Cerep

CYTOTOXICITY

IN VITRO ESSENTIAL CELL FUNCTION TESTING

Drug-induced toxicity is one of the major causes of failure during drug development and the major reason for removal of approved drugs from the market. Animal toxicity testing is an essential part of drug safety testing, but it is impractical in the early stages of drug discovery. Classic *in vitro* cytotoxicity assays are not very predictive of *in vivo* toxicity because they measure non-specific and late occurring cytototoxic events.

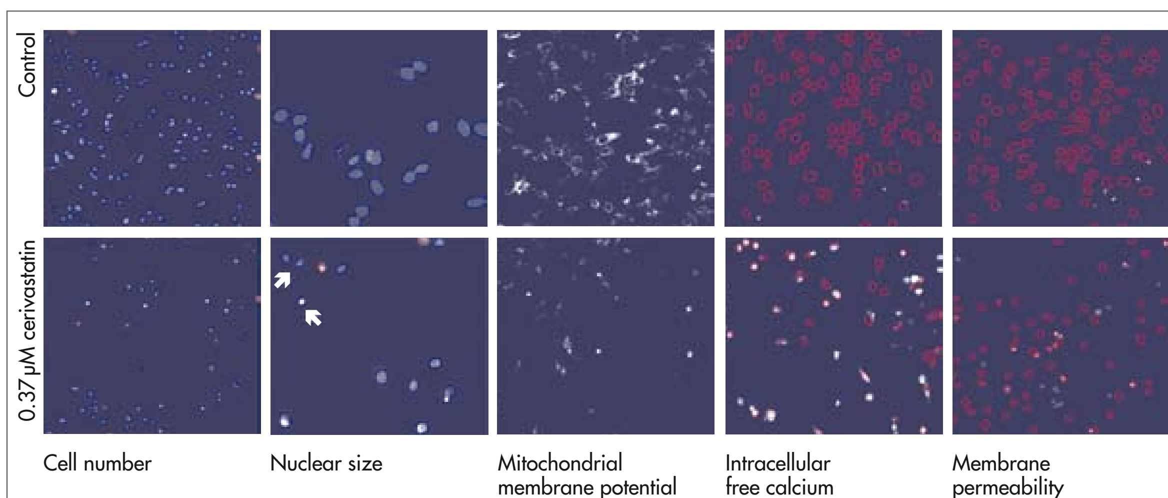
To fill this void in *in vitro* cytotoxicity testing, we offer a high content assay which measures sensitive cellular parameters such as mitochondrial membrane potential and intracellular free calcium, together with more classic parameters such as membrane permeability and cell proliferation, in live HepG2 cells. These end-points show a better correlation with clinical toxicity events than more classic cytotoxicity end-points such as alamarBlue reduction.

THE *IN VITRO* CYTOTOXICITY PANEL

The *in vitro* cytotoxicity panel consists of five end-points which are measured simultaneously in individual cells. The capacity to measure these end-points in the same cell can provide information about the mechanism of toxicity. The following table details the parameters measured and the biological basis of each.

End-point	Dye	Principle
Nuclear size	Hoechst	Nuclear shrinkage as a result of chromatin condensation is one of the hallmarks of apoptotic cell death.
Mitochondrial membrane potential	TMRM	The electrochemical gradient across the mitochondrial membrane is an indicator of the respiratory capacity of the cell and therefore of cellular energetics.
Intracellular calcium	Fluo-4	Uncontrolled increase in cytoplasmic calcium is a common pathophysiological event in cellular toxicity.
Membrane permeability	TOTO-3	Disruption of the cytoplasmic membrane is a common and late event in cellular toxicity.
Cell number	Hoechst	Decreased cell numbers are indicative of cell death and/or decreased cell proliferation, which is a very sensitive marker of toxicity.

