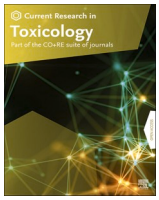
Current Research in Toxicology 3 (2022) 100092

Contents lists available at ScienceDirect

Current Research in Toxicology

journal homepage: www.journals.elsevier.com/current-research-in-toxicology



Confirmation of high-throughput screening data and novel mechanistic insights into FXR-xenobiotic interactions by orthogonal assays

Jon Hamm [a,*], Debabrata Mahapatra [b], Megan M. Knuth [b], Jaleh Abedini [a], Mary Lingerfelt [c], Sean Ekins [c], Seth W. Kullman [b,d]

[a] Inotiv, P.O. Box 13501, Research Triangle Park, NC 27709, USA
[b] Toxicology Program, Department of Biological Sciences, North Carolina State University, Raleigh, Campus Box 7633, Raleigh, NC 27695-7633, USA
[c] Collaborations Pharmaceuticals, Inc. 840 Main Campus Drive, Lab 3510 Raleigh, NC 27606, USA
[d] Center for Human Health and the Environment, North Carolina State University, Raleigh, NC, USA

ARTICLE INFO

Keywords:
ToxCast
Farnesoid X receptor
High-throughput screening
Teleost models
Medaka

ABSTRACT

Toxicology in the 21st Century (Tox21) is a federal collaboration employing a high-throughput robotic screening system to test 10,000 environmental chemicals. One of the primary goals of the program is prioritizing toxicity evaluations through *in vitro* high-throughput screening (HTS) assays for large numbers of chemicals already in commercial use for which little or no toxicity data is available. Within the Tox21 screening program, disruption in nuclear receptor (NR) signaling represents a particular area of interest. Given the role of NR's in modulating a wide range of biological processes, alterations of their activity can have profound biological impacts. Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that has demonstrated importance in bile acid homeostasis, glucose metabolism, lipid homeostasis and hepatic regeneration. In this study, we re-evaluated 24 FXR agonists and antagonists identified through Tox21 using select orthogonal assays. In transient transactivation assays, 7/8 putative agonists and 4/4 putative inactive compounds were confirmed. Likewise, we confirmed 9/12 antagonists tested. Using a mammalian two hybrid approach we demonstrate that both FXR agonists and antagonists facilitate FXRα-coregulator interactions suggesting that differential coregulator recruitment may mediate activation/repression of FXRα mediated transcription. Additionally, we tested the ability of select FXR agonists and antagonists to facilitate hepatic transcription of FXR gene targets Shp and Bsep in a teleost (Medaka) model. Through application of *in vitro* cell-based assays, *in silico* modeling and *in vivo* gene expressions, we demonstrated the molecular complexity of FXR:ligand interactions and confirmed the ability of diverse ligands to modulate FXRα, facilitate differential coregulator recruitment and activate/repress receptor-mediated transcription. Overall, we suggest a multiplicative approach to assessment of nuclear receptor function may facilitate a greater understanding of the biological and mechanistic complexities of nuclear receptor activities and further our ability to interpret broad HTS outcomes.

Introduction

Traditional animal-based approaches for testing chemical toxicity has resulted in a number of unmet needs, including the need to assess low-dose chronic exposures, defining appropriate mechanistic targets, establishing the significant economic and ethical expenses associated with *in vivo* testing, and evaluation of an extensive number of chemicals and chemical mixtures which lack appropriate data for health and safety. To address some of these issues, the National Research Council (2007) advocated the "use of mechanistically informative *in vitro* assays in human cell lines that quantify effects on toxicity pathways leading to human disease (NRC, 2007) ".

Abbreviations: Bsep, bile salt export pump; CDCA, chenodeoxycholic acid; DMSO, dimethyl sulfoxide; EPA, U.S. Environmental Protection Agency; FXR, Farnesoid X receptor; M2H, mammalian two-hybrid; qHTS, quantitative high-throughput screening; RXR, retinoid X receptor; Shp, small heterodimer partner; Tox21, Toxicology in the 21st Century.

