Development of an EROD Assay to Characterize the Induction of CYP1A Activity in *Xenopus laevis* Cells

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Abstract

The cytochrome P450-1A (CYP1A) family of monooxygenases is known to perform vital enzymatic functions in animals by detoxifying lipophilic environmental contaminants. The present study utilizes the ethoxy- and methoxyresorufin-*O*-deethylase (EROD and MROD) assays to quantify CYP1A protein activity in a kidney cell line derived from the African clawed frog *Xenopus laevis*, a species known to be highly insensitive to CYP1A-inducing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity. Twenty-four hours following TCDD treatment, this cell line exhibited an EROD EC_{50} of 62.8 ± 7.75 nM (SE) and MROD EC_{50} of 37.4 ± 3.03 nM (SE), approximately 4830-fold and 3850-fold higher than the EC_{50}’s of a mouse hepatocyte cell line, respectively. This corresponds to a maximal rate of EROD activity of 13.2 ± 1.37 (SE) and MROD activity of 6.86 ± 0.713 (SE) pmol resorufin·mg protein^{-1}·min^{-1} in the frog cell line at 400 nM TCDD. Characterizing the CYP1A activity in this amphibian species is vital to understanding its relative insensitivity to TCDD-like contaminants. This study lays the groundwork for future research investigating the individual activities of the two *Xenopus laevis* CYP1A proteins. Examining these differences will further the understanding of the function of CYP1As across the vertebrate taxa.

Objective

To develop CYP1A assays for frog and mouse cell lines (XLK-WG and Hepa1c1c7) which investigate TCDD-inducible CYP1A activity through ethoxy- and methoxyresorufin metabolism.

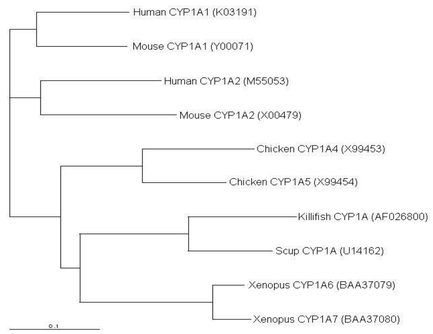

Figure 1. Phylogenetic analysis shows the relationship of the CYP1A paralogs from several vertebrate species. Genbank accession numbers of the CYP1A DNA sequences are shown for each analyzed sequence.

Cytochrome P450-1A’s

CYP1A’s in Mammals

- Mammals typically express two CYP1A paralogs, CYP1A1 and CYP1A2, which have been extensively characterized [4].
- Both CYP1As are inducible by TCDD and other aryl hydrocarbon receptor (AhR) ligands, in terms of CYP1A mRNA and protein [4].
- Each paralog is known to exhibit particular substrate preferences, and thus have different physiological and metabolic activities [7].
- These preferences have been characterized in fluorescence assays for CYP1A activity, such as the ethoxy- and methoxyresorufin-*O*-deethylase (EROD and MROD) assays [6].

CYP1As in Frogs

- The African clawed frog, *Xenopus laevis,* is also known to express two CYP1A paralogs: CYP1A6 and CYP1A7 [3].
- Both CYP1As are inducible by TCDD and other AhR ligands [1].
- However, frog CYP1As are remarkably less responsive to induction by typical AhR ligands, which reflects their relative insensitivity to their toxic effects compared to mammals [3].
- Frog CYP1A paralogs and their activities are not well characterized in the literature, so they may be functionally redundant, or alternatively exhibit substrate preferences.

