative toxicity of their compounds to those that have made it through the testing and evaluation process and are in the human patient population. This means that the risk associated with bringing a new compound forward can be assessed very early.

### Experimental

In the process, cells first are seeded into 96-well culture plates. The cells are allowed to grow and equilibrate for 48 h prior to being exposed to the test compounds. Test material is applied to the cells across a wide concentration range (1–300  $\mu\text{M}$ ). The range of the exposure concentrations was chosen because it represents those that bracket the typical blood concentrations required for therapeutic effects. Compounds that show no toxicity over this exposure range also are evaluated at concentrations extended to 1000  $\mu\text{M}$ . Negative and positive controls also are included. The higher concentrations sometimes are limited by solubility. The equilibrated cells are allowed to incubate with the



**Figure 1.** Key biochemical parameters essential to cell survival. GST = glutathione S-transferase; ROS = reactive oxygen species.

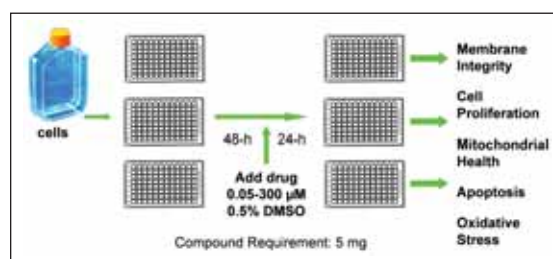
test compounds for 24 h. Following the exposure period, the cells or media are collected for analysis (Figure 1).

Analysts then measure the effects of test compounds on several biochemical endpoints associated with the general health of the cell. These include membrane integrity, mitochondrial function, cell proliferation, glutathione homeostasis, membrane lipid peroxidation and apoptosis (Figure 1 and Figure 2). Most cells in culture

express P-glycoprotein in the plasma membrane, where it functions as an efflux pump. For compounds with high affinity and low passive diffusion into the cells, this pump system can become the rate-limiting process for drug delivery. To evaluate the effect of P-glycoprotein pumps on cell toxicity, the pumps are inhibited and then treated with the test compound. Toxicity is evaluated and compared to toxicity measured with fully functional pumps. This added information helps identify situations where the *in vitro* assessment might underestimate toxicity; it also provides important information on issues related to uptake following oral administration and penetration of the blood–brain barrier. Finally, solubility is determined at the time compound is added to the cells and following the exposure period by Nepheloskan spectrometry. The overall testing paradigm evaluates 9–10 parameters that are monitored across seven exposure concentrations. All work is done in a medium-to high-throughput manner using 96-well plate readers, such as the Packard Fusion (Packard Instrument Company, part of PerkinElmer Life Sciences, Wellesley, Massachusetts, USA) or the PerkinElmer EnVision.

In an attempt to identify biochemical profiles associated with idiosyncratic toxicity, CeeTox has evaluated several compounds that have been withdrawn from the market due to unanticipated toxicity. By evaluating not only the drug associated with toxicity but also other members in the same group (as well as those with different therapeutic targets), it is possible to identify biochemical changes that might increase the probability of idiosyncratic toxicity.

The higher throughput nature of the *in vitro* screening is achieved by incor-



**Figure 2.** Cells are grown under standard culture conditions and seeded into 96-well culture plates 48 h prior to adding the test compounds. Following the exposure period, 9–10 different endpoints are evaluated. Seven exposure concentrations with six wells per concentration are used in each test.

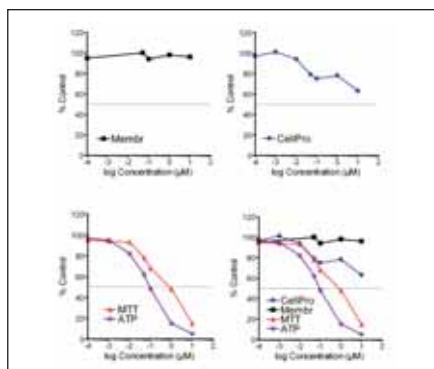
porating a sophisticated robotics platform, called PlateTrak, that is capable of performing the various tasks required by the assays employed in the toxicity panel. This system was designed by CeeTox scientists and assembled by CCS Packard (Carlsbad, California, USA). Following analysis of the raw data, concentration response curves are generated for each endpoint. Three graphs containing curves for acute toxicity, oxidative stress and apoptosis are generated automatically. A proprietary algorithm evaluates the shape of the curves, key inflection points and the relative significance of each endpoint to acute toxicity. The resultant analysis provides an estimate of the blood concentration (C<sub>tox</sub>) in a rat where toxicity first would be expected to occur. These data were validated against the standard rat 14-day repeat dose study.

