

# DETECTION OF METABOLIC TOXICITY IN A HOMOGENEOUS CELLULAR ASSAY

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## Abstract

A major concern in the pharmaceutical industry is the potential for metabolic toxicity of developmental compounds. However, existing assays are extremely cumbersome and time-consuming. Here, we present a cell-based, high throughput-screening assay that detects and differentiates between direct and indirect-acting toxins. A B-lymphoblastoid cell line, MCL-5, was created expressing 5 human P450's known to be important in metabolic toxicity, CYP1A1, 1A2, 3A4, 2A6 and 2E1 and epoxide hydrolase. Direct and indirect toxicity was measured with the MCL-5 cells in comparison to its control cell line, cH2 cells using the BD Oxygen Biosensor System (OBS) as the method of detecting cell viability. Experiments were annotated and IC<sub>50</sub>'s determined using the BD Gentest™ Multiwell Plate Manager (MPM)/ADMET Software Program. Data and the associated experiments were archived in MPM's internal database that provided easy retrieval. The MCL-5 cells were shown to be significantly more sensitive to compounds that required metabolic activation for toxicity such as NNK, aflatoxin and cyclophosphamide. Moreover, the metabolic toxicity of cyclophosphamide could be specifically inhibited by competitive inhibition of CYP2A6 by a non-toxic dose of coumarin. The OBS in combination with the MCL-5 and cH2 cell lines creates a simple, homogeneous and streamlined method for screening compounds for metabolic toxicity.

## Introduction

Toxicity of test compounds or their metabolites is a major reason for compound failure in clinical trials. Current methods for determining metabolite toxicity are cumbersome and expensive, such as *in vivo* animal studies or *in vitro* microsome studies that involves time-consuming material transfer. Moreover, animal studies do not always accurately predict metabolite toxicity in humans contributing to potential compound failure. Thus, the ability to detect compounds that are indirect-acting toxins in an easy, consistent and homogeneous assay, based on metabolism with human P450s, would greatly benefit the pharmaceutical industry. Using the MCL-5 cell line and the BD Oxygen Biosensor, we have created a screening tool designed to detect P450-dependent toxic metabolites.

The MCL-5 is a human lymphoblastoid cell line that has been stably transfected with human P450's, CYP1A1, CYP1A2, CYP2A6, CYP2E1 and CYP3A4, and human epoxide hydrolase. These P450's have been shown to play a crucial role in the formation of toxic metabolites (1). Analysis of compound toxicity after P450-specific metabolism is performed concurrently with a control cell line (cH2) that is derived from the same parental line as MCL-5 and transfected with 2 empty vectors. Analysis of toxicity is performed over time with the BD Oxygen Biosensor, a homogenous, fluorescence-based assay system previously shown to rapidly and accurately measure cyto-toxicity (2). The assay system relies on an oxygen-sensitive fluorescent dye such that fluorescence increases as cells consume oxygen. The system uses similar culture conditions for both cell types, a standard fluorometer, and does not require the addition of any components other than the test compounds.

This study demonstrates that MCL-5 cells and control cH2 cells are equally sensitive to direct-acting toxins (Methotrexate, Sodium Azide and Vinblastine) as well as to drugs that are P450 substrates but generate non-toxic metabolites (i.e. coumarin, caffeine, testosterone and chlorzoxane). MCL-5 cells were found to be more sensitive to drugs known to generate toxic metabolites from P450 metabolism relative to the cH2 control cells. We have shown differential sensitivity to NNK, acetaminophen (APAP), benzo-a,i-pyrene, dibenzo-a,i-pyrene and aflatoxin B1 among others, compounds known to be metabolized by the P450's present in the MCL-5 cells.

IC<sub>50</sub>'s were generated using the BD Gentest™ Multiwell Plate Manager (MPM)/ADMET Software Program by the non-parametric smoothing spline. Calculation of IC<sub>50</sub> is an integral part of screening possible lead compounds in the ADME-Tox phase of drug development and often results do not satisfy the requirements of classical statistical models. The smoothing spline gives accurate results over a wide range of assays and provides desirable statistical properties. The MPM/ADMET program allows for the direct importation of data from several plate readers, annotates and stores the experimental protocols and data.

The BD Gentest MCL-5 Metabo-Tox Assay Kit in combination with the BD Gentest MPM/ADMET Software program provides an easy and rapid method by which to screen large numbers of compounds for both acute and CYP-mediated toxicity.

## Methods

MCL-5 and cH2 cells were cultured in histidine-free RPMI medium 1640 supplemented with 9% v/v horse serum, 2mm histidinol and 100ug/mL hygromycin B according to the protocol supplied in the MCL-5 Metabo-Tox Assay Kit (Cat# 459600). Where noted, cells were treated 50 μM buthionine sulfoximine for 3 days prior to exposure to test compounds. Serial dilutions of biologically relevant concentrations of drug were solubilized in DMSO and prepared at 2x the final concentration in tissue culture media. Each sample (100 μL) was added to the plate (n=3) including a no drug control. After an initial read, an equal volume of cells was added to each well. Controls included a serial dilution of test compound in absence of cells. For each experiment, 40,000-45,000 cells were plated per well with the exception of methotrexate (30,000 cells/well). Cells were maintained at 37°C in a standard 5% CO<sub>2</sub> incubator.