* Corresponding author.
E-mail addresses: jonathan.hamm@inotivco.com, jhamm@ils-inc.com (J. Hamm), debabrata.mahapatra@inotivco.com (D. Mahapatra), mmknuth@ad.unc.edu (M.M. Knuth), jaleh.abedini@inotivco.com (J. Abedini), malingerfelt@alumni.uncg.edu (M. Lingerfelt), sean@collaborationspharma.com (S. Ekins), swkullma@ncsu.edu (S.W. Kullman).

https://doi.org/10.1016/j.crtox.2022.100092
Received 25 May 2022; Received in revised form 6 October 2022; Accepted 26 October 2022

In response, the U.S. government established the Toxicology in the 21st Century (Tox21) collaboration comprising the National Toxicology Program (NTP), the U.S. Environmental Protection Agency (EPA), the National Institutes of Health National Center for Advancing Translational Sciences (NCATS), and the U.S. Food and Drug Administration (FDA). Tox21 and the EPA ToxCast™ program have established a battery of *in vitro* assays and chemical libraries to achieve the National Research Council's objectives (Kavlock et al., 2009).

The emergence and increased use of such quantitative high-throughput screening (qHTS) approaches for cost effective and rapid profiling of environmental chemicals of potential health concerns has transformed the toxicity-testing landscape (Dix et al., 2007). Results from these studies have demonstrated selected ligand-receptor interactions and have provided a means to discern putative chemical mechanisms (Dix et al., 2007). However, it is recognized that these *in vitro* methods lack organismal complexity, and there are concerns about the ability to extrapolate results of these methods to human toxicity (Wang, 2018). To address this need, there is interest in developing *in vivo* translational methods for extrapolation of *in vitro* toxicity data (Krewski et al., 2010).

Many assays used in the ToxCast and Tox21 programs measure effects on nuclear receptor signaling. Nuclear receptors modulate physiological functions relevant to toxicity, and thus assays measuring chemical effects on nuclear receptors provide mechanistic data that enables predictive assessment of toxicity pathways relevant to human disease. Subsequently, targeted cell-based *in vitro* studies have been conducted to characterize the effects of drugs, food additives, environmental chemicals, consumer product ingredients, industrial chemicals, and environmentally relevant chemicals on receptor function (Dix et al., 2007).

Farnesoid X receptor alpha (FXRα, NR1H4) is a member of the nuclear receptor superfamily that has demonstrated importance in glucose metabolism, lipid and bile acid homeostasis and hepatic regeneration (Fang et al., 2021). The mechanism of FXRα-mediated gene transcription closely resembles that of other steroid hormones. These steps include high-affinity interaction between ligand and receptor, heterodimerization with the retinoid × receptor (RXR) and association with a canonical response element within target promoter regions. This process results in recruitment of coregulatory proteins, members of the Mediator (coactivator) complex, and RNA polymerase II to initiate both transactivation and transrepression of gene regulatory networks critical to cellular processes (Fang et al., 2021; Cai et al., 2021).

In this study, we used a variety of orthogonal methods to assess the activity of a subset of chemicals identified through Tox21 qHTS *in vitro* screens as FXRα agonists and antagonists. The aim of this study was to investigate both reproducibility of Tox21 data output and determine concordance with human and medaka FXR receptor activity profiles using *in vitro* luciferase reporter gene assays. We examined how the structurally and functionally diverse chemicals identified in the Tox21 screen, modify core nuclear receptor functions of FXRα with respect to receptor heterodimerization with human RXRα, recruitment of NR coactivators SRC-1 and PGC1α, and the ability to initiate/inhibit receptor transactivation. Molecular modeling was also employed to forecast and study the molecular interactions of the most potent chemicals once docked in the FXRα binding site. Lastly, we used the small aquarium fish medaka to investigate the ability of FXRα agonists and antagonists to induce or inhibit defined hepatic FXRα transcriptional targets (*Shp, Bsep*), *in vivo*. Overall, our data demonstrate the molecular complexity of ligand-mediated interactions with FXRα and suggest that FXRα transactivation may be a target site of action for diverse xenobiotics.

Materials and methods

Compound selection

The Tox21 10 K compound library (https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=246691) was compiled by EPA, the NTP, and the NCATS (Huang et al., 2011). The library consists of approximately 8,300 unique samples including drugs, food additives, environmental chemicals, consumer product ingredients and industrial chemicals. A cell-based human FXR β-lactamase (Bla) reporter gene assay adapted to qHTS format (Hsu et al. 2014) was used to measure chemical effects on FXRα activity. The qHTS data, processed using the tcpl data pipeline (Filer et al., 2016), were downloaded from the EPA website (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data) and compounds selected for this study were chosen based upon identifying select hFXRα agonists and antagonists that exhibited potent AC50 values (between 8.23 and 4.23 uM) reported from the Tox21 FXR-driven β-lactamase reporter assay. We also considered estimated human exposure potential when selecting test chemicals (Gangwal et al., 2012). Accordingly, we selected a set of 24 FXRα agonists and antagonists demonstrated to significantly transactive or inhibit hFXRα in addition to several inactive chemicals use here as negative controls for evaluation in select orthogonal assays (Table 1).