### An Example: Rotenone

Rotenone is a pesticide that elicits toxicity by inhibition of mitochondrial oxidative phosphorylation. This compound was screened in the CeeTox system and by analysis of each endpoint separately so it would be possible to demonstrate the power of multiple endpoints and dose-response in the interpretation of *in vitro* data.

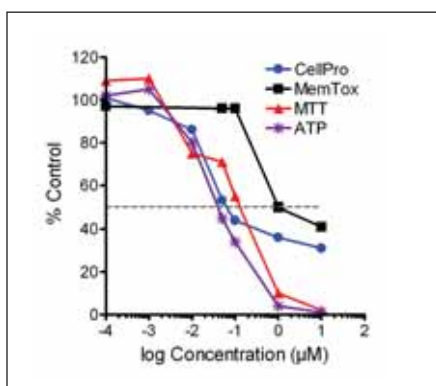
Rotenone was prepared in media and exposures were conducted for 6 h and 24 h. Following the 24-h exposure period, all parameters were affected and most cells were dead. In situations of extreme acute toxicity, a shorter time point can provide additional information on the sequence of biochemical events leading to cell death. The data in Figure 3 represent effects following a 6-h exposure.

The upper-left panel shows the concentration response curve for Rotenone-induced acute cell death as measured by the membrane leakage marker glutathione



**Figure 3.** Evaluation of Rotenone toxicity. Following a 6-h exposure to Rotenone, the cells display a differential response across the endpoints monitored. The best interpretation of the data can be achieved only when all endpoints are viewed together. All values represent the mean of 6–8 replicate wells. CellPro = cell proliferation; Membr = membrane leakage; MTT = 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide; ATP = adenosine triphosphate.

S-transferase (GST). If the assay chosen had been cell proliferation (Figure 3, upper-right panel), a clear concentration-related reduction in cell number would have been seen. However, with only these data it would not be possible to determine whether the reduction in cell number was due to acute cell death or a reduced rate of proliferation. By combining membrane leakage with cell proliferation it is clear that the reduction in cell number is not due to cell death. If only adenosine triphosphate (ATP) levels or mitochondrial function had been used to



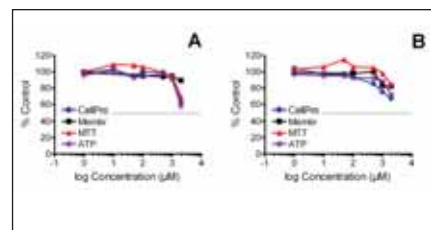
**Figure 4.** Evaluation of Rotenone toxicity. Following a 24-h exposure to Rotenone, several of the response curves show a similar response and no longer can be resolved from one another. All values represent the mean of 6–8 replicate wells. CellPro = cell proliferation; MemTox = membrane leakage assay; MTT = 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide; ATP = adenosine triphosphate.

evaluate the toxicity of Rotenone, then a pronounced concentration-dependent response would have been seen (Figure 3, lower-left panel). However, once again, with only these data it would not have been possible to know whether the reductions observed were the result of acute cell death, a reduced rate of cell proliferation or direct interference with the enzymes and systems used in the assays. It is only when multiple endpoints are combined with an extensive dose-response scenario that useful information can be obtained regarding the sequence of events and the most sensitive sub-cellular targets. In this example, the most sensitive endpoint was ATP depletion and mitochondrial function. The reduction in cellular energy contributed to a reduced rate of proliferation prior to acute cell death.

