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WuXi AppTec

WuXi Biology

Identify potential liability issues in early stages of drug discovery

# *In Vitro* Toxicity Services

www.wuxibiology.com

# Introduction to *In Vitro* Toxicity

Drug-induced toxicity is a primary cause of failure for drug candidates during preclinical and clinical testing and a significant reason for approved drugs being removed from the market. Initial toxicity tests, which largely rely on animal studies, are essential during the nonclinical phase of development. Despite the valuable safety data these animal models provide, they are often costly, low-throughput, and can lack precise predictability of human biology and pathophysiology due to species differences.

Given that most drug-induced toxicity occurs at the cellular level, *in vitro* models are becoming favored alternatives for estimating clinical responses. These models help decrease reliance on *in vivo* animal testing and increase throughput. The 2007 NRC report, "Toxicity Testing in the 21st Century (TT21C): A Vision and a Strategy[1] ", supports this shift, forecasting significant improvements in toxicity testing that will be more specific and predictive of human toxicity. *In vitro* toxicity testing studies are quicker, simpler, and more scalable, making them ideal for early-stage drug discovery to predict potential risks. This approach is not only cost-effective but also ethically superior as it significantly reduces the use of animals.

In response to the growing need for early-stage *in vitro* toxicity testing, WuXi Biology provides a suite of toxicity assays at the cellular level. Using advanced technologies like conventional and automated patch-clamp and high content screening (HCS), these assays can be applied to your lead ID and optimization strategy. The result is a comprehensive analysis of toxicity severity and specificity, guiding candidate compounds through the planning and execution of downstream *in vivo* tests.

# Table of Contents

# Cardiotoxicity

Drug-induced arrhythmia, particularly Torsade de Pointe arrhythmia (TdP), accounted for approximately 21.4% of drug withdrawals from markets between 1990 and 2012. For the past decade, cardiac safety screening studies have been performed following ICH S7B and ICH E14 guidelines. The ICH S7B guideline recommends an *in vitro* IKr (hERG) assay and an in vivo ECG assay to identify the potential for delayed repolarization (QT interval prolongation). However, while hERG is the chief channel associated with TdP risk, hERG screening alone does not reliably detect all potential cardiac adverse side effects. This over-simplified and hypersensitive approach can lead to unnecessary attrition of novel drug candidates due to false-positive results.

Recently, a new method to examine cardiotoxicity, the Comprehensive *in Vitro* Proarrhythmia Assay (CiPA), has been proposed to modify the current ICH S7B/E14 guidelines. In the preclinical stage, the core assays of CiPA include: 1. the assessment of drug candidate effects on multiple human ventricular ionic channels, 2. in silico reconstruction of human heart ventricular action potential to predict the proarrhythmic risk; and 3. the confirmation study using human stem-cell derived cardiomyocytes[2,3].

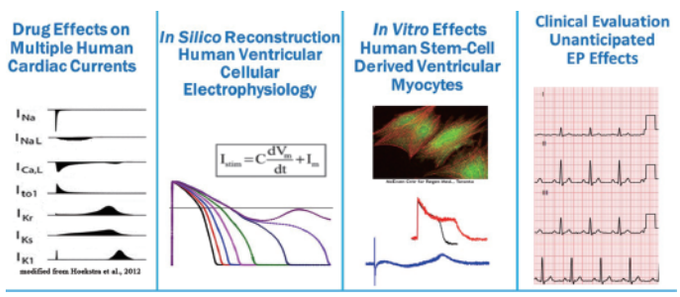



Figure 1. Comprehensive *in Vitro* Proarrhythmia (CiPA) Assay: Four components . https://cipaproject.org

| Ion Channel | Species | Assay Platform |
|---|---|---|
| hERG ($I_{Kr}$) | Human | Automated or Manual Patch Clamp |
| hCav1.2 ($I_{Ca}$, L-type) | Human | Automated or Manual Patch Clamp |
| hNav1.5 ($I_{Na}$ - peak current) | Human | Automated or Manual Patch Clamp |
| hNav1.5 ($I_{Na}$ - late current) | Human | Automated or Manual Patch Clamp |
| hKv4.3/hKChip2.2 ($I_{TO}$) | Human | Automated or Manual Patch Clamp |
| hKCNQ1/hmink ($I_{Ks}$) | Human | Automated or Manual Patch Clamp |
| hKir2.1 ($I_{K1}$) | Human | Automated or Manual Patch Clamp |

Table 1. Cardiac ion channel panel (CIPA recommended[3]).

WuXi AppTec provides a combination of assay platforms that include manual and automated patch-clamp and microelectrode array (MEA) to access drug candidate effects on multiple cardiac ion channels (Table 1) as well as stem cell-derived ventricular cardiomyocytes as CiPA recommended.

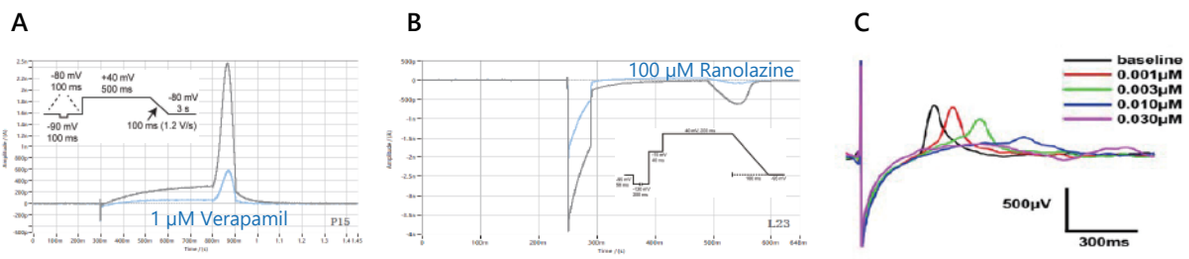



Figure 2. A and B: hERG and hNav1.5 current recorded with automated patch-clamp (Syncropatch) using CiPA recommended voltage protocols (inserted in the figures), respectively. 1 μM verapamil inhibited hERG current (A), while 100 μM ranolazine inhibited hNav1.5 late current (B). C: hiPSC-vCMs were used in the MEA system and the effects of dofetilide were recorded.

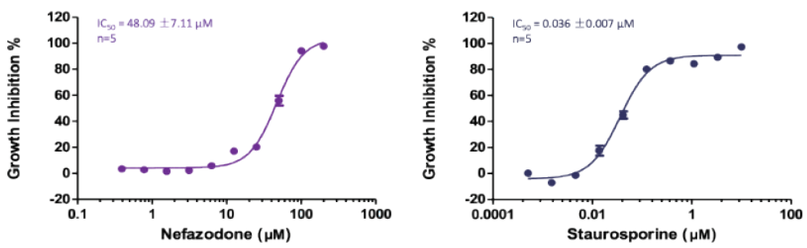
WuXi Biology collaborates with a third-party vendor for human-induced pluripotent stem cell-derived ventricle cardiomyocytes (hiPSC-vCMs). The purity of the ventricular cardiomyocytes is over 90%, which is the highest number seen in the literature. The action potential, main ion channel properties and development process of the hiPSC-vCMs have all been fully validated using the manual patch clamp system at WuXi AppTec[4,5]. WuXi Biology now offers a CiPA confirmatory electrophysiological test[3] using MEA (Maestro Pro, Axion) to analyze the effects of compounds on hiPSC-vCMs.

In addition, to satisfy the mechanistic study or high throughput study needs, WuXi AppTec Biology also provides traditional radioligand binding and fluorescence signal detection assays. The service for some other cardiac targets such as hKv1.5 (Ikur) and hCav3.2 (ICa, T) is also available.

