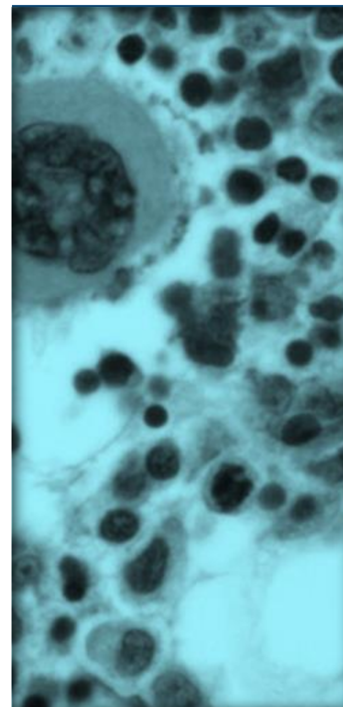
# *In vitro* Toxicology
# Cytotoxicity and Mechanism

## Introduction

ImQuest BioSciences offers a panel of *in vitro* assays to evaluate the cytotoxicity and mechanism of cytotoxicity of potential pharmaceutical products. *In vitro* cytotoxicity assays are used to predict the toxicity of compounds as well as to identify potential safety and development problems prior to *in vivo* safety and toxicology evaluations. Together with information on the drug candidate's efficacy and pharmaceutical properties, the results may be used to select lead compounds that will be given preference for progression to animal studies and IND-directed development. In addition, *in vitro* toxicity assays provide preliminary information about the mechanisms of toxicity likely to be observed during *in vivo* studies.

To assess the effects of test compounds on cell viability, cell proliferation and macromolecule synthesis, ImQuest's cytotoxicity evaluations are performed with a number of human immune system cell types and cell types representative of the major functional organ systems. Examples of these cell types include: peripheral blood mononuclear cells (PBMCs), monocyte-macrophages, dendritic cells, bone marrow progenitor cells, primary hepatocytes, induced pluripotent stem cell (iPS) cardiomyocytes, iPS neurons and renal proximal tubule cells (RPTEC).

Mechanism of cytotoxicity assays are used to evaluate the effect of test compounds on the cell cycle, mitochondrial respiration and function, apoptosis, membrane integrity and oxidative stress. These assays are used to analyze the predominant areas where a test compound may exert an adverse effect. Additional assays may be used to explore effects on cellular kinases, growth factors and signal transduction pathways.



Technological advances have increased the effectiveness of *in vitro* toxicology analysis:

- Pluripotent stem cells that can be directed to differentiate into various cell types
- New techniques to immortalize cells, such as telomerase overexpression
- Wide availability of primary cells
- 3D cell culture constructs
- Omic technologies that provide a global view of cellular activity

## Materials and Methods

***In vitro* Cytotoxicity Assays:** All cultures (Table 1) were incubated at 37°C and 5% $CO_2$ for 3 days with test compound in triplicate with the exception of bone marrow progenitor cells which were incubated up to 14 days.

Chemiluminescence, following CellTiter Glo staining, was measured using the Wallac 1450 Microbeta Trilux liquid scintillation counter to evaluate cytotoxicity in all cell types. Cytotoxicity in bone marrow cells was determined by the number of colony forming units (CFU) (> 30 cells).

**Table 1: Primary Human Cell Panel For *In Vitro* Cytoxicity Assays**

| Human Cell Type | Isolation and Culture |
|---|---|
| PBMCs | PBMCs isolated from whole blood by Ficoll hypaque gradient centrifugation were re-suspended in fresh tissue culture medium containing IL-2 with or without PHA. |
| Monocytes and Macrophages | Monocyte/macrophages were separated from PBMCs by plate adherence. Monolayers were washed to remove residual PBMCs and differentiated in culture for 5-7 days. Monocytes may also be obtained by magnetic bead separation based on cell surface antigen expression. |
| Dendritic cells | Dendritic cells were isolated from PBMCs using plate adherence. Cultures were maintained in the presence of cytokines followed by the addition of LPS (10 ng/mL) for 7 days. |
| Bone Marrow Cells | Bone marrow progenitor cells (Invitrogen) were suspended in supplemented IMDM and methylcellulose to a final concentration of 1%. |
| Hepatocytes | Fresh primary human hepatocytes (BioreclamationIVT) with a 0.2 mg/mL Matrigel overlay were incubated with fresh hepatocyte culture medium. |
| iPS Neurons | iPS neurons (Cellular Dynamics) were seeded in specialized medium (Cellular Dynamics) on plates pre-coated with poly-L-lysine, poly-L-ornithine and laminin. Cytotoxicity evaluations were initiated on day 14. |
| iPS Cardiomyocytes | iPS cardiomyocytes (Cellular Dynamics) were seeded in plating medium on plates coated with gelatin. Cells were cultured with specialize maintenance medium (Cellular Dynamics). |
| RPTEC Kidney Cells | Human primary renal proximal tubule cells (hRPTECs, Lonza) were propagated in supplemented REGM medium. |

## Mechanism of Cytotoxicity Assays

Macromolecular Synthesis Evaluation in PBMCs: PBMCs from pooled donors were cultured at 37°C with 5% $CO_2$ in triplicate in the presence or absence of compound. After incubation for 54 hours, 1 µCi of [methyl-$^3$H]-thymidine for DNA synthesis, [5-$^3$H]-uridine for RNA synthesis, or [3,4,5-$^3$H]-leucine for protein synthesis (New England Nuclear) was added to the culture and incubation was continued for an additional 18 hours. Cells were transferred to DEAE filtermats and washed with distilled water. Incorporated radioactivity was quantified with a Wallac scintillation counter.