Compound acquisition for orthogonal assay screening

Prioritized chemicals were either procured from EvoTec (South San Francisco, CA, USA) under EPA contract EP-D-12–034 or purchased from a commercial vendor (Sigma-Aldrich Corp., St. Louis, MO, USA). All chemicals were serially diluted in dimethyl sulfoxide (DMSO) to a final testing concentration ranging from 0.01 to 120 μM.

Human FXRα modeling

Molecular docking was conducted using either Protein Data Base (PDB) code 4qe6 (agonist mode) representing human FXRα bound with chenodeoxycholic acid 4OIV (Antagonist mode) representing FXRα bound to NDB (*N*-Benzyl-N-(3-(*tert*-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) Benzamide. X-ray crystal structures were preprocessed and curated using the Schrödinger Suite and the Protein Preparation Wizard (Schrödinger) module and the OPLS3 force field. All the missing side chains were generated using Prime (Schrödinger) and protein minimization was performed. The molecular docking procedure was performed using Glide software (Friesner et al., 2006) with XP scoring functions with a rigid protein and flexible ligand.

Cell culture

Hek293T cells were used for all transient transactivation and mammalian two hybrid assays (see below). Hek293T cells were grown in minimum essential medium containing 10 % heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ ml streptomycin. COS-7 cells (mammalian two-hybrid assay) were cultured in Dulbecco's Modified Eagle Medium containing 10 % fetal bovine serum. Cells were maintained in a humidified incubator at 37 °C with 5 % CO_2 and passaged when ~ 70–80 % confluent. Media was changed every other day and assays were conducted up to cell passage eight. All cell culture media and other necessary reagents were obtained from Thermofisher (Waltham, MA, USA).

Transient transactivation assay

The pSG5-Human FXRα and the (hsp27EcRE)2-tk-Luc (containing two imperfect IR-1 response elements) constructs were gifted from Dr. Jim Boyer, Yale Liver Center (New Haven, CT, USA). Coregulators for

Table 1
Selection of Test Chemicals.

Chemical Name	CASRN	Activity Decision	Percent of Control[2]	AC50 μM[2]	Notes
Agonists					
Acephate	30560-19-1	Inactive	0	–	Ranked #11 in Gangwal et al. (2012) human exposure estimate
Chenodeoxycholic acid	474-25-9	Active	108.8	27.5	Natural ligand
Cimicifugoside	27994-11-2	Active	24.6	5.0	
Crystal violet lactone	1552-42-7	Active	75.3	92.3	
Daunomycin hydrochloride	23541-50-6	Active	69.8	0.9	
Imazalil	35554-44-0	Active	24.8	24.2	Also active as an antagonist in the Tox21 qHTS data
Iprodione	36734-19-7	Inactive	0	–	Ranked #8 in Gangwal et al. (2012) human exposure estimate
Maleic hydrazide	123-33-1	Inactive	0	–	
Phenolphthalein	77-09-8	Active	60.9	39.8	
Prometon	1610-18-0	Inactive	0	–	
Propazine	139-40-2	Inactive	0	–	Ranked #21 in Gangwal et al. (2012) human exposure estimate
Triphenylphosphine	603-35-0	Inactive	0	–	
Antagonists					
Actinomycin D	50-76-0	Active	105.8	6.75e-4	
Bifenthrin	82657-04-3	Active	46.1	13.3	Ranked #2 in Gangwal et al. (2012) human exposure estimate
Bisphenol B	77-40-7	Active	46.9	57.2	
Colchicine	64-86-8	Inactive	0	–	
Diuron	330-54-1	Inactive	0	–	Ranked #41 in Gangwal et al. (2012) human exposure estimate
Emetine dihydrochloride	316-42-7	Active	108.4	2.5	
Phenolphthalein	77-09-8	Active	60.9	39.8	
Podofilox	518-28-5	Active	60.7	1.47e-2	
Tricaprylin	538-23-8	Inactive	0	–	
Clorphocinone[1]	3691-35-8	Active	94.6	5.3	Reported in the literature to be an FXR antagonist. Tox21 qHTS data became available during experimentation.
Ivermectin[1]	70288-86-7	Active	NA	NA	Reported in the literature to be an FXR antagonist.
Moxidectin[1]	113507-06-5	Active/Inactive	NA	NA	Reported in the literature to be an FXR antagonist. Tox21 qHTS data became available during experimentation.