# General Cytotoxicity

Cytotoxicity testing, required by the FDA and/or CFDA for IND submission, is typically conducted during the nonclinical discovery phase. It's widely accepted that *in vitro* cytotoxicity testing methods should precede animal dosing for acute oral systemic toxicity evaluation. Early identification of potential cytotoxicity can significantly conserve time and resources by filtering out potentially toxic compounds before crucial animal studies. Moreover, cell-based assays are easily scalable for medium to high throughput screening, aiding in making go/no-go decisions.

## • Cell Viability: ATP Production Measured in Active Cells



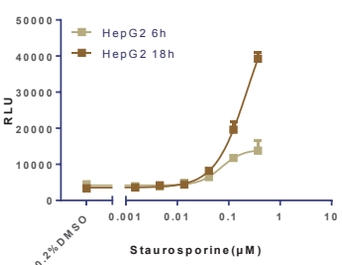

(A) In a 384-well HepG2 cell viability assay, a 24-hour treatment of Nefazodone demonstrated a concentration-dependent reduction in cell viability (n=5).

(B) Staurosporine exhibited a concentration-dependent decrease in HepG2 cell viability after a 72-hour treatment in 384-well plates (n=5).

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) is a homogeneous method for determining viable cell counts based on ATP quantitation, serving as an indicator of metabolically active cells. WuXi Biology has fully validated this assay across a broad spectrum of cell types, in both 96- and 384-well formats, utilizing EnVision (Perkin Elmer) as the luminescent signal reader.

## • Apoptosis: Caspase Assay

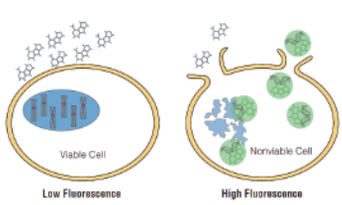
The activation of the caspase cascade is a crucial event in the apoptotic pathway. WuXi Biology employs the Caspase-Glo® 3/7 Assay kit (Promega) to measure the activities of caspase-3 and caspase-7. The assay, fully validated on HepG2 Cells using EnVision as the luminescence signal reader, is available in both 96- and 384-well formats.



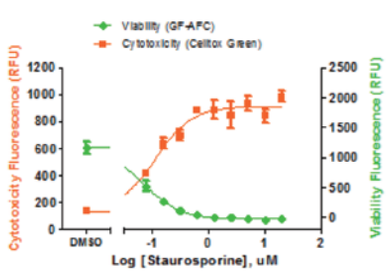

Staurosporine has been observed to enhance caspase activity. In a 96-well plate assay, staurosporine increased caspase activities in HepG2 cells in a concentration-dependent manner. The EC50 values were 0.07 μM and 0.23 μM for 6-hour and 18-hour staurosporine treatments, respectively.

## • CellTox™ Green Cytotoxicity Assay

The CellTox™ Green Cytotoxicity Assay (Promega) detects changes in membrane integrity that occur due to cell death. It employs a proprietary asymmetric cyanine dye that is excluded from viable cells but preferentially stains the DNA of dead cells. There is no significant increase in fluorescence in viable cells. Thus, the fluorescent signal generated by dye binding to dead-cell DNA is proportional to cytotoxicity. This assay, available in both 96- and 384-well formats for various cell types, uses Envision as the signal detector. It can be paired with the CellTiter-Fluor™ Cell Viability Assay or the CellTiter-Glo® Luminescent Cell Viability Assay.




CellTox™ Green Dye binds DNA of cells with impaired membrane integrity.




LN-18 cells were treated with staurosporine at specified concentrations in a 384-well assay plate for 24 hours. After applying the CellTox™ Green Reagent, fluorescence indicative of cytotoxicity was measured. Then the CellTiter-Fluor Reagent was applied and the fluorescence signal was measured. These measurements yielded similar EC50 values.

# Mitochondrial Toxicity

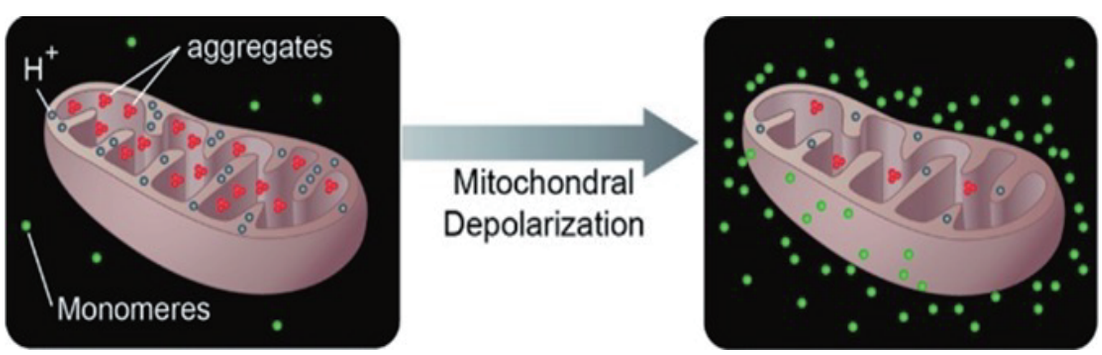
Mitochondria play a pivotal role in cellular energy (ATP) production and maintaining homeostasis. Mitochondrial dysfunction is increasingly implicated as a major contributor to drug-induced toxicity, leading to the discontinuation of prominent drugs, including troglitazone, cerivastatin, and nefazodone. In addition to post-market drug withdrawals, mitochondrial liabilities have also been associated with many drugs carrying a black box label for hepatic and cardiac toxicity.

WuXi AppTec offers a set of *in vitro* assays to access mitochondrial toxicity from different endpoints

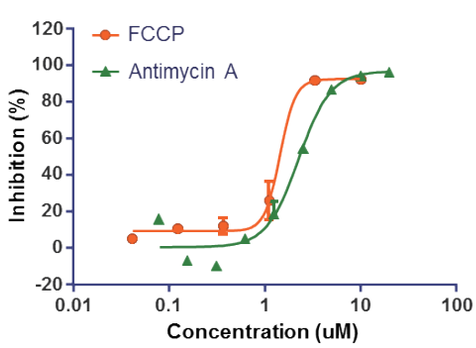
## • Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential (MMP) is tightly interlinked to many mitochondrial processes so it is a key indicator of mitochondrial function and cell health. The dissipation of MMP is considered an early indicator of apoptosis.

WuXi Biology offers a plate-based HCS assay to detect the MMP, using the Acumen Cellista (TTP Labtech) with MITO-ID® Membrane Potential Cytotoxicity Kit (ENZO Life Sciences). The assay is available in both 96- or 384-well format, and in a wide range of cells.




The MITO-ID® Membrane Potential Cytotoxicity Kit utilizes a cationic dual-emission dye that exists as green fluorescent monomers in the cytosol, and accumulates as orange fluorescent aggregates in the mitochondria. Cells exhibit a shift from orange to green fluorescence as mitochondrial function becomes increasingly compromised.



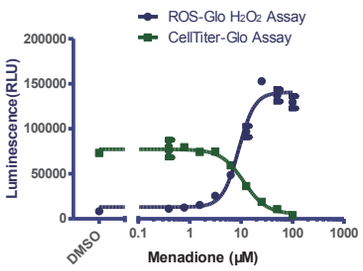

| Compound | $IC_{50}$ (μM) | Literature (μM)[6] |
|---|---|---|
| FCCP | 1.12 ± 0.55 | 1.31 |
| Antimycin A | 2.56 ± 0.63 | 4.51 |

After an hour of treatment, two reference compounds, FCCP and Antimycin A, demonstrated MMP inhibition in a 384-well assay plate using HepG2 cells (n=4). The IC50 values are consistent with those reported in the literature.

