

An *In vitro* Model for the Prediction of Chemical Metabolism and Toxicity

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Introduction

The use of cell lines for drug and chemical toxicity screening, although very attractive, has serious restrictions because of the limited expression of cytochrome P450 isozymes and limited information about toxic mechanism.

In order to circumvent these issues, we have generated a cell line that reports on a specific pathway of oxidative stress. The **antioxidant response element (ARE)** is a transcriptional regulatory element involved in the activation of cytoprotective genes coding for a number of antioxidant proteins and detoxifying enzymes. This cell line measures Nrf2 mediated activation of the ARE via a luciferase reporter.

Additionally, we have developed novel adenoviral vectors that allow the expression of single or multiple human P450s in any cell line of interest using the foot-and-mouth disease virus peptide 2A sequence that permits the expression of **multiple proteins from a single promoter system**. Using this approach we have expressed combinations of CYP1A1, CYP2A6, CYP2D6 & CYP3A4 in CHO cells and the ARE reporter cell line.

We have exemplified utility in the **antioxidant responsive element (ARE) cell line**. These cells were exposed to either 7-ethoxycoumarin or butylated hydroxyanisole and ARE activity was only induced in cells expressing CYP1A1 and CYP2A6, where the metabolites are formed.

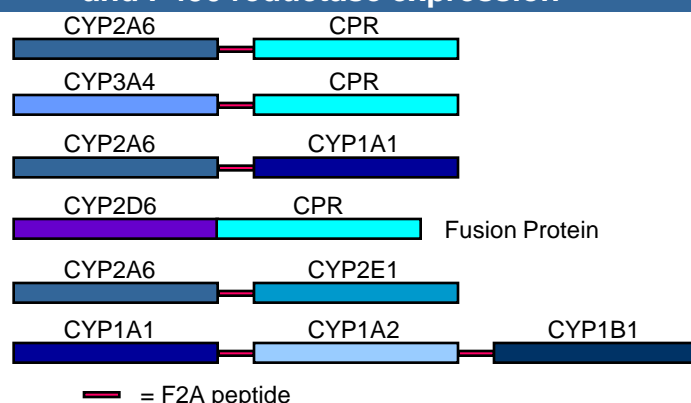
These data demonstrate the power of this experimental approach to give a more accurate assessment of toxicity associated with exposure to chemical agents or their metabolites.

Experimental Design

A. Strategy for the development of a heterologous system for the high expression of human P450's in culture

The 2A peptide of foot-and-mouth disease virus (FMDV) can direct production of separate proteins from one polyprotein open reading frame in a variety of higher eukaryotic systems. The following outlines the vector constructs that have been engineered to drive the expression of the human P450's (Figure 1).

Figure 1: Constructs utilised for multiple P450s and P450 reductase expression



B. Generation of a cell line expressing various combinations of P450s and reporter transgene

The above constructs have been used to generate recombinant adenoviruses, which were then infected into the developed reporter cells individually or in a group of different P450s to express various combinations of P450 and reporter in cultured cells.

1. Expression of multiple P450s and CPR in cells

Introducing the adenoviral constructs into cells resulted in high levels of expression of individual P450s or up to four P450s (3A4, 2D6, 2A6 & 1A1) together with Cytochrome P450 reductase (CPR) in several cell lines. Figure 2 shows the expression of multiple P450s and CPR in CHO cells. Expression of fused CYP2D6-CPR generated a ~ 120 kDa protein.