A longer exposure time (24 h) caused several of the response curves to come together, and differentiation between the endpoints becomes more difficult (Figure 4). An added advantage to multiple endpoint analysis is redundancy; false positive and false negative data are reduced dramatically. A concern voiced by many pharmaceutical scientists working in early discovery relates to false positive data, who are worried that the *in vitro* systems employed could be too sensitive and could thus falsely flag a potential blockbuster drug as toxic. The incidence of false positive data is less than 1%, based on the CeeTox multiple endpoint and dose-response approach.

### Database of Known Toxicity Profiles

CeeTox has developed an extensive database consisting of known drugs and chemicals. This database has not demonstrated toxicity profiles that are inconsistent with animal or human effects and therefore provides a high degree of confidence. Two examples include the well-known and highly effective medicines ibuprofen (Figure 5A) and acetaminophen (Figure 5B). These compounds showed low toxicity in the system across the exposure concentrations and time tested. The predicted (Ctox) blood level of toxicity for acetaminophen was estimated to be 800 µM in the absence of significant metabolism. In humans, the therapeutic concentration of acetaminophen is 60–130 µM. Sustained serum concentrations of 900 µM for 4 h or 300 µM for 12 h puts the patient at risk for liver damage (8). Note that



**Figure 5.** Ibuprofen (A) and acetaminophen (B) toxicity profiles. The use of multiple endpoints reduces false positive and negative results. The *in vitro* toxicity data for ibuprofen and acetaminophen demonstrate profiles of safe drugs. All values represent the mean of 6–8 replicate wells. CellPro = cell proliferation; Membr = membrane leakage; MTT = 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide; ATP = adenosine triphosphate.

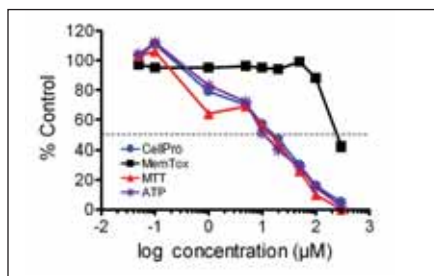
the cells used here essentially have no constitutive cytochrome P450 activity. Therefore, toxicity is based upon an assessment of the parent compound and does not address the potential issue of reactive metabolite formation. False negative data are not a concern, as these compounds will continue in the development process. However, it is important to evaluate metabolic activation, protein binding and interaction with membrane transporter systems when negative data are obtained in order to improve the compound's overall safety profile.

The tissue from which a cell is derived does not determine the target organ for toxicity *in vivo*. An example of this can be seen in Figure 6. This compound had no acute toxicity up to and including the 100 µM exposure concentration. In comparison, cell proliferation was reduced in a significant concentration-related manner. ATP and mitochondrial function could not be differentiated from the loss of cells. These data indicate a cytostatic mechanism of toxicity. This compound produced bone marrow hypocellularity *in vivo*. The key to predicting target organ toxicity is to choose biochemical endpoints that are unique to a specific organ. CeeTox currently is working on an *in vitro* model to predict target organ toxicity using unique biochemical targets. By selecting pathways that are essential to cell health and highly-specific for a single organ, a high level of predictive information regarding the target organ for toxicity can be obtained.

### Identifying Idiosyncratic Toxicity

An important component of the database is the addition of drugs that have been

## In Vitro Toxicity Testing



**Figure 6.** Target organ toxicity is not limited to the tissue type from which the cells were derived. In this example, the rat hepatoma cells respond to a cytostatic agent. This type of profile can result in bone marrow hypocellularity *in vivo*. CellPro = cell proliferation; MemTox = membrane leakage; MTT = 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide; ATP = adenosine triphosphate.

withdrawn because of idiosyncratic toxicity. Idiosyncratic toxicity is a term used to describe adverse effects in the human population that meet the following criteria: 1) toxicity is observed only in a small patient population, 2) effects do not appear to be dose- or time-dependent, 3) the target of toxicity is different from the pharmacological target and 4) effects are not reproducible in animals. The ability to assign a risk factor or probability that a new drug candidate could be associated with idiosyncratic toxicity would be extremely valu-

able. CeeTox has evaluated several drugs, including troglitazone, grepafloxacin, serzone and cerivastatin (CEV), that have been withdrawn from the market. The idea was to screen these drugs using the *in vitro* system described in order to identify biochemical changes that were similar among the drugs withdrawn.