## • Mitochondrial Reactive Oxygen Species (ROS) Assay

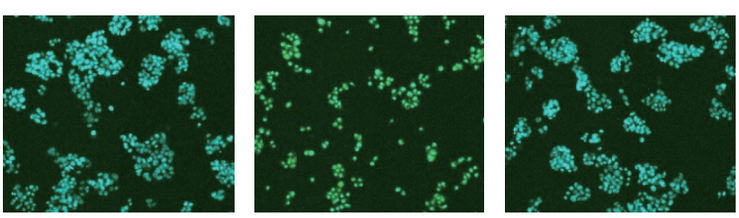
Mitochondrial dysfunction typically leads to elevated free radical production. The primary source of free radical generation is the mitochondrial respiratory chain. Inhibition of this process is often associated with increased levels of reactive oxygen species (ROS).

**$H_2O_2$ Assay:** Among the various ROS generated in cell culture, $H_2O_2$ is convenient to assay due to its long half-life in cultured cells. A change in $H_2O_2$ can indicate a general shift in ROS levels. WuXi Biology has validated a plate-based assay using either the ROS-Glo™ $H_2O_2$ assay kit (Promega) or the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) to detect $H_2O_2$ levels. The assay is offered in both 96- and 384-well formats.



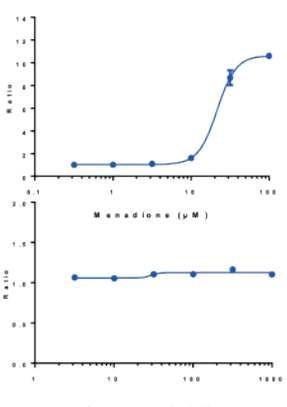

In this representative example, the ROS-Glo™ $H_2O_2$ assay and the CellTiter-Glo® Luminescent Cell Viability assay were performed simultaneously on HepG2 cells in a 384-well format. The cells were treated with the ROS-generating compound menadione and the $H_2O_2$ substrate. Following a two-hour incubation at 37°C, half the volume of the supernatant was used for ROS-Glo™ $H_2O_2$ detection, while the remaining cells were lysed for CellTiter-Glo® detection. The luminescence signal from both assays was measured using EnVision. Menadione demonstrated an EC50 of 8.96 μM in the $H_2O_2$ assay and an IC50 of 11.28 μM in the viability assay.

**CellROX® Green Assay:** The CellROX Green Reagent (Life Technologies) is a fluorogenic probe used for measuring oxidative stress in live cells. This cell-permeable dye exhibits weak or no fluorescence in a reduced state, but upon oxidation by reactive oxygen species (ROS), it displays bright green photostable fluorescence. As a DNA dye, it binds to DNA upon oxidation, localizing its signal primarily in the nucleus and mitochondria.



DMSO Control | 100 μM Menadione | 1000 μM Streptomycin

HepG2 cells were treated with test articles in a 96-well plate for 24 hours. The cells were stained with CellROX Green Reagent to detect ROS and Hoechst 33342 to stain the cell nucleus. Fluorescence images were captured with CQ1. The CellROX Green Reagent and Hoechst 33342 emit green and blue fluorescence, respectively. Menadione was tested as a positive control and Streptomycin was tested as a negative control.



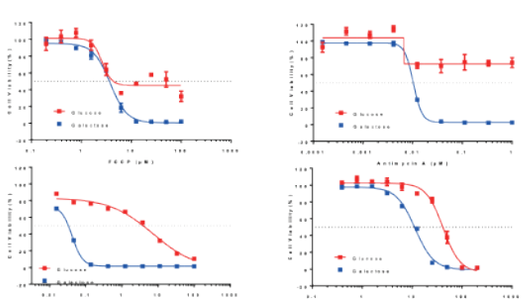

## • Glucose/Galactose Assay

Many highly proliferative cells produce almost all ATP through glycolysis, despite the presence of abundant O2 and fully functional mitochondria, a phenomenon known as the Crabtree effect7. Oxidation of galactose to pyruvate through glycolysis yields no net ATP, thus forcing cells to rely on mitochondrial oxidative phosphorylation (OXPHOS) to generate sufficient ATP for survival. Therefore, substituting glucose with galactose in the cell media increases the cells' dependence on mitochondrial oxidative phosphorylation, thereby augmenting their susceptibility to mitochondrial damage. By comparing the differential toxic effects on cells grown in glucose and galactose, it's possible to differentiate between mitochondrial toxicity and non-specific cytotoxicity.

We utilize HepG2 cells cultured in either glucose (25 mM) or galactose (10 mM), with cytotoxicity assessed using CellTitre-Glo™ (Promega). A mitochondrial toxicant is indicated by a change in IC50 value greater than three-fold in the glucose media compared to the galactose media.

The data illustrates the impact of a 24-hour treatment with the mitochondrial uncoupler, FCCP, and the electron transport inhibitors antimycin, rotenone, and nefazodone.

For instance, nefazodone exhibits significantly higher toxicity to cells grown in galactose media, aligning with data suggesting that it strongly inhibits respiration by impairing complex I of the mitochondrial electron transport system (ETS).

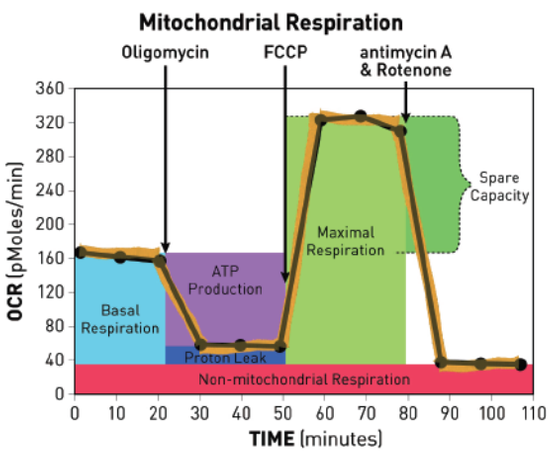


## • Seahorse Cellular Mitochondrial Stress Assay

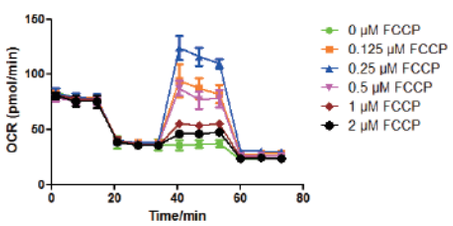
The Agilent Seahorse XF Cell Mito Stress Test quantifies cellular metabolism by measuring the rate of change in oxygen concentration and pH, enabling the calculation of the Oxygen Consumption Rate (OCR, indicative of mitochondrial respiration) and the Extracellular Acidification Rate (ECAR, indicative of glycolysis).

Sequential compound injections allow for the measurement of basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration.

**XFe96 Analyzer**



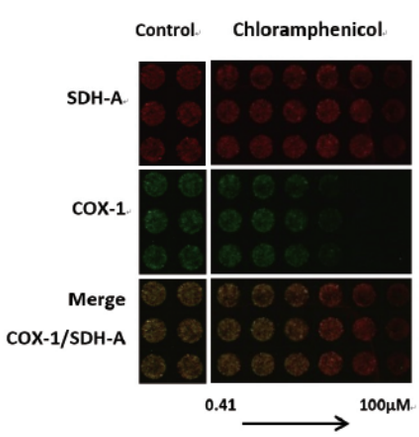

| Compound(s) | Electron Transport Chain (ETC) Target | Effect on OCR |
|---|---|---|
| Oligomycin | ATP synthase (complex V) | Decrease |
| FCCP | Inner mitochondrial membrane | Increase |
| Rotenone/antimycin | Complex I and III (respectively) | Decrease |

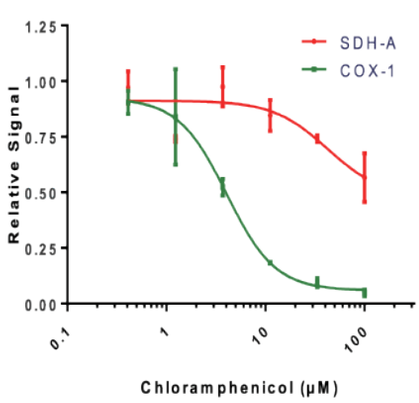


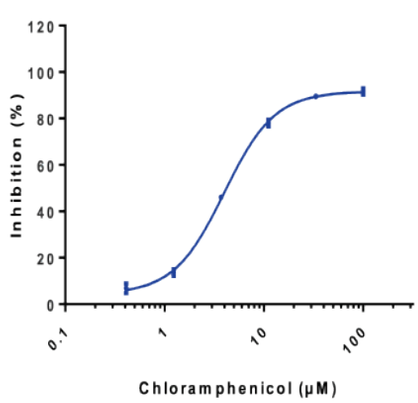

## • MitoBiogenesis Assay

Determining the level of mitochondrial biogenesis relative to cellular protein synthesis provides key insights into potential mitochondrial toxicity. This is particularly crucial for the development of new antiviral and antibiotic drugs due to the similarity between mitochondrial biogenesis and bacterial/viral replication. Numerous such drugs can induce severe mitochondrial toxicity.

