

A Rapid Homogenous Method for Metabolic Toxicity Screening

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Abstract

Drug toxicity, particularly that arising from toxic metabolite formation is a major reason for compound failure in clinical trials. We present here a cell-based, high-throughput assay that can rapidly detect the potential toxicity of parent compounds as well as their metabolites. The BD Oxygen Biosensor System (OBS) (JBS 2000 June 5/31/14) was used in conjunction with the MCL-5 human lymphoblast cell line (BD Gentest) (Chem Res Toxicol 1991 Sep-Oct;4(5):566-72) which contains 5 human P450s (CYPs 1A2, 1A1, 3A4, 2A6 and 2E1). General toxins, CYP substrates with non-toxic products and CYP substrates with known toxic metabolites were tested using the MCL-5 lymphoblast cell line in comparison to its control cell line ch2, transfected with empty DNA vectors. Cellular oxygen consumption was monitored using the OBS and IC50s were derived using a 4-parameter or spline curve fitting analysis using the BD Biosciences MMP ADMET software. MCL-5 and ch2 cell lines showed comparable sensitivity to acute toxins or to CYP substrates that do not have toxic metabolites. In contrast, MCL-5 cells were significantly more sensitive to agents that required metabolic activation such as NNK, aflatoxin, cyclophosphamide, fofosamide, and benzo a,j pyrene. CYP-dependent acetaminophen toxicity was increased after treatment with buthionine sulfoximine suggesting a protective role for glutathione. The OBS in combination with the MCL-5 cell lines creates a simple, homogeneous and streamlined method for screening large numbers of compounds for both acute and CYP-mediated toxicity.

Introduction

Toxicity of compounds or their metabolites is a major reason for the failure of a compound in clinical trials. Current methods for determining compound toxicity are cumbersome and expensive, such as in vivo animal studies or in vitro assays such as MTT, which involves the addition of toxic chemicals. Moreover, animal studies do not accurately predict metabolite toxicity in humans leading to expensive compound failure. The ability to rapidly detect potential compounds that are either acutely toxic or become toxic after they are metabolized in a high throughput screening assay would greatly benefit the pharmaceutical industry. We have created a rapid, high throughput assay to screen for compound toxicity using the CYP-containing MCL-5 cell line and the BD Oxygen Biosensor (1).

The metabolically competent MCL-5 cells are a human lymphoblastoid cell line that have been stably transfected with human CYPs 1A1, 1A2, 2A6, 2E1 and 3A4, and human epoxide hydrolase (2). Analysis of compound toxicity after P450-form specific metabolism is performed concurrently with a control cell line (ch2) that is derived from the same parental line as MCL-5 and has been transfected with empty vector. Analysis of toxicity is performed over time with the BD Biosciences Oxygen Biosensor, which is a homogeneous, fluorescence based assay system previously shown to rapidly and accurately measure cytotoxicity (1). The assay system relies on a stabilized oxygen sensitive fluorescent dye such that fluorescence increases as cells consume oxygen. This plate-based system can rapidly determine cell toxicity in real time using a standard fluorometer without addition of any components other than the test compound.

This study establishes that the metabolically competent MCL-5 cells and the control ch2 cells are equally sensitive to four general toxins with a wide range of mechanism of action (Glucose, Methotrexate, Sodium Azide and Vinblastine) as well as drugs that are substrates for CYPs that have no toxic metabolites (coumarin, caffeine, testosterone, and chlorzoxazone). MCL-5 cells are more sensitive to drugs known to have toxic metabolic products after metabolism by particular P450s as compared to the control ch2 cell line. We have shown differential sensitivity to NNK, acetaminophen (APAP), benzo-a,j pyrene, cyclophosphamide, fofosamide, and aflatoxin B1 which are known to be metabolized by different P450s present in the MCL-5 cells. NNK is metabolized by 2A6 & 2E1. Acetaminophen is metabolized by 2E1, 1A2 & 3A4. Benzo-a,j pyrene and benzo-a,j pyrene are metabolized primarily by 1A1 and 3A4 and Aflatoxin B1 is metabolized by both 1A2 & 3A4. The specificity of the assay is demonstrated by blockade of cyclophosphamide cytotoxicity by coumarin.