For example, CEV targets HMG-CoA and is used in the treatment of high cholesterol. Cerivastatin is one of several HMG-CoA drugs known collectively as statins. Liver toxicity is associated with these drugs, but severe skeletal muscle toxicity (rhabdomyolysis) resulted in the recall of CEV from the market in 2001. Pravastatin appears to have a lower incidence of liver toxicity and has not been associated with skeletal muscle toxicity. Both drugs are administered as the pharmacologically-active hydroxyl acid forms (9). Studies by Shitara (10, 11) indicate that 80% of CEV uptake into the hepatocyte is dependent on active transport mechanisms, and both drugs are substrates of the uptake transporter OATP-C. Cerivastatin is considerably more lipid soluble than pravastatin and therefore passive diffusion also could be involved with CEV uptake.

The *in vitro* toxicity profiles indicated a modest reduction in cell proliferation and

ATP levels. The lack of a dose-related reduction at high exposures suggests a saturable uptake mechanism that is consistent with the known mechanism of uptake for both CEV and pravastatin. The response profiles for the acute toxicity markers would indicate that CEV (Figure 7A) and pravastatin (Figure 7B) are safe drugs but that pravastatin has a cleaner response profile. CEV reduced cell proliferation, and it has been suggested that this drug has cytostatic properties (12, 13). However, an evaluation of the markers associated with chronic or indirect toxicity (glutathione depletion and apoptosis) showed a significant loss in total reduced glutathione levels and an increase in apoptosis, as indicated by caspase 3 activation (Figure 7A, middle and

bottom panels). In comparison, the effects produced by more hydrophilic pravastatin essentially were undetectable in the test system (Figure 7B). The lower toxicity associated with pravastatin could be due to reduced cellular uptake via passive diffusion; however, the test cells do not possess OAT transporters for uptake. Information in the published medical literature suggests that the mechanisms associated with CEV toxicity are linked to energy depletion and apoptosis (14, 15, 16); these findings are consistent with ours. Moreover, the reduced mitochondrial function, loss of total glutathione and apoptosis represent a pattern of change that has been observed to varying degrees with other drugs associated with idiosyncratic toxicity. The number and combination of endpoints affected and the shape of the dose-response curves provide a novel means of assigning risk to new drug candidates, as well as those currently on the market.

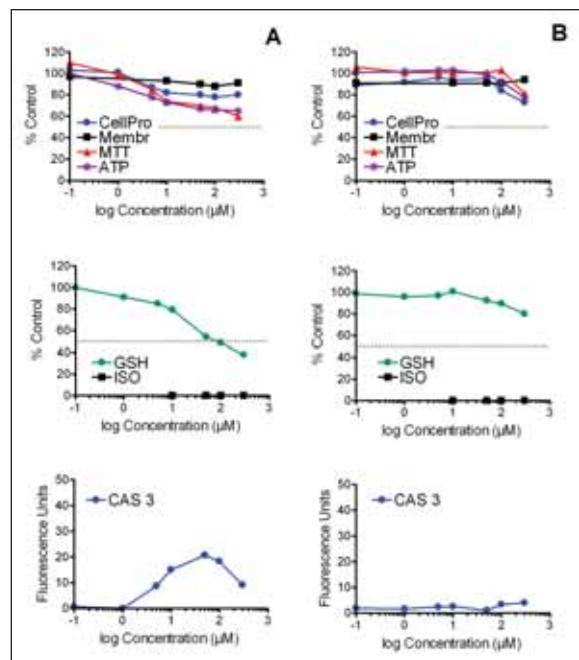
Many potential mechanisms exist for idiosyncratic effects. As the database of known idiosyncratic compounds is expanded, the consistent occurrence of biochemical changes produced by members of a drug class that have been shown to be toxic (but not by the compounds that are considered safe) increases the significance of the biochemical event, which in turn increases the predictive power.