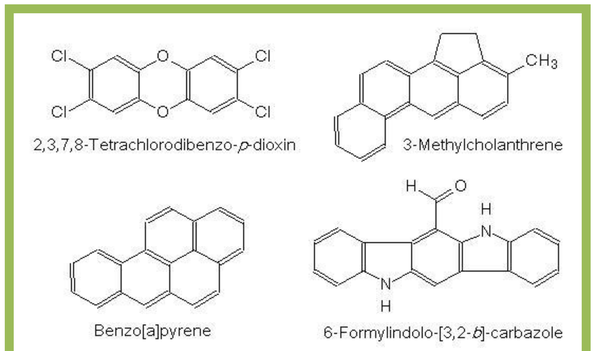

Figure 2. A wide variety of compounds are capable of inducing CYP1A activity. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the highly carcinogenic contaminant in Agent Orange, the defoliating agent used during the Viet Nam war, and is the most toxic polychlorinated dibenzodioxin. 3-Methylcholanthrene (3-MC) is a polycyclic aromatic hydrocarbon, commonly used in carcinogenesis studies. Benzo[*a*]pyrene (BaP) is a mutagenic and carcinogenic polycyclic aromatic hydrocarbon which arises from incomplete combustion reactions. 6-Formylindolo-[3,2-*b*]-carbazole (FICZ) is a recently characterized [5] endogenous ligand of the AhR, and a highly potent CYP1A inducer.

Materials and Methods

Cells

XLK-WG kidney cells from *Xenopus laevis* and Hepa1c1c7 liver cells from *Mus musculus* were grown in RPMI-1640 with 20% fetal bovine serum or α-MEM with 10% fetal bovine serum respectively. When 90% confluent, cells were trypsinized and divvied into treatment wells on a 96-well plate at ~40,000 cells per well.

Treatment

After 24 hours of acclimation to the 96-well plate, the media was replaced. Cells were treated for 24 hours with a range of TCDD concentrations to induce CYP1A protein. XLK-WG cells were dosed with 10 concentrations from 0.012 to 400 nM TCDD, while Hepa1c1c7 cells were dosed with 10 concentrations from 0.0004 to 12 nM TCDD. DMSO was added to control groups.

Assay

Treated cells were washed with PBS, and a 2µM solution of 7-ER in a sodium phosphate buffer was added to treatment and control wells. After 30 minutes, plates were read for resorufin fluorescence using a fluorescence detector (Molecular Devices Gemini EM) with excitation and emission wavelengths of 530 and 586 nm, respectively. Reactions were halted with ice cold fluorescamine in acetonitrile (150 µg/mL). Protein fluorescence was detected with excitation and emission wavelengths of 400 and 460 nm, respectively. A duplicate standard curve was generated to assess the treatment wells for resorufin and protein concentrations.

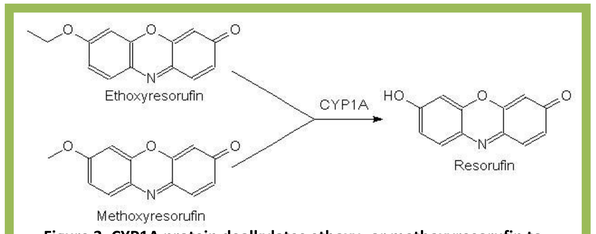

Figure 3. CYP1A protein dealkylates ethoxy- or methoxyresorufin to produce resorufin. After TCDD treatment, cells were dosed with sodium phosphate buffered 2µM solutions of ethoxy- or methoxyresorufin. These substrates are metabolized by the CYP1A proteins to yield the fluorescent detectable product, resorufin, and thus the fluorescence of the treatment well reflects the activity of the cell’s CYP1A proteins, measured in picomoles of resorufin produced per milligram of total protein per minute [2].

EROD and MROD CYP1A Activity

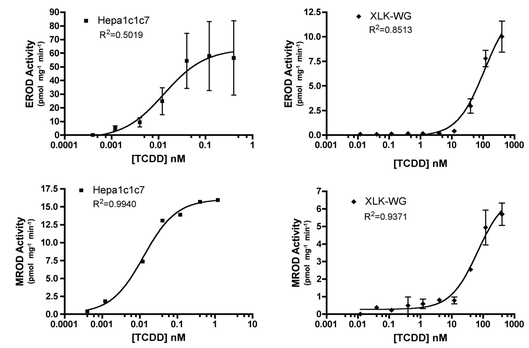

Figure 4. Hepa1c1c7 cells and XLK-WG cells exhibit distinct TCDD-inducible maximal EROD and MROD activities. Activity values were normalized to the lowest reading and logarithmically scaled for a dose-response curve. TCDD is a less potent inducer of EROD and MROD activity in XLK-WG cells than in Hepa1c1c7 cells, i.e. the maximal activities are much lower in the frog than in the mouse. In addition, both XLK-WG and Hepa1c1c7 cells exhibit a lower maximal activity in MROD assays than in EROD assays, indicating that there are substrate preferences for both mouse and frog CYP1As. Maximal activities for these responses are located in Table 1.