[1] Chlorphocinone, Ivermectin, Moxidectin were reported to be antagonists in Hsu et al. 2016.[2]Data retrieved from the US EPA CompTox Chemicals Dashboard located at: https://comptox.epa.gov/dashboard/.

transient transactivation and mammalian 2-hybrid constructs were gifted from Dr. Donald McDonnell, Duke University (Durham, NC, USA). Medaka FXRα2 was cloned as described in Howarth et al., 2010. Transient transactivation assays used HEK293T cells transfected with full-length human and medaka FXR constructs. The FXRα agonist GW4064 (3-(2,6-Dichlorophenyl)-4-(3′-carboxy-2-chlorostilben-4-yl) oxymethyl-5-isopropylisoxazole, Millipore-Sigma) was used as a positive control to verify that the assays reported FXRα activation activity by test chemicals. Experiments with reported Tox21 FXRα agonists and antagonists were conducted in cells seeded in 96-well plates at 2.5×10^4 cells per well 24 h prior to transfection. Cells were transfected at 90–95 % confluency using Lipofectamine 2000 (Thermofisher, Waltham, MA) with DNA diluted in Opti-MEM I Reduced Serum Medium (Thermofisher, Waltham, MA) as per the manufacturer's recommendations. Transactivation assays contained 20.0 ng/well of human FXRα or medaka FXRα2 construct transiently transfected into HEK293T cells with 60.0 ng/well (hsp27EcRE)2-tk-Luciferase reporter, pCDNA-RXR 20 ng/well and pGC1a 40 ng/well as coregulators and 15 ng of Renilla luciferase, which served as an internal luciferase control (Promega Corporation, Madison, WI). Transfected cells were treated with selected Tox21 chemicals diluted in DMSO to concentrations ranging from 0.1 uM to 120uM with a final DMSO concentration of 0.1 %. At 24 h post-exposure the Dual-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA) was used to passively lyse the cells and test for luciferase activity following the manufacturer's protocols. Luciferase activities were measured using a Wallac MicroBet TriLuc luminometer (Perkin Elmer Life Sciences, Waltham, MA, USA). Control reactions included empty pSG5 vector and DMSO as a vehicle control. Luciferase readings were normalized to the internal Renilla control, and FXRα response was normalized to an empty vector control. All experiments were replicated three times in groups of three technical replicate wells. Data were modeled using log transformed nonlinear regression algorithm with Prism 8.0 (GraphPad Software, Inc, CA).

Mammalian two-hybrid assays

Protein-protein interactions between FXRα, its heterodimer partner RXRα, and members of the nuclear receptor coactivator family SRC1 or PGC1a were assessed using a mammalian two-hybrid system (Clontech, Mountain View, CA, USA). Assays were conducted with chimeric FXRs containing the herpes simplex VP16 activation domain fused to full-length human FXR (pVP16-FXR; pVP16-h FXRα or pVP16-mFXRα2). NR coregulators consisted of fusion proteins containing a complete NR Box of the hSRC-1 (pM-SRC1 aa 241–386), the NR box of hPGC1α or full-length hRXRα fused to the yeast Gal4 DNA-binding domain. COS-7 cells were seeded into 96-well plates 24 h pretransfection as described above. Cells were transfected with 70 ng pVP16-FXR, 50 ng pM-coregulator (SRC1, PGC1α, RXR), 200 ng 5XGal4-TATA-Luc reporter (containing response elements for the yeast Gal4 DNA-binding domain), and 15 ng *Renilla* using Lipofectamine 2000 as described above. All assays were conducted in either the presence or absence of cotransfected full-length RXR, SRC-1 or PGC1a (50 ng/well) to assess if exogenous protein expression would further facilitate FXRα coregulator/coactivator interactions. Negative controls consisted of transfections containing empty pM, pVP16 or both empty pM and pVP16 vectors. All experiments were replicated three times in groups of three technical replicate wells.