## Summary

Many new technologies are available for the evaluation and identification of toxicity early in drug development. However, the endgame is biochemical function. If a compound impairs or disrupts key processes of cell homeostasis, it has a high probability of producing the same event *in vivo*. The key to predictive *in vitro* toxicity screening is a well-defined test system, multi-endpoint analysis combined with detailed dose-response, *in vivo* validation, a comparative database and a consistent means to analyze the data (17, 18). It is clear that a basic biochemical approach to assessing the toxicity of new chemical entities greatly improves the data sets used to make decisions during the design and development of a new drug candidate.

## References

1. UBS Warburg Report, Charles River Laboratories. February 28, 2003, pp. 7–8.
2. J. Gilbert, P. Henske and A. Singh, *The Busi-*



**Figure 7.** Toxicity profiles for CEV and pravastatin. CeeTox has identified changes in cell proliferation, glutathione levels and caspase 3 activation as markers that several drugs classified as producing idiosyncratic toxicity have in common. GSH = reduced glutathione; ISO = 8-isoprostane; CAS = caspase 3.

## In Vitro Toxicity Testing

- ness & Medicine Report 21(1), 73–83 (2003).
- N. Weil, *Bio-IT World* September, pp. 11 (2004).
  - E. Razvi, *Drug & Market Development*, July 2003, pp. 1–8. Accessed at [www.bioportfolio.com/reports/DMD\\_ADME.htm](http://www.bioportfolio.com/reports/DMD_ADME.htm).
  - C. Davie, J. Pierre-Valentin, C. Pollard et al., *J. Cardiovasc. Electrophysiol.* 11, 1302–1309 (2004).
  - D.K. Walker, *Br. J. Clin. Pharmacol.* 58, 601–608 (2004).
  - A. Joshi, T. Dimino, Y. Vohra et al., *J. Electrocardiol.* 37(suppl), 7–14 (2004).
  - RxMed medical advisory board. Accessed at <http://www.RxMed.com>.
  - P. Thomayant, R. Subramanian, X. Fang et al., *Drug Metab. Disp.* 30, 505–512 (2002).
  - Y. Shitara, M. Hirano, Y. Adachi et al., *Drug Metab. Disp.* 32, 1468–1475 (2004).
  - Y. Shitara H. Sato and Y. Sugiyama, *Annu. Rev. Pharmacol. Toxicol.* Oct 12, 44 (2004).
  - Y. Ito, M. Kawasaki, H. Yokoyama et al., *Circ. J.* 68, 784–790 (2004).
  - M. Igarashi, H. Yamaguchi, A. Hirata et al., *J. Cardiovasc. Pharmacol.* 40, 277–287 (2002).
  - T.E. Johnson, X. Zhang, K.B. Bleicher et al., *Toxicol. Appl. Pharmacol.* 200, 237–250 (2004).
  - T. Kubota, K. Fujisaki, Y. Itoh et al., *Biochem. Pharmacol.* 67, 2175–2186
  - S. Kaneta, K. Satoh, S. Kano et al., *Atherosclerosis* 170, 237–243 (2003).
  - G.L. Cockerell, J.M. McKim and S.L. Vonderfecht, *Toxicol. Pathol.* 30, 4–7 (2002).
  - T. Koppai, *Drug Discovery*, April, 47–50 (2004).

**James M. McKim, Jr., Paul C. Wilga, Jeffrey F. Pregoner and Diane K. Petrella** are all with CeeTox Inc., in Kalamazoo, Michigan, USA. James M. McKim, Jr., can be reached at Tel. 269-372-3351; e-mail [james.mckim@ceetox.com](mailto:james.mckim@ceetox.com). **PD**

© Reprinted from PHARMACEUTICAL DISCOVERY, January 2005 AN ADVANSTAR PUBLICATION Printed in U.S.A.

Copyright Notice Copyright by Advanstar Communications Inc. Advanstar Communications Inc. retains all rights to this article. This article may only be viewed or printed (1) for personal use. User may not actively save any text or graphics/photos to local hard drives or duplicate this article in whole or in part, in any medium. Advanstar Communications Inc. home page is located at <http://www.advanstar.com>.



**CeeTox, Inc.**  
4717 Campus Drive, Suite 500  
Kalamazoo, MI 49008  
P: 269.353.5555  
F: 269.544.1077  
[Kristy.sheets@ceetox.com](mailto:Kristy.sheets@ceetox.com)