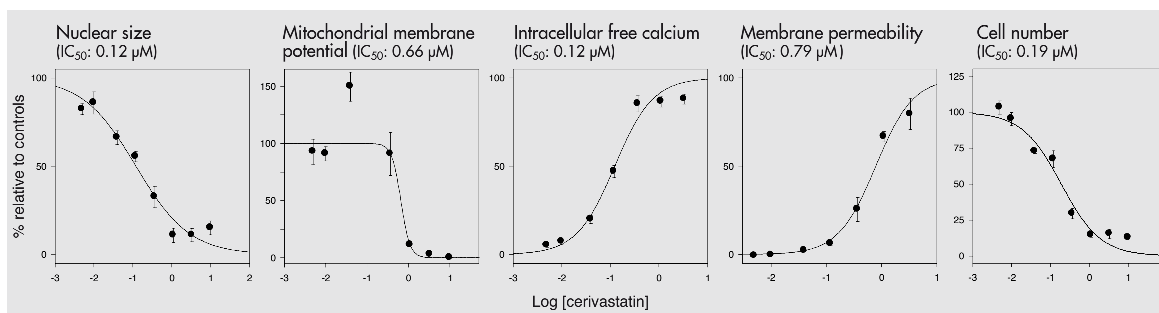
The *in vitro* cytotoxicity panel measures five-end points, three of which decrease in the presence of a cytotoxic agent (cell number, nuclear size, and mitochondrial membrane potential) and two parameters increase in the presence of a cytotoxic agent (intracellular free calcium and membrane permeability). Next page are representative graphs of these five parameters. HepG2 cells were treated with cerivastatin for 72 hours.



This figure shows images corresponding to five of the end-points in cells treated with control (1% DMSO) and 0.37µM cerivastatin.

Note the changes in shape and fluorescence in control vs. treated cells: the numbers of cell decreases upon treatment with cerivastatin, nuclei of cells treated with cerivastatin shrink (white arrows), and the mitochondrial membrane potential decreases with cerivastatin. Conversely, cerivastatin increases the release of intracellular calcium and the movement of the TOTO-3 dye through damaged cell membranes. Note the increased fluorescence in cells treated with cerivastatin in the intracellular calcium and membrane permeability parameters.

The fluorescence signals were measured using quantitative image analysis. An example of the dose-response curves and IC_{50} obtained for cerivastatin is shown below ▼



EXPERIMENTAL PROTOCOL

HepG2 cells (passage 1-15) are plated in 96-well poly D-lysine-coated plates at 3,000 cells/well in MEM alpha growth medium 16-24 h prior to the start of the assay. One hour prior to the addition of test compounds, the cells are equilibrated with assay medium containing 1% fetal bovine serum. Test compounds are added as a 5x solution and the cells are incubated for 72 h. At the end of the incubation period, the cells are loaded for one hour with a dye cocktail containing Hoechst, TMRM, Fluo-4 and TOTO-3.

Plates are scanned with an automated fluorescent microscope. Image-analysis software is used to quantitate surface area (nuclear size) and fluorescence intensity (rest of the end-points) in defined cellular areas.

Raw data are normalized and expressed as % of effect relative to the untreated controls, and IC_{50} curves are generated when applicable. The data are also normalized using the reference compound (cerivastatin), whose maximum effect at any concentration will be considered the 100% effect, and will define the top or bottom of the IC_{50} curves.

The default test concentrations are 1, 30, and 100 µM. The recommended dose-range is 0.05-100 µM, but other concentrations can be accommodated for IC_{50} determinations. Cerivastatin is used as a positive control, since it shows maximal responses for all 5 parameters evaluated.

- SMALL AMOUNT OF COMPOUND REQUIRED
  The *in vitro* cytotoxicity panel requires ~1-3 mg of compound to test at a top concentration of 100 µM (assuming a MW of approximately 500).
- RAPID TURNAROUND TIME
  Results are delivered within two weeks upon receipt of the compounds at the testing site of Cerep. Data are made available on line as soon as they are validated.

IC_{50}s (µM) of various reference compounds for the 5 measured end-points in the assay panel ▼