Induction of CYP1A Activities

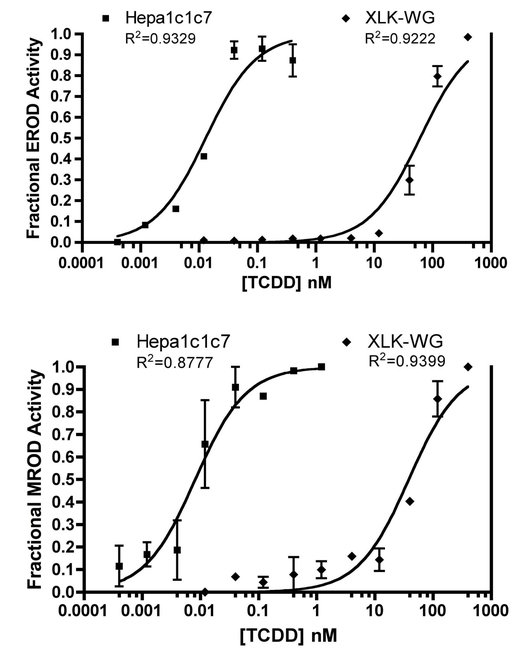

Figure 5. Hepa1c1c7 and XLK-WG cell lines exhibit distinct TCDD induction profiles for CYP1A activity. Fractional EROD and MROD activities were scaled logarithmically as a sigmoidal dose response curve, and TCDD EC_{50} values were calculated for both XLK-WG and Hepa1c1c7 cells. It is apparent that TCDD is a less potent inducer of EROD and MROD activity as indicated by the considerably higher TCDD concentration needed to generate 50% of the maximal response in frog cells than in mouse cells. The EC_{50}’s for these responses are located in Table 1.

EC_{50}’s and Maximal Activities

Table 1. The TCDD EC_{50} and maximal values for EROD and MROD activity in XLK-WG and Hepa1c1c7 cells, and a fold-change comparison.

	XLK-WG Cells	Hepa1c1c7 Cells	Fold Change
EROD EC_{50} (nM)	62.8±7.75 (n=6)	0.013±0.0004 (n=3)	~4830
MROD EC_{50} (nM)	37.4±3.03 (n=2)	0.00972±0.005 (n=1)	~3850
EROD Maximum (pmol resorufin) / (mg protein)·(min)	13.2±1.37 (n=6)	63.2±13.0 (n=3)	4.79
MROD Maximum (pmol resorufin) / (mg protein)·(min)	6.86±0.713 (n=2)	16.1±0.424 (n=1)	2.35

Conclusions

- *Xenopus laevis* cells exhibit TCDD-inducible EROD and MROD activities, though considerably less than those seen in a mouse cell line.
- Maximal TCDD-inducible EROD activity was lower in the frog cell line by a factor of 4.79 compared to the mouse cell line, while maximal TCDD-inducible MROD activity was lower in the frog cell line by a factor of 2.35 compared to the mouse cell line. These results are indicative of a species difference in dose-dependent CYP1A induction, and possibly in CYP1A protein activity.
- Comparison of fractional EROD activity in the frog and mouse cell lines shows a ~4800-fold disparity in the EC_{50} TCDD concentrations, and a ~3800-fold disparity in EC_{50} TCDD concentrations for MROD activity, again indicative of species differences.
- Combined, these findings show that methoxyresorufin is a less robust CYP1A substrate compared to ethoxyresorufin in both species. Further tests may examine and compare the activities of the individual CYP1A paralogs in each species to further characterize CYP1A activity in *X. laevis*.

http://www.iacuc.arizona.edu/training/xenopus/images/xenopus.jpg

Acknowledgments

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