Our mitobiogenesis assay utilizes Odyssey (LI-COR) with the MitoBiogenesis™ In-Cell ELISA Kit (IR) (Abcam). The assay has been validated on HepG2 cells and is available in both 96- and 384-well formats.




The assay concurrently measures the levels of two mitochondrial proteins: the mitochondrial DNA-encoded COXI and the nuclear DNA-encoded SDH-A. Therefore, the specific inhibition of mitochondrial DNA-encoded protein synthesis by chloramphenicol can be easily detected.

Chloramphenicol inhibits mitochondrial biogenesis. (A) HepG2 cells were seeded at 1200 cells/well in a 384-well plate. Chloramphenicol inhibits COX-I protein synthesis relative to SDH-A protein synthesis. (B) The overall mitochondrial biogenesis inhibition was calculated from the ratio of measured COX-I to SDH-A protein levels.

# Lipotoxicity

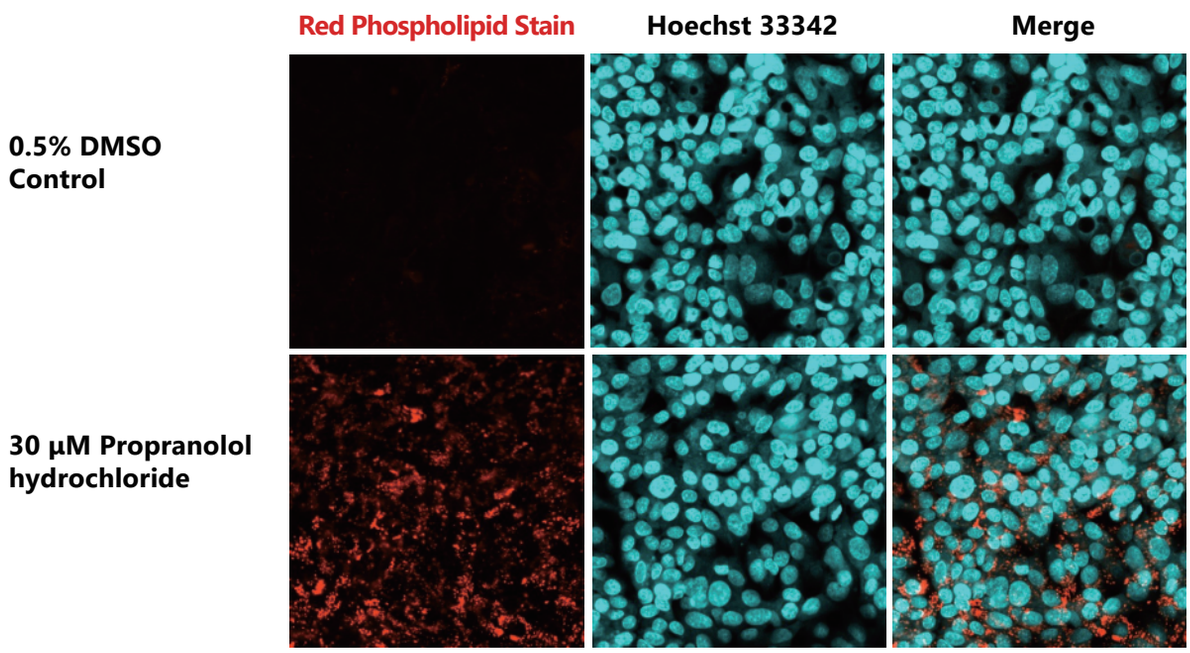
Phospholipidosis is a lysosomal storage disorder characterized by the accumulation of excessive phospholipid complexes within lysosomal membranes. Certain cationic amphiphilic drugs (CADs), including antibiotics, antidepressants, antihistamines, and other prescription drugs, can induce phospholipidosis, a condition recognized by the US FDA as an adverse drug reaction[8] .

Steatosis refers to the cytoplasmic accumulation of neutral lipids. Some drugs can interfere with hepatic lipid processing, leading to the accumulation of triglycerides within liver cells, potentially causing harmful liver inflammation or steatohepatitis.

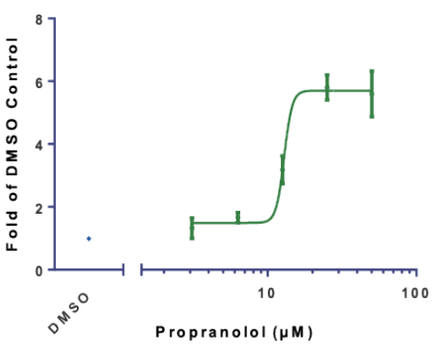
While both drug-induced phospholipidosis and steatosis are often reversible and not significantly harmful, prolonged exposure to a particular drug can lead to long-term toxic effects. Hence, it's crucial to evaluate drug-induced cellular lipotoxicity leading to phospholipidosis and/or steatosis during early drug discovery to minimize potential risks.

WuXi AppTec now offers *in vitro* high-content screening (HCS) assays on HepG2 cells using HCS LipidTOX™ Stains (Thermo Fisher Scientific), with either the CQ1 (confocal quantitative image cytometer, Yokogawa Electric Corporation) or Acumen Cellista (TTP Labtech) as the image reader. Phospholipidosis is detected with the LipidTOX™ Red phospholipid stain, while steatosis is detected with the LipidTOX™ Green neutral lipid stain.