Figure 2: Expression of multiple P450s & P450 reductase in CHO cells

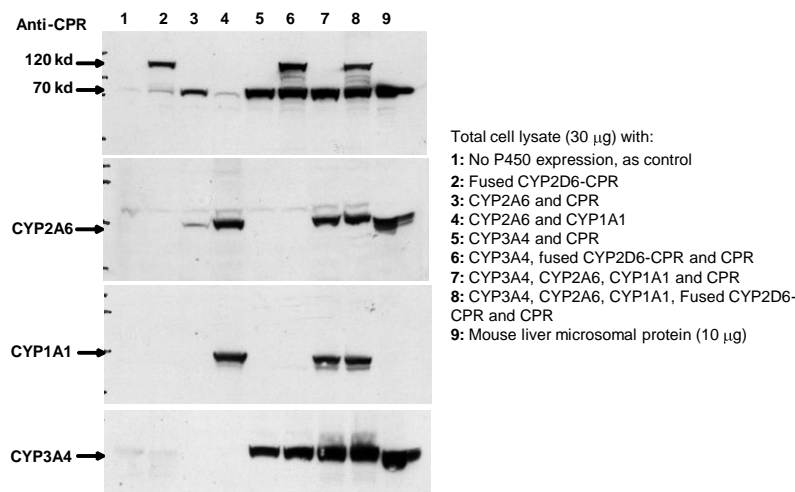


Table 1: P450 enzyme activities in infected ARE/MCF-7 reporter cells

Enzyme activity	MCF-7/ARE	MCF-7/ARE with P450 expression [#]
CPR (nmol/min.mg protein)	23.1	79.1
CYP3A4 1'-hydroxymidazolam (pmol/min.mg protein)	0.1	4.4
CYP2D6 1'-hydroxybufuralol (pmol/min.mg protein)	0	16.2
CYP2A6 pmol/hr/10 ⁵ cells	0	349.0
CYP1A1 pmol/hr/10 ⁵ cells	0	44.2

Cells were treated with P450 substrates in culture medium for 1.0 h. At the end of the incubation, metabolites in the medium were determined. Cell lysate was prepared and protein concentration measured.

[#]: MCF-7/ARE cells with four P450s (3A4, 2D6, 2A6 and 1A1) and CPR expressed

Western blotting (Figure 2) and analysis of enzyme activities (Table 1) confirmed that all P450s and CPR are functional and expressed at high levels.

Figure 3: Direct induction of ARE by *tert*-butylhydroquinone

2. Generation of a cell line expressing various combinations of P450s in addition to a reporter transgene (luciferase).

In the ARE reporter cell line without P450 metabolising enzyme expression, a ~16 fold increase in luciferase transgene induction was achieved with the known inducer *tert*-butylhydroquinone (tBHQ). This demonstrates direct activation of the ARE pathway by tBHQ where no metabolism is necessary. The parent compound butylated hydroxyanisole (BHA) has no effect alone (see Figure 4).

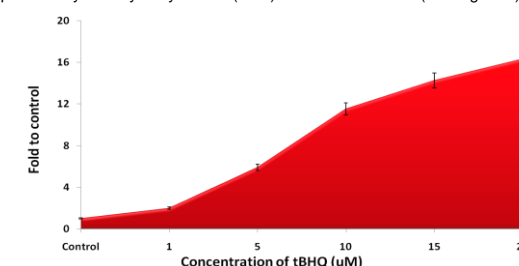
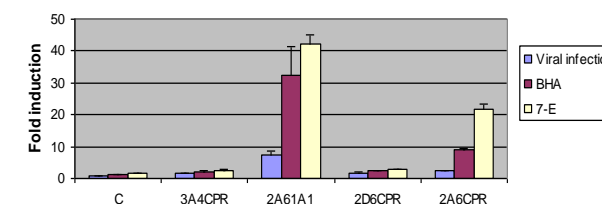


Figure 4: Induction of ARE by P450-dependent metabolites of the chemicals butylated hydroxyanisole & 7-ethoxycoumarin

In the ARE reporter cell line transfected with specific CYP450s, high levels of P450 expression were achieved in the ARE reporter cell line (see table 1). When both butylated hydroxyanisole (BHA - 20µM) and 7-ethoxycoumarin (7-E - 100µM) were applied to control infected cells, transgene induction (luciferase expression) was barely detectable. Similarly, very low levels of induction were achieved when CYP2D6 and CYP3A4 were expressed. However, when CYP2A6 was expressed alone induction of 9-fold and 22-fold were achieved with BHA and 7-E respectively. The induction further increased to 28-fold and 43-fold when CYP1A1 was co-expressed with CYP2A6. This demonstrates that parent compounds, BHA and 7-E do not activate the ARE system. However, metabolism via the specific P450s CYP2A6 and CYP1A1 leads to activation of the pathway through metabolite production.



A heterologous expression system has been developed that will allow assessment of both parent and P450-dependent metabolite toxicity.

This expression system is applicable to other cell lines.

Scientific and Practical Significance

- We have produced a cell line co-expressing multiple P450s and a reporter transgene.
- This cell line can be used as a model for the drug metabolism seen in human hepatocytes whilst retaining greater consistency and rapid growth characteristics in culture.
- The inserted reporter gene provides a rapid assay to assess the toxic effects of test agents and metabolites.
- This cell line will provide an economical method for large scale toxicity testing while reducing the requirement for animal testing.
- These initial results provide proof of concept for further development of additional transgenic cell lines that can improve the relevance of this *in vitro* screen to man.

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