Apoptosis: HeLa cells were incubated with compound for three days. Subsequently, apoptosis was quantified fluorometrically using the Apo-ONE Homogeneous Caspase-3/7 kit (Promega). Fluorescence was measured at 485/530 nm.

Membrane integrity: Compound was incubated with L929 cells for three days at 37°C with 5% $CO_2$. Membrane integrity was quantified fluorometrically by LDH release from non-viable cells using the CytoTox-ONE Homogeneous Membrane Integrity kit (Promega); fluorescence was measured at 560/590 nm.

Cellular proliferation: Unstimulated or PHA-stimulated human PBMCs were incubated with compound. After 48 hours, BrdU (Cell Proliferation kit, Millipore) was added to the cells and incubation was continued for an additional 24 hours. Cells were fixed and permeabilized; DNA was denatured with fixative/denaturing solution. Detection of anti-BrdU antibody was measured by absorbance at 450/540 nm.

Oxidative Stress: HeLa cells were incubated with compound. After one hour, oxidative stress was quantified by luminescence measurement utilizing the GSH/GSSG Glo kit (Promega). Total glutathione and oxidized glutathione were measured using a Wallac scintillation counter.

## Results

Evaluation of *In vitro* Cytotoxicity: The cytotoxicity of the test compounds using nine different cell types was evaluated at six concentrations (Table 2).

- $TC_{50}$ values for staurosporine, a potent protein kinase inhibitor, ranged from 0.02 μM in PHA-stimulated PBMCs to 1.6 μM in hepatocytes.
- The toxicity of AZT to bone marrow progenitor cells resulted in a $TC_{50}$ of 1.9 μM. AZT treatment results in neutropenia and anemia when used as a therapeutic agent.
- The $TC_{50}$ calculated for cisplatin in RPTEC cells was 0.003 μM. One of the observed side effects of cisplatin when used as a chemotherapeutic agent is nephrotoxicity.
- Doxazosin mesylate, a known inducer of apoptosis in cardiomyocytes, yielded a $TC_{50}$ of 23.6 μM.

**Table 2: *In vitro* Cytotoxicity Assays**

| Cell Type | Compound | $TC_{50}$ (μM/μg/mL) |
|---|---|---|
| PHA-Stimulated PBMCs | Staurosporine | 0.02 |
| Unstimulated PBMCs | Staurosporine | 0.04 |
| Monocyte/Macrophages | Staurosporine | 0.05 |
| Dendritic | Staurosporine | 0.13 |
| Hepatocytes | Staurosporine | 1.60 |
| iPS Neurons | Staurosporine | 0.78 |
| RPTEC Kidney | Cisplatin | 0.003 |
| Bone Marrow Progenitor | Azidothymidine | 1.90 |
| iPS Cardiomyocytes | Doxazosin | 23.6 |

Evaluation of Mechanisms of Toxicity: The effect of staurosporine on macromolecular synthesis was determined in fresh human PHA-stimulated PBMCs treated for 72 hours with six concentrations of staurosporine. DNA, RNA and protein synthesis was measured using tritiated precursor incorporation assays. Staurosporine affected DNA, RNA and protein synthesis similarly in PHA-stimulated PBMCs with $TC_{50}$ values of 5, 7 and 3 nM, respectively.

Test compounds were evaluated for effects on apoptosis, membrane integrity, cellular proliferation and oxidative stress. The $ED_{50}$ values calculated from these assays are summarized in Table 3.

**Table 3: Mechanism of Toxicity Evaluations**

| Assay Evaluation | Cell Type | Compound | $ED_{50}$ (μM/ng/mL) |
|---|---|---|---|
| Apoptosis | HeLa | Staurosporine | 0.64 |
| Membrane Integrity | L929 | TNF-α | 0.02 |
| Cellular Proliferation | PHA-P stimulated PBMCs | Doxorubicin | 7.54 |
| Oxidative Stress | HeLa | Menadione | 261 |

- Apoptosis was induced in HeLa cells treated with staurosporine, a compound know to induce apoptosis by activation of caspase 3, at concentrations greater than 0.64 µM.
- Tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine linked to programmed necrosis, reduced membrane integrity of L929 cells at concentrations greater than 0.02 ng/mL.
- Doxorubicin, a DNA intercalating agent that inhibits DNA synthesis, reduced cell proliferation of stimulated PBMCs at concentrations greater than 7.54 µM.
- Menadione, a polycyclic aromatic ketone reported to trigger cell death through ROS-mechanisms involving PARP activation, induced oxidative stress at concentrations greater than 261 µM.

## Summary

As a key component of the ImQuest *SUCCESS* platform, ImQuest BioSciences has assembled and validated the Toxi*SENS* panel of *in vitro* and *ex vivo* toxicity and mechanism of toxicity assays to evaluate the toxicity and off-target effects of new therapeutic entities. The results may be used to identify potential safety and development problems before animal safety toxicology studies and to provide a focused rationale for the continued development of a therapeutic or prevention product. Prior to IND-enabling *in vivo* toxicity studies, *in vitro* and *ex vivo* toxicity and mechanism of toxicity assays may be used to prioritize lead compounds, select lead compounds for pilot *in vivo* efficacy studies, or assist in the design of *in vivo* safety studies. The results presented herein illustrate the usefulness of such assays as screening and decision making tools.

## References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Move your drug or biologic development program forward more efficiently and expeditiously with ImQuest's Toxi*SENS* platform. Many aspects of compound toxicity are affected by the pharmaceutical properties and formulation of a product. Thus, Toxi*SENS* evaluations are performed in parallel with our Pharma*SENS* platform studies to assist with the development of a drug delivery strategy that will yield enhanced efficacy and reduced toxicity.

ImQuest BioSciences, a preclinical CRO, specializes in services for the development of drugs, vaccines and biologics for the treatment and prevention of infectious disease, cancer and inflammatory disease.