The BD Biosciences Oxygen Biosensor assay system in combination with the MCL-5 and ch2 cell lines from BD Gentest provides an easy and rapid method by which to screen large numbers of compounds for both acute and CYP-mediated toxicity. This novel methodology will facilitate the selection of compounds that may have a better chance of being successful in clinical trials. The assay will be available soon as a kit from BD Gentest (MCL-5 METABO-TOX ASSAY KIT, Cat # 459600).

Methods

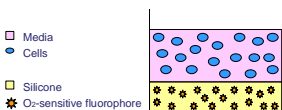
BD Oxygen Biosensor Cytotoxicity Assays

Serial dilutions of biologically relevant concentrations of drug were prepared at twice the final concentration in tissue culture media. Each drug concentration (100 uM) was added across the plate (n=3-4), reserving one column for no-drug (media-only) controls. After an initial reading, an equal volume of cells was added to each well of the assay system. For each experiment, 40,000 to 45,000 cells per well were added for all drugs tested except methotrexate (30,000 cells/well). Cells were maintained at 37°C in a standard 5% CO2 incubator. Cells were cultured in Hsrdine-free RPMI medium 1640 supplemented with 5% v/v horse serum, and 2mM histidinol and hygromycin B (100ug/ml).

IC50 values were derived using linear interpolation, or from standard dose response curves in which the mean normalized fluorescence (0.4 wells per time point) was plotted against the corresponding drug concentration. Standard dose response curves were fitted using a four-parameter curve fitting analysis. The derived IC50 drug concentration corresponds to a response halfway between the upper and lower asymptotes of the four-parameter logistic model using the equation shown below. According to this equation, the estimated IC50 is the determined value of the parameter, β₂. The IC50 values were taken from curves derived at 72 or 96 hours except methotrexate (at 120 hrs) as previously determined (1).

$$f(x) = \beta_1 + \frac{\beta_2 - \beta_1}{1 + \left(\frac{x}{\beta_3}\right)^{\beta_4}}$$

HOW DOES IT WORK?



Schematic of the BD Oxygen Biosensor System. The Oxygen Biosensor System consists of a fluorophore embedded in a silicone matrix that is permeable to oxygen but is impermeable to aqueous solutions. The presence of oxygen in the matrix suppresses the fluorophore but as the concentration of oxygen is depleted through cellular respiration the fluorescent signal increases.

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Direct Acting Cytotoxicity

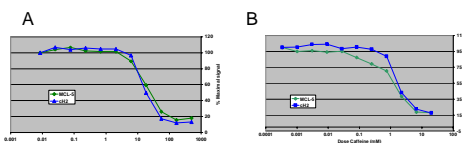


Table 1

	ch2	MCL-5
Acute toxins		
Vinblastine	16 +/- 0.5 uM	18 +/- 1.1 uM
Sodium Azide	2.3 +/- 0.2 uM	4.0 +/- 0.8 uM
Methotrexate	21 +/- 1.5 nM	23 +/- 1 nM
Coumarin	106 +/- 5 nM	116 +/- 9 nM
CYP Substrates with Nontoxic metabolites		
Caffeine	1.6 +/- 0.2 mM	1.3 +/- 0.2 mM
Testosterone	53 +/- 3 uM	76 +/- 5 uM
Chlorzoxazone	474 +/- 13 uM	592 +/- 30 uM
Coumarin	204 +/- 70 uM	238 +/- 60 uM

Figure 1. BD Oxygen Biosensor Cytotoxicity Assay. These data show that the MCL-5 and ch2 cells are equally sensitive to vinblastine (Figure A), a direct acting toxin, and to caffeine (Figure B), a substrate for CYP1A1 and CYP1A2. Table 1 shows IC50 data for additional direct acting toxins and CYP substrates. MCL-5 and ch2 cells are equally sensitive to direct acting compounds with a wide range of cytotoxic mechanisms. P450 substrates that generate non-toxic metabolites are equally toxic to both cell lines at a high enough dose. Data in Figures are shown as the percent maximal normalized to the lowest dose of drug. IC50 values are the concentration of drug to give half the maximal signal.