IC<sub>50</sub> values were derived using the MPM/ADMET software program (Cat# 457000) using a non-parametric smoothing spline or four-parameter logistic fit. The IC<sub>50</sub>'s derived were compared to those IC<sub>50</sub>'s derived by linear interpolation, a standard calculation for IC<sub>50</sub>. The IC<sub>50</sub> values were taken from curves at 72 or 96 hours except for methotrexate (120 hrs.) as previously determined (1).

Figure 1. Schematic of the BD Oxygen Biosensor System

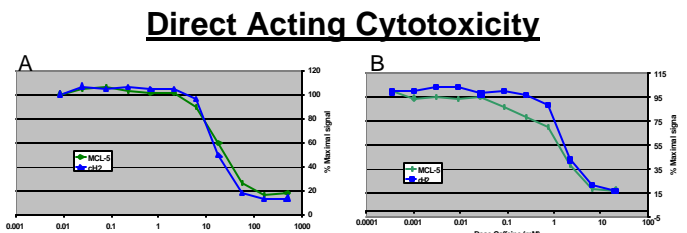
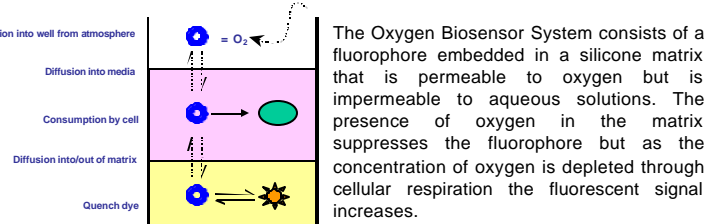


Figure 2. Direct-Acting Cytotoxicity. These data show that the MCL-5 and cH2 cells are equally sensitive to vinblastine (A), a direct acting toxin, and to caffeine (B), a substrate for CYP1A1 and CYP1A2 at 72 hours of incubation. Data is shown as percent of maximal signal.

	cH2	MCL-5
<b>Acute toxins</b>		
Vinblastine	16 +/- 0.5 μM	18 +/- 1.1 μM
Sodium Azide	2.3 +/- 0.2 μM	4.0 +/- 0.8 μM
Methotrexate	21 +/- 1.5 nM	23 +/- 1 nM
Glucose	106 +/- 5 mM	116 +/- 8 mM
<b>CYP Substrates with Nontoxic metabolites</b>		
Caffeine	1.6 +/- 0.2 mM	1.3 +/- 0.2 mM
Testosterone	53 +/- 3 μM	76 +/- 5 μM
Chlorzoxzone	474 +/- 13 μM	592 +/- 30 μM
Coumarin	204 +/- 70 μM	208 +/- 65 μM

Table 1. IC<sub>50</sub> Summary Table for Direct-Acting Toxins. MCL-5 and cH2 cells are equally sensitive to a variety of direct acting compounds and P450 substrates that do not generate toxic metabolites, and IC<sub>50</sub> values derived as described in methods.

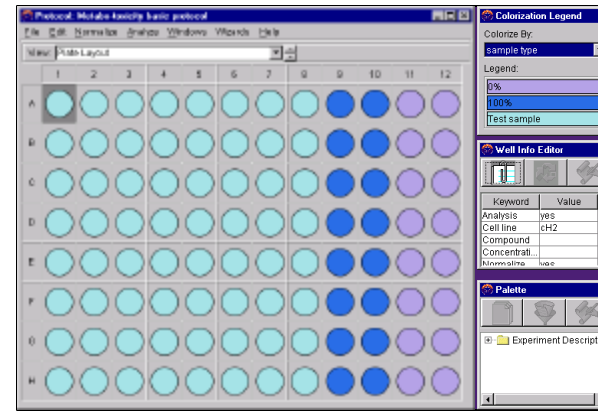


Figure 3. Implementation of MPM/ADMET Software Program. MPM/ADMET can be used to easily and quickly describe and record protocols and experiments. It can acquire data directly from several plate readers or any data in an excel format. The data can be automatically normalized with pre-set normalization schemes, graphed and analyzed for IC<sub>50</sub> by a number of different curve-fitting options (3). The data and its analysis can be saved to an internal searchable database and used to generate reports.

