Whole Zebrafish Cytochrome P450 Microplate Assays for Assessing Drug Metabolism and Drug Safety

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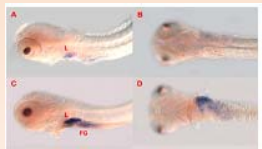
Introduction

Cytochrome P450 (CYP) enzymes, particularly CYP3A4 and CYP2D6 catalyze the majority of known drug-metabolizing reactions and many clinically relevant drug-drug interactions are associated with inhibition and/or induction of a specific CYP enzyme. Because of their genetic and physiological similarity to humans, zebrafish show promise as an efficient, predictive animal model for assessing drug metabolism and drug safety. In an earlier study, we have demonstrated that zebrafish exhibit dose-responsive drug toxicity comparable to effects in mouse and human. Several zebrafish CYP genes with high homology to humans or showing a similar catalyzing reaction with mammals, including CYP3A65, CYP1A1, A19, B19, 2K6, 3C1, 2J1 and CYP26D1 have been cloned and characterized. A CYP3A orthologue designated CYP3A65 has been shown homologous to the human CYP3A subfamily. Recently, we have successfully developed microplate-based whole zebrafish CYP3A4 and CYP2D6 functional activity assays for assessing drug metabolism and drug safety. These microplate-based CYP assays use human CYP specific substrates and show high sensitivity and specificity. We found that CYP3A4 functional activity was upregulated in zebrafish treated with 2, 4 -dichlorophenoxyacetic acid (2, 4-D), rifampicin, and dexamethasone, similar to the response of CYP3A4 in humans. Our results were also consistent with a recent report showing that low doses of dexamethasone (10 μM) enhanced the transcription of zebrafish CYP3A65; whereas high doses of dexamethasone (100 μM) did not have such an effect, as measured by whole mount *in situ* hybridization. Zebrafish CYP2D6 functional activity was upregulated by dexamethasone, but downregulated by 2, 4-D and ethanol. These findings underscore the high degree of CYP conservation in zebrafish. Our whole zebrafish CYP assays provide sensitive and robust tools for moderate throughput CYP screening *in vivo* in drug discovery and development. Other microplate-based whole zebrafish CYP functional activity assays including CYP1A2, 2C9, and 2C19 are in development.

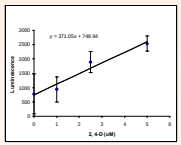
Cytochrome P450 (CYP) enzymes, particularly CYP3A4 and CYP2D6, catalyze the majority of known drug-metabolizing reactions

Modification of CYP activities can profoundly affect therapeutic efficacy and can lead to life-threatening toxicity

This project was aimed at developing an *in vivo* whole zebrafish CYP microplate assay for assessing drug metabolism and drug safety

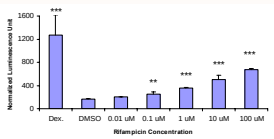


A human CYP3A orthologue designated CYP3A65 was expressed in untreated zebrafish (arrow) assessed by whole-mount *in situ* hybridization. A and B: 72 hpf; and C and D: 84 hpf. A and C: lateral view; and B and D: ventral view. L = liver; and FG = foregut. (Tseng et al., 2005)



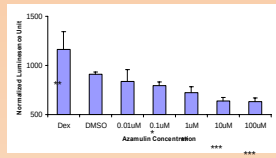
Dose-dependant increase in CYP3A4 functional activity in zebrafish treated with various concentrations of 2, 4-dichlorophenxyacetic acid (2, 4-D). 2 dpf zebrafish were treated for 24 hrs. Results represent SD ± SD from 5 measurements.

Rifampicin Induces CYP3A4 in Zebrafish



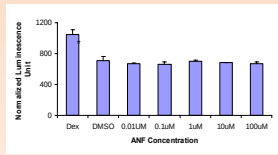
CYP3A4 induction by rifampicin using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with rifampicin for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. ***p* < 0.01 and ****p* <0.001 as compared with 0.1% DMSO control. Zebrafish treated with
50 μM dexamethasone (Dex) was used as a positive control.