In vivo exposures

Medaka (*Oryzias latipes*; Cab strain) embryos were collected from breeding tanks, cleaned, and staged according to previously established methods (Iwamatsu, 2004). Fertilized embryos were distributed to six-well tissue culture plates containing five mL of 1X embryo rearing medium (17.1 mM NaCl, 272 μM $CaCl_2$-$2H_2O$, 402 μM KCl, and 661 μM $MgSO_4$-$7H_2O$, pH 7.4) at a density of 15 embryos/well. Embryos were allowed to grow for 14 days, reaching a weight of 0.8–0.9 mg. During grow out, embryos were fed Otohime B1 larval diet (Reed Mariculture, Campbell, CA) and embryo rearing media was replaced every 48 hrs to maintain water quality and minimize changes to pH and ammonia accumulation. When larvae reached 14 days of growth, FXR agonists and antagonists were added to a final aqueous concentration of 10 μM (diluted in DMSO, final concentration 0.1 %), and exposed for a total of 48 hrs. Exposures were maintained in a 26 °C incubator on a rotating shaker with embryo rearing medium changed and re-dosed at 24 hrs. Larvae were fed for the duration of the experiment and observed repeatedly for physical distress. After 48 hrs of exposure, larvae were collected, anesthetized by immersion in ice-cold water, and processed for RNA extraction, cDNA synthesis, and quantitative real-time PCR. All antagonist assays were conducted in the presence of 125 nM GW4064 and 0.25 μM selected FXRα antagonists. These conditions were optimal for alleviating observed cytotoxicity.

Gene expression by real-time PCR

Total RNA was isolated from treated whole medaka larvae using the Zymo RNA Isolation kit (Zymo Research, Irvine, CA, USA) and reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using the QuantiTect SYBR Green PCR kit (Qiagen, Germantown, MD, USA). Relative mRNA abundance of known FXR gene targets were assessed. Expression of mRNA was normalized against expression of the housekeeping gene β-actin. Primers used for medaka small heterodimer partner (Shp) and bile salt export pump (Bsep) are as follows (Bsep: forward 5′-TGAACTGCTGGAGAGGAAGG-3′, reverse 5′-GCGACGTTGATGGAGGATTC-3′; Shp: forward 5′-AGCCAA-GACTGCCTCAACTA-3′, reverse 5′- TGAGCCAGCCCCAGAATAAA-3′; β-actin forward 5- TCCACCTTCCAGCAGATGTG-3′, reverse 5′-AGCATTTGCGGTGGACGAT-3′). Fold gene induction following treatments was calculated as 2ΔΔCt, where ΔCt represents the differences in cycle threshold numbers between gene target and β-actin, and ΔΔCt represents the relative change in these differences between control and treatment groups (Pfaffi, 2001). Values were plotted as a percentage and compared to the percentage induction of 0.125 μM GW4064, a potent FXRα agonist.

Statistics

Statistical significance of results from both the transient transactivation and mammalian two-hybrid assays was ascertained by one-way analyses of variance. These analyses were followed by Tukey's honest significant difference post-hoc tests, sigmoidal dose–response calculation with variable slopes, and nonlinear regression analyses. All analyses were run in GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Real-time PCR data were tested for significant differences within individual treatment using unpaired *t*-test ($\alpha = 0.05$).

Results

Transactivation studies

Transactivation studies were conducted to validate activity of 24 putative Tox21 human FXRα and medaka FXRα2 ligands as receptor agonists or antagonists (Table 2, **Supplemental Table 3**). All assays were preformed using a standard receptor transactivation assay comprised of full-length constructs for hFXRα or mFXRα2 (in pSG5), a IR-1 HRE-luciferase reporter assay, human PCG1α (in pSG5), and *Renilla* luciferase (pRL-CMV, Promega Corp., Madison, WI, USA) for normalization in HeK293 cells. AC50 values for FXRα agonists (0.1 μM-125 μM) were determined relative to GW4064 as a positive control (Fig. 1). Of the 12 chemicals selected for evaluation of agonist activity, 6/6 putative agonists were confirmed to exhibit human FXRα transactivation activity 5/6 Tox21 inactive compounds were confirmed, and one inactive compound from the Tox21 dataset proved active in this confirmation assessment (Table 1) Tested agonists exhibited a wide spectrum of FXRα potencies, with AC50 values ranging from 9.8 μM for cimicifugoside to $>$ 100 μM for crystal violet lactone and phenolphthalein (Table 2, Fig. 1**A**). Of the the six inactive compounds, five including: acephate, propazine, maleic hydrazide, prometon and triphenylphosphine, consistently failed to facilitate FXRα transactivation and were thus confirmed inactive (Table 2). Conversely, while iprodione was designated inactive in the Tox21 dataset, we observed significant transactivation with human FXRα (AC50 37.25).