Drug	Nuclear size	Mitochondrial membrane potential	Intracellular calcium	Membrane potential	Cell numbers	Alamar Blue	Drug safety
cerivastatin	0.30	0.40	0.30	0.70	0.70	>100.00	Withdrawn 2001
amiodarone	2.70	3.76	4.03	2.95	7.57	9.10	Liver 1
astemizole	3.84	2.76	4.26	3.66	6.89	5.80	Withdrawn 1999
tamoxifen	5.36	2.74	10.80	6.16	6.95	87.00	Liver 2
chlorpromazine	10.80	10.80	9.52	10.80	7.91	16.00	Liver 2
cyclosporin A	30.80	4.63	13.40	31.10	29.20	43.00	Liver 2
ketoconazole	59.80	NC	79.30	11.60	24.50	>100.00	Liver 1
labetalol	75.60	33.40	83.90	35.80	NC	>100.00	Liver 1
imipramine	47.10	NC	91.40	36.30	78.80	89.00	Liver 2
danazol	40.40	22.40	>100.00	27.60	>100.00	9.20	Liver 1
flurazolidone	NC	31.30	>100.00	>100.00	48.80	39.00	Liver 1
flutamide	89.00	64.50	>100.00	>100.00	71.00	44.00	Liver 1
flufenamic acid	83.40	NC	>100.00	>100.00	75.00	>100.00	LML
betaine HCl	>100.00	>100.00	>100.00	>100.00	>100.00	85.00	LML
isoproterenol	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	LML
praziquantel	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	LML
primidone	>100.00	>100.00	>100.00	>100.00	>100.00	>100.00	LML

NC — *IC_{50} Not Calculable*
Liver 1 — *Severe hepatotoxic liability (>1% clinical incidence of elevated liver enzymes plus 2 of the following: jaundice, documented reports of 3+ cases of acute liver failure/fulminant hepatitis, BW/BBW for hepatotoxicity).*
Liver 2 — *Variable market liability for hepatotoxicity (0.1-1% clinical incidence of elevated liver enzymes plus 2 of the following: jaundice, anecdotal repots of severe hepatitis, precaution/adverse effect listed in PDR.*
LML — *Low market liability.*

Even when drugs might have the same *in vitro* cytotoxic potency, their toxicity can be more clearly reflected in a particular cell function end-point. A good way to rank compounds is to use the most sensitive end-point or the end-point with the lowest IC_{50} value. Some drugs can show a more marked effect (or lack of effect) on a particular parameter. For example, results for cyclosporin A show that mitochondrial membrane potential is 3 times more sensitive than any other parameter; for tamoxifen, calcium is a relatively insensitive parameter; for labetalol, membrane potential and permeability are more sensitive than nuclear size and calcium.

The *in vitro* cytotoxicity panel also provides a more sensitive method than classical cytotoxicity assays (like alamar Blue reduction) to detect drugs that might have low margins of safety.

FAQS

. Why is 72 hours the chosen timepoint?
Early timepoints such as 24 or 48 h may not fully assess the cytotoxic potential of a test compound. Some compounds may require a longer incubation period (72 h) to trigger physiological changes in the cells.

. How are the data processed?
The raw data for the compound-treated cells are normalized to the negative control (1% DMSO) and cerivastatin-treated cells.

. What does % effect mean for each of the different parameters?
. Cell number: % reduction (a negative value may indicate cell proliferation relative to the control cells)
. Intracellular calcium: % increase
. Nuclear size: % reduction
. Membrane permeability: % increase
. Mitochondrial membrane potential: % reduction (negative values may indicate mitochondrial hyperpolarization, P-gp inhibition, or cell shrinkage).

. How is cytotoxic defined?
A cytotoxic compound will decrease the cell number, nuclear size, and mitochondrial membrane potential. Concurrently, a cytotoxic compound will increase intracellular calcium and membrane permeability. A general guideline for defining a compound as cytotoxic is: 40% effect for cell number, nuclear size and mitochondrial membrane potential and a 20% effect for intracellular free calcium and membrane permeability.

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QUESTIONS OR CONCERNS?

Please contact us: sales@cerep.com

FRANCE
Le Bois l'Evêque
86600 CELLE L'EVESCAULT
tel. +33 (0)5 49 89 30 00

(Headquarters)
155 boulevard Haussmann
75008 PARIS
tel. +33 (0)1 45 64 44 60

USA
15318 N.E. 95th Street
REDMOND, WA 98052
tel. +1 (425) 895 8666

JAPAN
Namiki Shoji Co., Ltd.
Kenseishinjuku Bldg. 5-5-3
Shinjuku, Shinjuku-ku
TOKYO, 160-0022
tel. +81 (0)3 3354 4026
fax +81 (0)3 3352 2196

CHINA
Ai Di Sheng (Edison) Road 326,
302-1 room
Zhangjiang Hich-Tech Park
SHANGHAI
tel. +86 18702160370

sales@cerep.com
www.cerep.com