Azamulin Inhibits CYP3A4 in Zebrafish



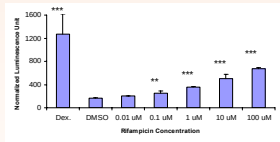
CYP3A4 inhibition by azamulin using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with azamulin for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. * *p* < 0.05, ***p* < 0.01 and ****p* <0.001 compared with 0.1% DMSO control.

ANF Has No Effect on CYP3A4 in Zebrafish



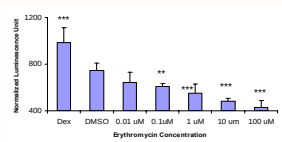
α-Naphthoflavone (ANF) effect on CYP3A4 using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with ANF for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. No statistical differences were observed in zebrafish treated with any concentration of ANF compared with those treated with DMSO. **p* < 0.001 compared with 0.1% DMSO control.

Rifampicin Induces CYP3A4 in Zebrafish



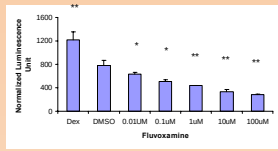
CYP3A4 induction by rifampicin using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with rifampicin for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. ***p* < 0.01 and ****p* <0.001 as compared with 0.1% DMSO control. Zebrafish treated with
50 μM dexamethasone (Dex) was used as a positive control.

Erythromycin Inhibits CYP3A4 in Zebrafish



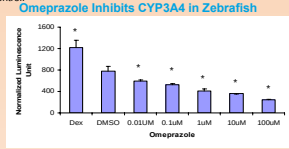
CYP3A4 inhibition by erythromycin using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with erythromycin for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. ***p* < 0.01 and ****p* <0.001 compared with 0.1% DMSO control.

Fluvoxamine Inhibits CYP3A4 in Zebrafish



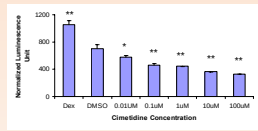
CYP3A4 inhibition by fluvoxamine using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with fluvoxamine for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.01 and ***p* <0.001 compared with 0.1% DMSO control.

Omeprazole Inhibits CYP3A4 in Zebrafish



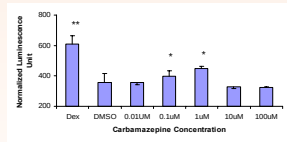
CYP3A4 inhibition by omeprazole using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with omeprazole for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control.
**p* <0.001 compared with 0.1% DMSO control.

Cimetidine Inhibits CYP3A4 in Zebrafish



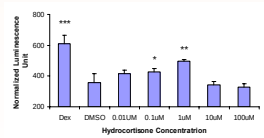
CYP3A4 inhibition by cimetidine using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with cimetidine for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.01 and ***p* <0.001 as compared with 0.1% DMSO control.

Carbamazepine Induces CYP3A4 in Zebrafish



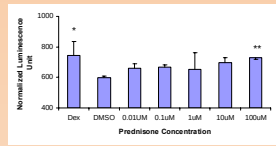
CYP3A4 induction by carbamazepine using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with carbamazepine for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.05 and ***p* < 0.001 compared with 0.1% DMSO control.

Hydrocortisone Induces CYP3A4 in Zebrafish



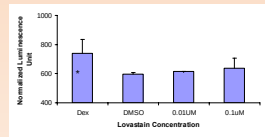
CYP3A4 induction by hydrocortisone using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with hydrocortisone for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.05, ***p* < 0.01 and *** *p* < 0.001 compared with 0.1% DMSO control.

Prednisone CYP3A4 Induction in Zebrafish



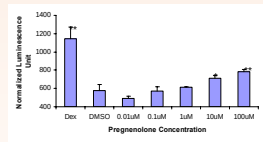
CYP3A4 induction by prednisone using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with prednison for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.05 and ***p* < 0.01 compared with 0.1% DMSO control.

Lovastain (Mevinolin) Does Not Induce CYP3A4 in Zebrafish



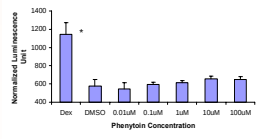
CYP3A4 induction by prednisone using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with lovastain for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.05 as compared with 0.1% DMSO control. All zebrafish were dead when treated with 1, 10 or 100 μM Lovastain. There was no statistical difference between zebrafish treated with DMSO and zebrafish treated with 0.01 and 0.1 μM Lovastain.