## P450-Mediated Cytotoxicity

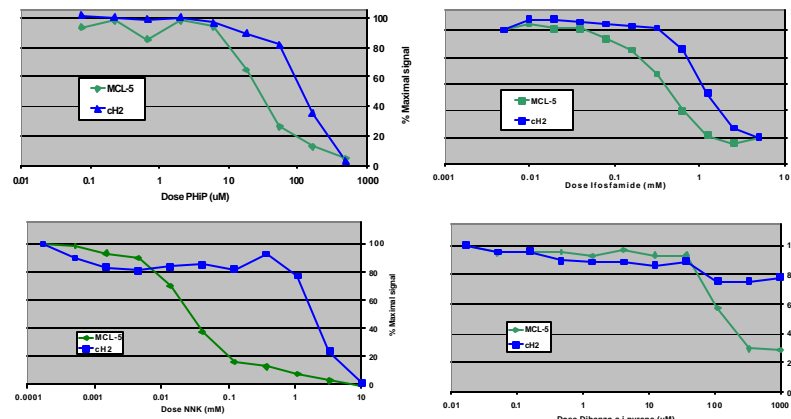


Figure 4. Increased Toxicity in MCL-5 with compounds that generate toxic P450 metabolites. Compounds PHiP, Ilofosamide, NNK, and dibenzo-a,i-pyrene are known to produce P450-dependent cytotoxic metabolites. MCL-5 cells are shown to be significantly more sensitive to these compounds than the control cH2 cell line. Data is presented as percent maximal signal and IC<sub>50</sub> values are shown in Table 2.

Test Substrate	cH2 cells	MCL5 Cells	Major P450's
Benzopyrene	8% toxicity >20 μM	1.6 μM	1A1, 3A4
Aflatoxin	N/D	47.9 nM	3A4, 1A2, 1A1
NNK	2.3 mM	0.040 mM	2A6, 2E1
Dibenzopyrene	20% toxicity > 1 mM	70 μM	1A1, 3A4, 1A2
Acetaminophen (50 μM BSO pretreat)	0.8 mM	0.49 mM	2E1, 3A4, 1A2
PhiP	106 μM	26 μM	1A1
Ilofosamide	1.3 mM	0.5 mM	3A4, 2A6
Cyclophosphamide	4.7 mM	2.4 mM	3A4, 2A6

Table 2. Summary Table of IC<sub>50</sub> Values for Metabolism-dependent Toxicity. IC<sub>50</sub> values for compounds were generated using the MPM/ADMET Software Program by four-parameter logistic fit or nonparametric smoothing spline analysis or by linear interpolation as described in "Materials and Methods". The results for some compounds tested in cH2 cells are expressed as percent toxicity, the IC<sub>50</sub> being greater than the highest concentration tested in the assay.

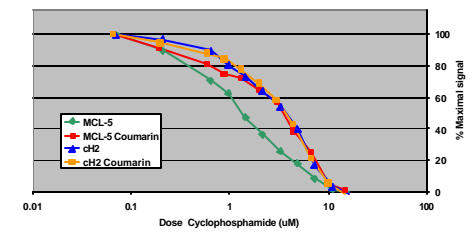


Figure 5. Competitive inhibition of CYP2A6-mediated Toxicity. Cyclophosphamide is metabolized by a number of P450's including CYP2A6, to produce toxic metabolites. Here, we demonstrate that P450 mediated toxicity of Cyclophosphamide can be specifically inhibited by Coumarin. Coumarin was shown to competitively inhibit the production 4-hydroxy-cyclophosphamide, a toxic metabolite, by competition for CYP2A6 (4). Cells treated with a non-toxic amount of coumarin (50 μM) reduced the toxicity of cyclophosphamide in MCL-5 cells to that observed in control cH2 cells.

Treatment	APAP	Vinblastine
cH2 cells	3.76 +/- 0.26	16 +/- 0.52
BSO-treated cH2 Cells	3.94 +/- 0.34	13 +/- 0.96
MCL-5 cells	1.35 +/- 0.03	18 +/- 1.13
BSO-treated MCL-5 cells	0.69 +/- 0.04	17 +/- 1.5

Figure 6. Depletion of cellular GSH levels increases sensitivity to acetaminophen (APAP) in MCL-5 cells. MCL-5 and cH2 cells were pre-exposed to 50 μM BSO (buthionine sulfoximine) for 72 hours and then challenged with APAP or Vinblastine. CYP-competent MCL-5 cells were significantly more sensitive to APAP after BSO treatment whereas no change in IC<sub>50</sub> occurred with Vinblastine, a direct acting toxin. No change in APAP sensitivity was apparent in the BSO treated cH2 control cells or in their sensitivity to other toxins. (IC<sub>50</sub> data are expressed in mM for APAP or μM for Vinblastine).

## Conclusion

1. The MCL-5 Metabo-Tox Assay Kit provides a consistent, homogeneous method for detection of potential metabolic toxicity.
2. The MCL-5 cell system is based on human P450's known to be involved with the potential production of toxic metabolites.
3. Use of the BD Oxygen Biosensor System provides a wealth of real-time, kinetic information in a simple, one-plate, homogeneous assay system which requires no additional components other than the test compounds.
4. The assay can discriminate between direct-acting toxins and indirect-acting toxins.
5. The use of the MPM/ADMET Software Program facilitates annotation of experiments and data and provides rapid analysis for curve-fitting and IC<sub>50</sub> generation.

This assay system can be used to rapidly screen compounds and may better predict the success of a compound in clinical trials.

## References

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