P450-Mediated Cytotoxicity

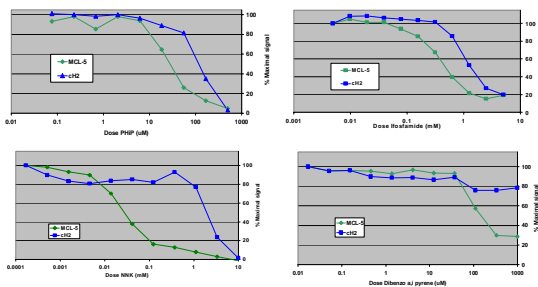


Figure 2. BD Oxygen Biosensor Cytotoxicity Assay Compounds PHIP, Ifofosamide, NNK, and benzo-a,j pyrene are and are known to produce P450-dependent cytotoxic metabolites. MCL-5 cells are shown to be significantly more sensitive to these compounds than the control ch2 cell line. IC50 values are shown in Table 2.

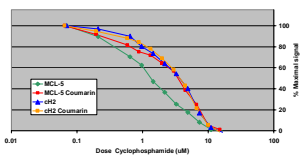


Figure 3. Competitive inhibition of CYP-mediated Toxicity by Coumarin. Cyclophosphamide is metabolized by a number of P450's including CYP2A6. Coumarin has been shown to competitively inhibit the production of the toxic metabolite 4-hydroxy-cyclophosphamide by competition for CYP2A6 expressed in lymphoblast cells (4). In these studies, we demonstrate that the presence of a non-toxic amount of coumarin (50 uM) reduced the toxicity of cyclophosphamide in MCL-5 cells to that observed in control ch2 cells. This demonstrates the specificity of the assay for CYP activity to produce a toxic effect.

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 4. Depletion of cellular GSH levels increases sensitivity to acetaminophen (APAP) in MCL-5 cells. MCL-5 and ch2 cells were pre-exposed to 50 uM 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 IC50 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. (IC50 data are expressed in mM for APAP or uM for Vinblastine).

Test Substrate	ch2 cells	MCL5 Cells	Major P450's
Benzopyrene	8% toxicity >20uM	1.6uM	1A1, 3A4
Aflatoxin	N/D	47.9nM	3A4, 1A2, 1A1
NNK	2.3 mM	0.040 mM	2A6, 2E1
Dibenzopyrene	20% toxicity > 1 mM	70 uM	1A1, 3A4, 1A2
Acetaminophen (50uM BSO pretreat)	0.8 mM	0.49 mM	2E1, 3A4, 1A2
PhIP	106 uM	26 uM	1A1
Ifofosamide	1.3mM	0.5 mM	3A4, 2A6
Cyclophosphamide	4.7 mM	2.4 mM	3A4, 2A6

Table 2. IC50 Values for Metabolism Dependent Toxins using the BD Oxygen Biosensor Assay. IC50 values for compounds were generated using the 4-parameter or spline curve fitting analysis or by linear interpolation as described in "Materials and Methods". MCL-5 cells contain 5 human P450s and human epoxide hydrolase. ch2 cells represent the control lymphoblast cells that contain no transfected P450s. Those compounds for which an IC50 was not obtained, results were expressed as percent toxicity, the IC50 being greater than the highest concentration tested in the assay.

Conclusions

1. The BD Oxygen Biosensor greatly facilitates the determination of potentially toxic compounds. It is easy to use and provides a wealth of real-time, kinetic information in a simple, one-plate, homogeneous assay system.
2. The assay can discriminate between direct-acting toxins and indirect-acting toxins.
3. The BD Oxygen Biosensor, combined with the metabolically competent MCL-5 cells from BD Gentest, provides a powerful method to rapidly and easily screen for CYP-induced metabolic toxicity.

This assay system can be used to screen large numbers of compounds prior to costly animal studies and may better predict the success of a compound in clinical trials

References

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