Pregnenolone Induces CYP3A4 in Zebrafish



CYP3A4 induction by pregnenolone using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with pregnenolone for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.01 and ** *p* < 0.001 compared with 0.1% DMSO control.

Phenytoin Does Not Induce CYP3A4 in Zebrafish

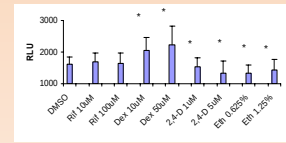


CYP3A4 induction by phenytoin using microplate-based whole zebrafish CYP3A4 functional assay. 2 dpf zebrafish were treated with phenytoin for 24 hrs and zebrafish CYP3A4 functional activity was measured using a CYP3A4 chemiluminogenic substrate. Zebrafish treated with 50 μM dexamethasone (Dex) was used as a positive control. **p* < 0.001 compared with 0.1% DMSO control. There were no statistical differences between zebrafish treated with DMSO and those treated with any concentrations of phenytoin.

Table: Assessment of Compound CYP3A4 Inhibition/Induction in Zebrafish

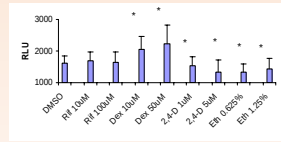
Compound	CYP3A4 Inhibition or Induction in Zebrafish	CYP3A4 Inhibition or Induction in Mammals	Correct Prediction
Azamulin	Inhibition	Inhibition	Yes
Disopyramide	Inhibition	Inhibition	Yes
Erythromycin	Inhibition	Inhibition	Yes
Fluvoxamine	Inhibition	Inhibition	Yes
Omeprazole	Inhibition	Inhibition	Yes
Cimetidine	Inhibition	Inhibition	Yes
Rifampicin	Induction	Induction	Yes
Carbamazepine	Induction	Induction	Yes
Hydrocortisone	Induction	Induction	Yes
Prednisone	Induction	Induction	Yes
Lovastain	None	Induction	No
Pregnenolone	Induction	Induction	Yes
Phenytoin	None	Induction	No
α-Naphthoflavone	None	None	Yes

CYP2D6 in Zebrafish Treated with Drugs for 24 hrs



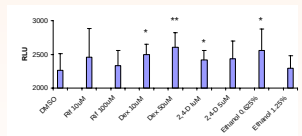
CYP2D6 functional activity in zebrafish. 2 dpf zebrafish were treated with drugs for 24 hrs and CYP2D6 functional activity was measured using a CYP2D6 chemiluminogenic substrate. Zebrafish CYP2D6 was upregulated by treatment with dexamethasone (Dex), 2, 4-dichlorophenxyacetic acid (2, 4-D), and ethanol. **p* < 0.05 compared with 0.1% DMSO control. RLU = Relative Luminescence Units.

CYP2D6 in Zebrafish Treated with Drugs for 24 hrs



CYP2D6 functional activity in zebrafish. 2 dpf zebrafish were treated with drugs for 24 hrs and CYP2D6 functional activity was measured using a CYP2D6 chemiluminogenic substrate. Zebrafish CYP2D6 was upregulated by treatment with dexamethasone (Dex), 2, 4-dichlorophenxyacetic acid (2, 4-D), and ethanol. **p* < 0.05 compared with 0.1% DMSO control. RLU = Relative Luminescence Units.

CYP1A2 in Zebrafish Treated with Drugs for 24 hrs



CYP1A2 functional activity in zebrafish. 2 dpf zebrafish were treated with drugs for 24 hrs and CYP1A2 functional activity was measured using a CYP1A2 chemiluminogenic substrate. Zebrafish CYP1A2 was upregulated by treatment with 10 μM dexamethasone (Dex), 1μM 2, 4-dichlorophenxyacetic acid (2, 4-D), and 0.625% ethanol. **p* < 0.05 and ** *p* <0.01 compared with 0.1% DMSO control. RLU = Relative Luminescence Units; Rif = Rifampicin

Conclusions

- Overall prediction success rate for CYP3A4 inhibition and induction in zebrafish was 86%; 100% for CYP3A4 inhibition and 71% for CYP3A4 induction
- Zebrafish exhibits comparable CYP drug metabolism profiling as in mammals.
- Whole zebrafish CYP microplate assays is a predictive, reproducible animal model for assessing drug metabolism and drug safety