## • Phospholipidosis Assay

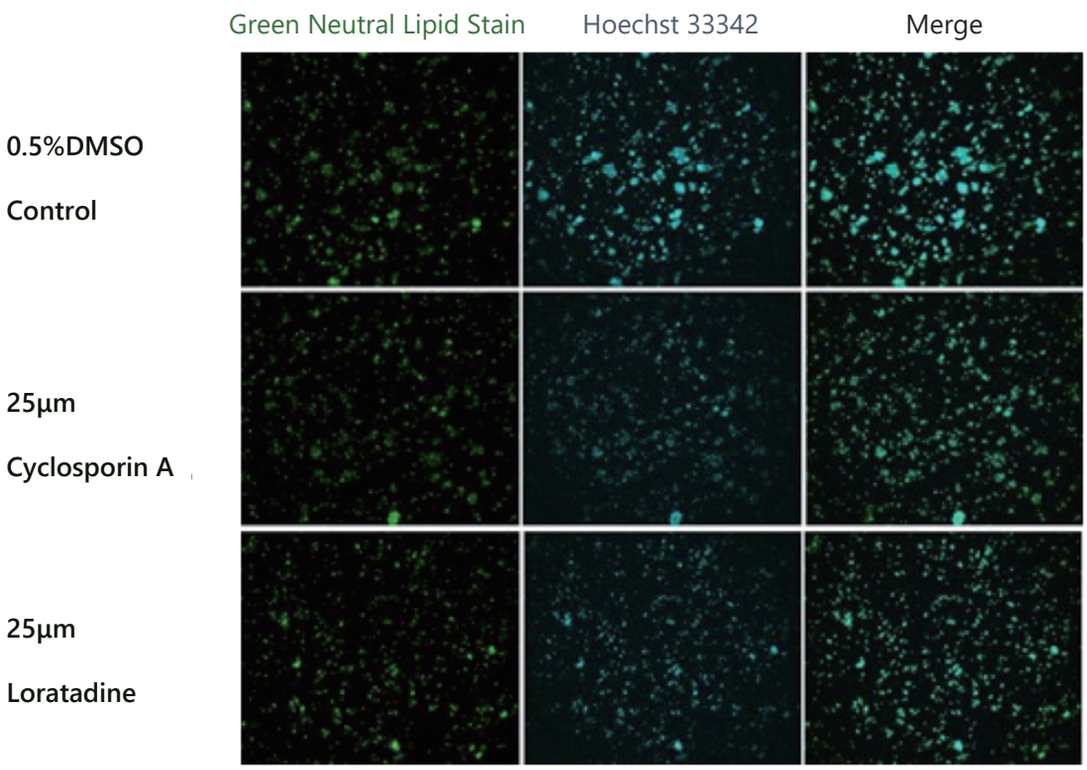



HepG2 cells were exposed to test articles and LipidTox reagent in a 96-well plate for 48 hours. Hoechst 33342 nuclear staining was used as a control. Fluorescence images were captured with CQ1. LipidTox red phospholipidosis and Hoechst 33342 emit red and blue fluorescence in the same field, respectively.

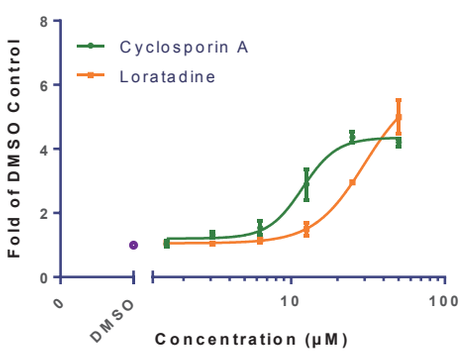



Results for red fluorescence were normalized to those of blue fluorescence (Hoechst). Propranolol concentration-dependently increased phospholipid staining.

- **Steatosis Assay**




HepG2 cells were exposed to the test articles in a 96-well plate for 48 hours. Fixed cells were stained with LipidTOX™ Green neutral lipid stain and the Hoechst 33342. Fluorescence images were captured with CQ1. The Green Neutral Lipid Stain and Hoechst 33342 emit green and blue fluorescence in the same field, respectively.




Results for green fluorescence (LipidTox Green Neutral Lipid Stain) were normalized to those of blue fluorescence (Hoechst). Both Cyclosporin A and Loratadine concentration-dependently increased the Green Neutral Lipid Stain, suggesting the accumulation of steatosis.

# Hepatotoxicity

The liver plays a crucial role in transforming and clearing chemicals, making it vulnerable to toxicity from these agents. Drug-induced liver injury (DILI) can be caused by hundreds of widely prescribed drugs and is a leading cause of drug development and registration failure, withdrawal of approved drugs, and cautionary labeling that restricts drug usage.

Traditionally, the evaluation of compound-induced hepatotoxicity has relied on *in vivo* studies; however, these are limited to a small number of late-stage compounds and their predictive value is not sufficiently high. A recent survey demonstrated that animal models provided low positive predictive values for human liver toxicity, with rates of only 33% (rats), 27% (dogs), and 50% (monkeys)[9].

Given that the most severe DILI is due to hepatocellular injury, *in vitro* hepatotoxicity testing could provide valuable information to predict potential risks.

WuXi Biology provides multiplexed *in vitro* assays to evaluate potential hepatotoxicity at the cellular level. These assays utilize HepG2 (human liver hepatocellular carcinoma) cells or human primary hepatocytes. When these cells are used as *in vitro* models, all assays listed in the General Cytotoxicity, Mitochondrial Toxicity, and Lipotoxicity sections of this brochure can be employed to assess potential hepatotoxicity. The combination of these assays will yield valuable information from various endpoints to analyze the mechanism and severity of hepatotoxicity.

## 2D Culture Hepatotoxicity Testing Using HepG2 Cells or Human Primary Hepatocytes

Recommended HepG2 hepatotoxicity first tier assays:

- Cell Viability Assay: a very sensitive marker to detect general toxicity;
- Mitochondrial Membrane Potential Assay: an indicator of poor respiratory capacity and cell health;
- Mitochondrial Reactive Oxygen Species (ROS) assay: ROS increase may result in significant cell structure damage and cause oxidative stress;
- Caspase assay: measuring Caspase-3/7 activities.

Assay Features and Advantages:

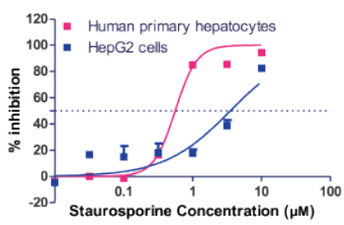
- Plate-based assay (96-or 384-well plate) read by plate reader or high-content image reader;
- Medium to high throughput with high sensitivity;
- Low cost with a short turnaround time;
- Short-term (24h) and long-term (10~14d) toxicity assays available.

IC50 values of several reference compounds in the HepG2 cell viability assay.

| | Cell Viability Assay (HepG2 cells) | | | | |
|---|---|---|---|---|---|
| Compound | Nefazodone | Rotenone | Staurosporine | Staurosporine | Tamoxifen |
| Treatment | 24h | 24h | 24h | 72h | 72h |
| $IC_{50}$ (μM) | 48.09±7.11 | 4.49±1.05 | 5.96 ± 2.32 | 0.036 ± 0.007 | 10.55±0.59 |
| n | n=5 | n=5 | n=5 | n=5 | n=5 |

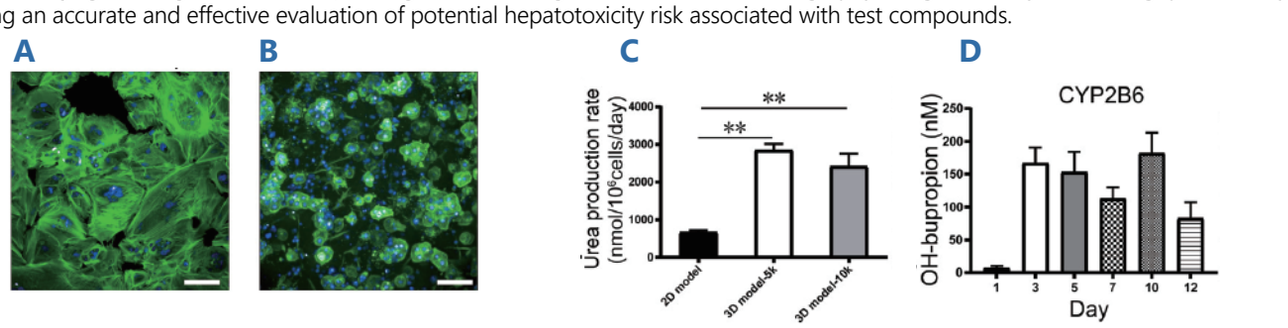
The effects of chlorpromazine and staurosporine on the viability of Human Primary Hepatocytes.

| | Cell Viability Assay | |
|---|---|---|
| | Human Primary Hepatocytes | |
| Compound | Chlorpromazine | Staurosporine |
| Treatment | 24h | 24h |
| $IC_{50}$ (μM) | 32.53 | 0.56 |



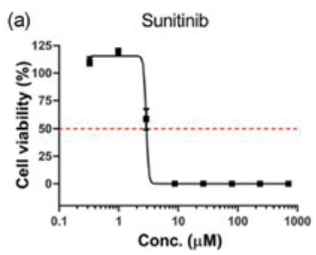

## 3D Culture Hepatotoxicity Testing Using Human Primary Hepatocytes

An *in vitro* 3D human primary liver cell model has been developed using human primary hepatocytes and an integrated biomimetic array chip (iBAC, Beijing Daxiang Biotech, China) for organoid culturing. This 3D model offers high physiological fidelity and throughput, thereby providing an accurate and effective evaluation of potential hepatotoxicity risk associated with test compounds.

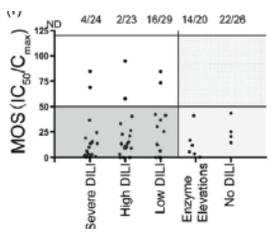



The 2D model of monolayer human primary hepatocytes appeared well-spread and flat (A), while the 3D model formed small spheroids (B). The 3D model demonstrated superior liver function, as evidenced by urea production, compared to the 2D model (C). CYP450 enzymatic activities were maintained for an extended period in the 3D model (D). These data suggest that the 3D model of primary human hepatocytes can more accurately mimic the physiological functions of liver cells *in vitro*.

In a study evaluating 122 FDA-approved clinical drugs with a known clinical toxicity classification, the *in vitro* 3D human primary liver cell model demonstrated a high positive predictive value (up to 75.7%)[10].




The concentration response curve of a representative drug (sunitinib).




Margin of safety (MOS, ratio of the IC50 and Cmax)

| DILI drugs | Non-DILI drugs | TP | TN | FP | FN | Threshold | Sensitivity | Specificity | Predictivity |
|---|---|---|---|---|---|---|---|---|---|
| 76 | 46 | 24 | 44 | 2 | 52 | 10x | 31.6% | 95.6% | 63.6% |
| 76 | 46 | 39 | 40 | 6 | 37 | 25x | 51.3% | 87.0% | 69.1% |
| 76 | 46 | 48 | 37 | 9 | 28 | 50x | 63.2% | 80.4% | 71.8% |
| 76 | 46 | 54 | 37 | 9 | 22 | 100x | 71.1% | 80.4% | 75.7% |

Abbreviations: FN, false negative; FP, false positive; iBAC, integrated biomimetic array chip; PHH, primary human hepatocyte; TN, true negative; TP, true positive.

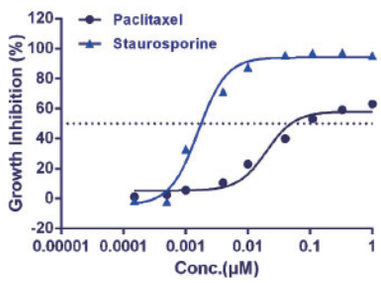
Performance of the 3D model for predicting hepatotoxicity

# Nephrotoxicity

The kidney's primary function is to filter and excrete soluble waste while retaining essential biochemicals. Its function as a selective filter makes it particularly susceptible to toxic injury. Renal tubular cells, especially proximal tubule cells, are highly vulnerable to drug toxicity due to their significant role in the filtration process, which exposes them to high levels of circulating toxins. Consequently, our assays use human-derived cellular systems from the renal proximal tubule epithelial cell line (HK-2) to maximize nephrotoxicity predictive power. HK-2 cells, derived from normal adult human kidneys and immortalized by transduction of the E6/E7 genes via HPV-6, are used.

WuXi AppTec has developed several nephrotoxicity assays for high-throughput screening, offering early assessment of nephrotoxic effects and the potential for kidney-specific cellular injury. Typically, all assays listed in the General Cytotoxicity and Mitochondrial Toxicity sections above, including the Cell Viability Assay, Apoptosis assay, etc., can be used to assess nephrotoxicity.

Validation data for the CellTiter-Glo viability assay on HK-2 cells is presented. The assay was performed with nine different concentrations in triplicate, and the concentration response curve of paclitaxel is shown. Two reference compounds, paclitaxel and staurosporine, were tested, yielding IC50 values of 20 nM and 16 nM, respectively.




# Hematotoxicity

The bone marrow and blood cells are common targets of toxicity from drugs. Hematotoxicity can result from direct cytotoxicity to the various blood cell types or from targeting of sensitive blood-forming stem cells/progenitor cells in the bone marrow. *In vitro* models for investigating the hematotoxicity potential of new test compounds are critical in early preclinical drug development. Hematopoietic stem/progenitor cells express the cluster of differentiation marker CD34.

WuXi Biology has developed a cell-based assay to Evaluate Hematotoxicity using Bone-marrow-derived Stem/progenitor cells Differentiating into hematopoietic lineage Lineages, as described by Mahalingaiah et al (2018)[11]. The 96-well or 384-well CTG-3D assay (Promega) uses the CD34+ human bone marrow cells, or rSca1+ and c-Kit+ mouse bone marrow cells.

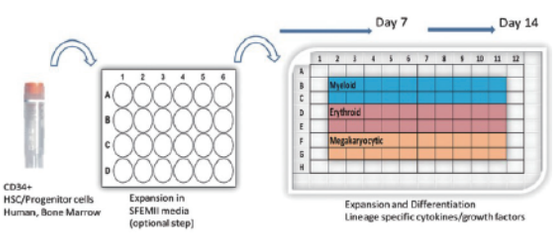
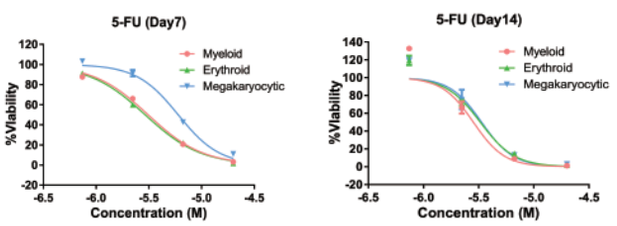



Plate map/design for seeding CD34+ cells for growth and differentiation into myeloid, erythroid, and megakaryocytic lineages.

The IC50 obtained is consistent to the literature[11].




| IC50 (μM) | Megakaryocyte | Myeloid | Erythroid |
|---|---|---|---|
| Day 7 | 6.00 | 3.11 | 2.92 |
| Day 14 | 2.83 | 3.36 | 3.27 |

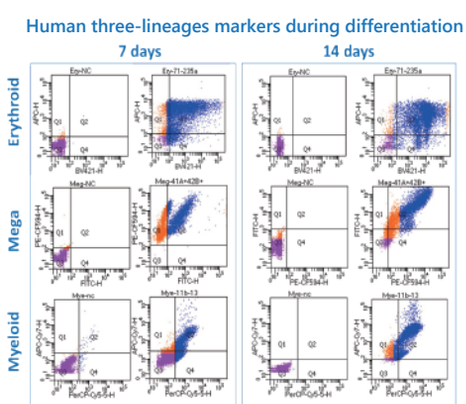



Figure legend: Percentage of differentiation markers during specific-lineage differentiation process.

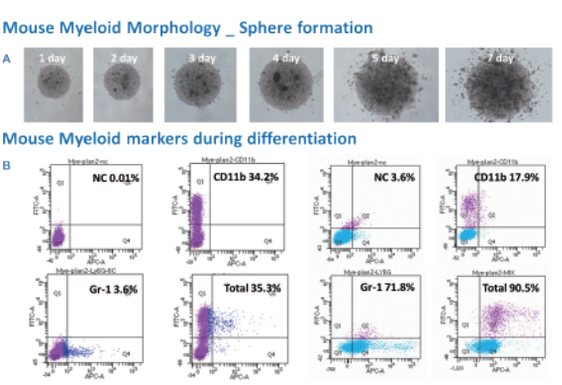



Figure legend: A. Sphere formation during mouse myeloid expansion. B. Percentage of differentiation markers during mouse myeloid differentiation process.

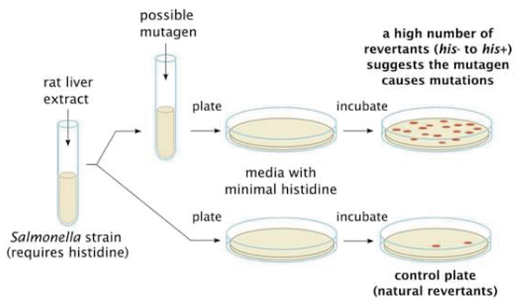
# Genotoxicity

When DNA is exposed to specific chemicals, mutations and other types of damage can occur, potentially leading to cancer and/or teratogenic effects. Given the severity of these effects, it is crucial to examine whether new or existing chemicals intended for human use have any impact on DNA. This genotoxic potential forms an integral part of the basic toxicological information package used in the decision-making and risk assessment process of drug development. As no single test can detect all relevant genotoxic endpoints, regulatory agencies recommend a battery of tests for genotoxicity.

## • Bacteria Reverse Mutation Assay (Ames Assay)

The Ames assay uses amino-acid-requiring strains of Salmonella typhimurium and Escherichia coli to detect point mutations, which involve the substitution, addition, or deletion of one or a few DNA base pairs. The principle of the assay is that it identifies mutations that revert the existing mutations in the test strains, thereby restoring the bacteria's functional ability to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent test strain.

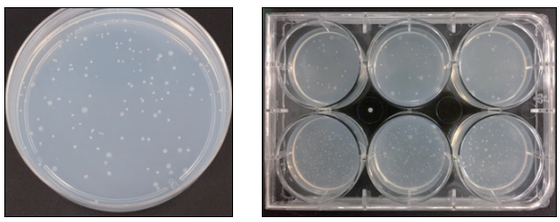
WuXi Biology offers the Ames Assay following the OECD (471) guidance[12]. The five strains of bacteria are used in the assay: TA98, TA100, TA1535 and TA1537, and *E. coli WP2 uvrA (pKM101).*




General Procedure of the Ames Assay

## • Mini Ames Assay

The mini Ames assay is a modification of the standard Ames test that uses 6-well plates and 20% of the typical Ames assay medium. The goal is to create a rapid screening test that uses small quantities of the test compound while still providing results consistent with the standard Ames assay. The mini Ames assay is widely used as an early compound screen during lead optimization or preclinical candidate selection.



A Petri dish used in a standard Ames (left) and the 6-well plate used in the mini Ames (right).

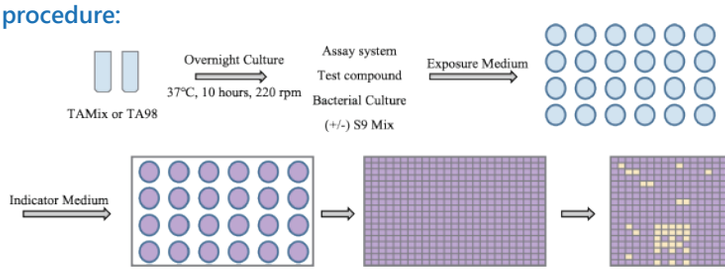
| | TA98 | | TA100 | |
|---|---|---|---|---|
| | +S9 | -S9 | +S9 | -S9 |
| DMSO | 6 | 13 | 36 | 26 |
| 2-Aminoanthracene (2 µg/well) | 172 | | 268 | |
| 2-Nitroflurene (4 µg/well) | | 224 | | |
| MNNG (1 µg/well) | | | | 671 |
| Fold Response | 28.7 | 17.2 | 7.4 | 25.8 |

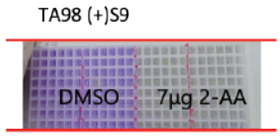
Relative mutagenic potential of reference compounds as detected by Salmonella typhimurium TA98 and TA100. The numbers in the table are means in three wells.

## • Ames II Assay

The Ames II assay is a microplate format of the Ames test, utilizing TAMix (a combination of Salmonella typhimurium strains TA7001, TA7002, TA7003, TA7004, TA7005, and TA7006) and TA98. Due to standardized kits, quality-controlled bacterial strains, the micro-well format, and colorimetric readout, this assay allows for the automation of substantial parts of the process, thereby requiring considerably less test article and allowing for high-throughput. WuXi Biology has validated the assay using the assay principle and kit from Xenometrix[13] .

### Assay procedure:



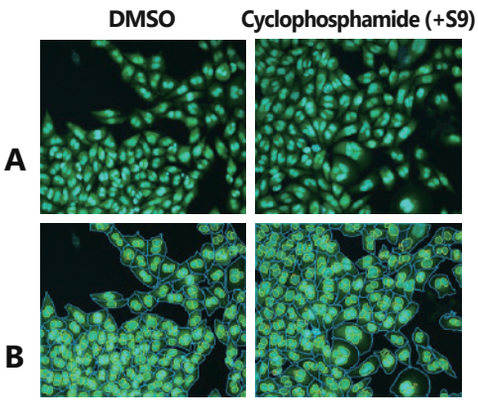




For a given dose, a revertant response greater than two folds from baseline level was classified as positive.

| Strain | Treatment | Positive Control | Concentration (µg/mL) | Revertant folds |
|---|---|---|---|---|
| TA98 | (+) S9 | 2-Aminoanthracene | 7 µg/mL | 19 |
| | (-) S9 | 4-nitroquinoline-N-oxide | 3 µg/mL | 27 |
| TAMix | (+) S9 | 2-Aminoanthracene | 7 µg/mL | 6 |
| | (-) S9 | 4-nitroquinoline-N-oxide | 3 µg/mL | 27 |

## • HCS *In Vitro* Micronucleus Assay

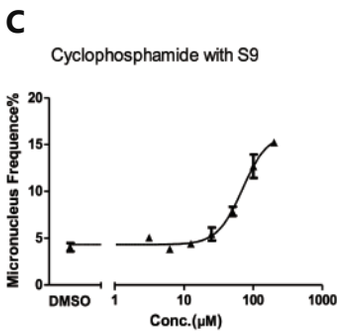
Micronucleus formation is a characteristic sign of genetic toxicity, and the micronucleus assay serves as a genotoxic effects screening tool. The *in vitro* micronucleus assay is part of the recommended regulatory testing battery for genotoxicity. This assay identifies clastogenic and aneugenic compounds (i.e., compounds causing chromosome breakages or the loss of an entire chromosome) by visualizing and quantifying micronuclei in the cytoplasm. WuXi Biology offers a plate-based high-content screening (HCS) *in vitro* micronucleus assay in line with OECD (487) guideline[14]. The assay uses an automated fluorescence microscopy platform, CQ1, in combination with automated pattern recognition and analysis software to detect micronucleus formation in CHO-K1 cells.




Representative images showing the effects of Cyclophosphamide (+S9) on the micronucleus formation in CHO-K1 cells.

A: original fluorescence images captured with CQ1. The cells were stained with calcein-AM (green) and Hoechst 33342 (blue).

B: the analyzed image of the same field in the CQ1 analysis software.




| Compounds | Data Source | Maximum % of Micronucleated cells | Maximum % of Cytotoxicity |
|---|---|---|---|
| Bleomycin Sulfate | Literature | 30 | 51.2 |
| | WuXi AppTec | 31.0 | 64.3 |
| Cyclophosphamide | Literature | 27 | 46.7 |
| | WuXi AppTec | 16.9 | 54.2 |

C: Cyclophosphamide (+S9) concentration - dependently increased micronucleus frequency.

D: The comparison of WuXi AppTec data and literature data15.

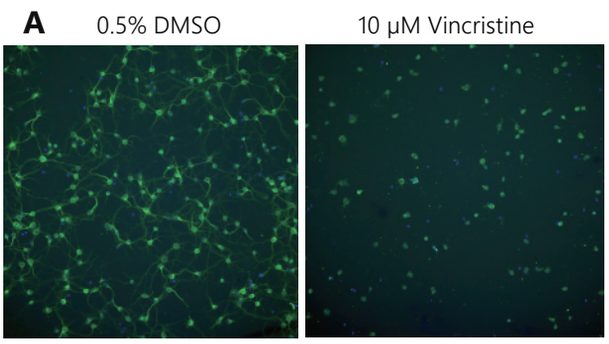
# Neurotoxicity

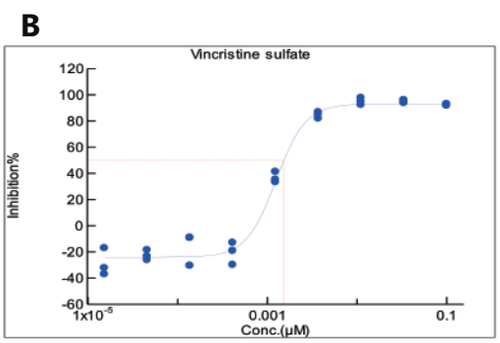
The central nervous system (CNS) is frequently considered the primary target organ of systemic toxicity. Due to the often elusive nature of neurotoxicity, many project closures related to neurotoxic issues occur during the clinical phase. In vitro models can provide rapid toxicological screening of chemicals for their potential to induce neurotoxicity. Cell cultures derived from nervous system tissue have proven to be powerful tools for understanding cellular and molecular mechanisms of nervous system development and function, and have been utilized to decipher the mechanism of action of neurotoxic chemicals.

## • Neurite Outgrowth Assay

Neurite outgrowth refers to the extension of axonal projections from developing neuronal cell bodies, facilitated by cytoskeletal reorganization. This process is crucial for normal neuronal development and allows neurons to arrange themselves into intricate functional networks connected synaptically. Given its complex regulation, neurite outgrowth offers numerous potential targets for chemical perturbation. The neurite outgrowth assay is particularly useful for hazard identification and screening of chemical-induced changes, especially when the action sites are unknown.

WuXi Biology has created a high-content analysis assay utilizing their HCS Platform across various cellular models, such as NGF-stimulated PC12 cells, primarily cultured fetal rat neurons, and iPSC-derived neurons. This cell-based screening assay serves as an effective tool for evaluating potential neurotoxicity.




A: The provided images depict the impact of vincristine on the neurite outgrowth of primarily cultured rat hippocampal neurons. The cells were stained with beta III tubulin (green) and Hoechst 33342 (blue), with images captured via the CX7 platform.

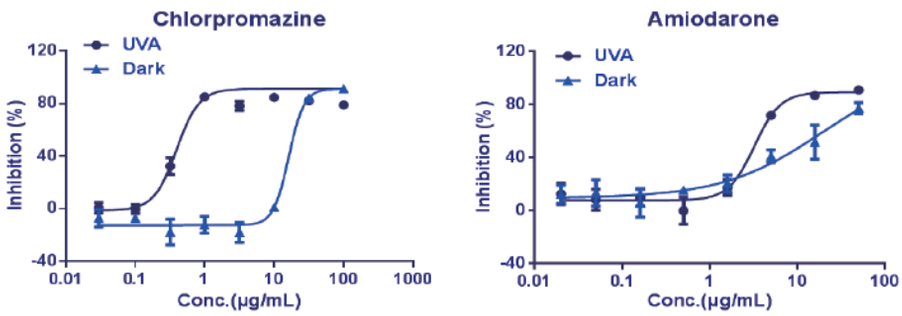
B: Vincristine was observed to decrease neurite length in a concentration-dependent manner.

# Phototoxicity

Phototoxicity refers to a toxic response elicited or amplified by light exposure after a substance is applied to the body, or it can be triggered by skin irradiation following systemic administration of a substance. Regulatory guidelines for phototoxicity are covered under the ICH S10 guideline [16].

## • *In Vitro* 3T3 NRU Phototoxicity Test

The *in vitro* 3T3 NRU phototoxicity test is utilized to identify the phototoxic potential of a test substance, which is induced by the excited chemical after exposure to light. The test measures photo-cytotoxicity by the relative reduction in cell viability when exposed to the chemical in the presence of light versus its absence. WuXi Biology Group has fully validated the test using the permanent mouse fibroblast cell line, Balb/c 3T3, following the protocol outlined in the OECD 432 guideline, *In Vitro* 3T3 NRU Phototoxicity Test17. The light source used was SOL 500 (Dr. Hönle AG), and data was collected using the SpectraMax2 (Molecular Devices) plate reader.



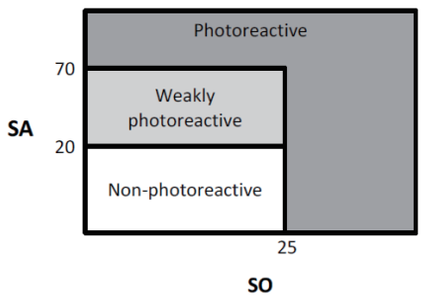

| Test article | $IC_{50}$ Dark | $IC_{50}$ UVA | PIF | Phototoxicity |
|---|---|---|---|---|
| | (μg/mL) | (μg/mL) | | PIF |
| Chlorpromazine | 13.21± 4.29 | 0.35±0.07 | 38.2 | + |
| Amiodarone hydrochloride | 17.30±2.61 | 2.79±0.59 | 6.2 | + |

The results for two phototoxic compounds, chlorpromazine and amiodarone hydrochloride, are presented. For result evaluation, the Photo-Irritation-Factor (PIF) was calculated using the following formula: PIF = $IC_{50}$(-UV)/$IC_{50}$(+UV). In this study, the PIF values of the two compounds align with the reference data in the OECD (432) guideline. A test material is considered to pose a potential phototoxic hazard if the PIF value is 5 or greater.

## • Reactive Oxygen Species (ROS) Assay for Photoreactivity

The Reactive Oxygen Species (ROS) assay is an efficient, high-throughput system utilized for anticipating the phototoxic potential of pharmaceutical substances. Apart from light absorption and distribution to light-exposed tissue, the generation of reactive species from chemicals post-absorption of UV-visible light is identified as a crucial factor in causing direct phototoxic reactions.

This assay is a multi-well plate-based study conducted using a quartz reaction container. This setup aims to decrease sample volume, enhance assay productivity, and produce high-uniform irradiation. The test has been fully validated by the WuXi Biology Group, adhering to the protocol outlined in the OECD 495 guideline, Reactive Oxygen Species (ROS) Assay for Photoreactivity18. SOL 500 (Dr. Hönle AG) was employed as the light source, while the SpectraMax2 (Molecular Devices) plate reader was used for data collection.




Criteria for judgment17

| Compound name | Solvent | Working concentration (μM) | Test Compounds | | Comment |
|---|---|---|---|---|---|
| | | | Singlet oxygen (SO) (mean) | Superoxide anion (SA) (mean) | |
| Quinine hydrochloride | DMSO | 200 | 255.13 | 242.17 | Photoreactive |
| Sulisobenzone | DMSO | 200 | -5.23 | -12.27 | Non-photoreactive |
| Benzocaine | DMSO | 200 | -5.30 | -9.07 | Non-photoreactive |
| Erythromycin | DMSO | 200 | -3.33 | 10.83 | Non-photoreactive |
| Acridine | DMSO | 200 | 147.67 | 241.17 | Photoreactive |
| Diclofenac | DMSO | 200 | 101.70 | 132.57 | Photoreactive |
| Ketoprofen | DMSO | 200 | 143.53 | 88.47 | Photoreactive |

The expected ROS prediction for proficiency chemicals

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18. OECD Guideline for the Testing of Chemicals 495: Reactive Oxygen Species (ROS) Assay for Photoreactivity (2019).

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