Assessment of Medaka FXRα2 transactivation with the same 12 chemicals (Table 2) was consistent with observed human activities confirming 6/6 active agonists and 5/6 inactive compounds. Again, we observed transactivation activity with iprodione. Which was determined inactive in Tox21 screens. Observed potencies ranged between 30.3 μM for daunomycin hydrochloride to $>$ 100 μM for both cimicifugoside and phenolphthalein illustrating a narrower range of potencies than observed with human FXRα. Crystal violet lactone exhibited a noticeable increase in activity in this system with an AC50 of 50.76 μM compared to human FRXα (AC50 $>$ 100 μM). Conversely, cimicifugoside was only weakly active with mFXRα2 (AC50 $>$ 100 μM) compared to human FXRα (AC50 = 9.8 μM), indicating some evolutionary divergence in ligand specificity. Conversely, both human FXRα and medaka FXRα2 exhibited comparable transactivational activity with chenodeoxycholic acid, a secondary C24 bile acid common to both species (Howarth et al., 2010a; Howarth et al., 2010b). Additionally, phenolphthalein was relatively inactive with both medaka mFXRα2 and human FXRα which is contrary to data reported in the Tox21 dataset (Huang et al., 2011). The five chemicals identified as putative inactive compounds in the Tox21 screen including acephate, propazine, maleic hydrazide, prometon and triphenylphosphine were confirmed as inactive with medaka FXRα2 consistent with observed activities with human FXRα.

FXRα antagonists were assessed by quantifying inhibition of 100 nM GW4064-induced responses in human FXRα transient transactivation assays. From a total of 12 selected FXRα antagonists (nine identified in Tox21 screening, three selected from Gangwal et. al., 2012 based on the potential for human exposure), 8/9 Tox21 antagonists were confirmed active with human FXRα in this study. The activity for active antagonists varied widely, with AC50 values ranging between 2.35 μM for ivermectin up to 55.1 μM for phenolphthalein. Fig. 2**A** and Table 2 illustrate functional potencies of selected antagonists, demonstrating potential differences between partial and full antagonists. One antagonist, podofilox, that demonstrated human FXRα activity in the Tox21 screen proved to be inactive in our transactivation assays. We next confirmed that both Colchicine and tricaprylin are inactive as human FXRα antagonists, however the aryl urea herbicide diuron exhibited significant transactivation activity with human FXRα contrary to reported inactivity in the Tox21 dataset (Fig. 2**A**, Fig. 2**B**).

With medaka FXRα2 we were able to confirm 7/9 active antagonists tested (Table 2**)**. Consistent with human FXRα, chlorphocinone, ivermectin, and moxidectin proved to be potent and efficacious antagonists in the medaka assay (AC50s between 5.5 and 11.2) (Fig. 2**B**, Table 2). Bifenthrin, phenolphthalein, emetine dihydrochloride, and bisphenol B also exhibited antagonist activity with mFXRα2. We observed no transactivation with actinomycin D and similar to human FXRα,

Table 2
Activity of FXR Agonists and Antagonists with Human FXRa, and Medaka FXRa2.

Activity Human FXRa AGONISTS/ANTAGONISTS		Tox21 Results Human FXRa		Current Study Human FXRa		Current Study Medaka FXRa2	
CASRN	CHEMICAL	Activity Human FXRa	AC50 μM	Activity Human FXRa	AC50 μM	Activity Medaka FXRa2	AC50 μM
AGONISTS							
23541-50-6	Daunomycin hydrochloride	Active	6.19	Active	38.31	Active	30.29
474-25-9	Chenodeoxycholic acid	Active	4.61	Active	40.08	Active	41.44
27994-11-2	Cimicifugoside	Active	5.52	Active	9.8	Active	>100
1552-42-7	Crystal violet lactone	Active	4.29	Active	>100	Active	50.76
77-09-8	Phenolphthalein	Active	4.91	Active	>100	Active	>100
35554-44-0	Imazalil	Active	4.31	Active	78.81	Active	80.48
36734-19-7	Iprodione*	Inactive	-	Active	37.25	Active	73.37
603-35-0	Triphenylphosphine	Inactive	–	Inactive	–	Inactive	–
30560-19-1	Acephate	Inactive	–	Inactive	–	Inactive	–
139-40-2	Propazine	Inactive	–	Inactive	–	Inactive	–
1610-18-0	Prometon	Inactive	–	Inactive	–	Inactive	–
123-33-1	Maleic hydrazide	Inactive	–	Inactive	–	Inactive	–
ANTAGONISTS							
518-28-5	Podofilox*	Active	7.52	Inactive	–	Inactive	–
316-42-7	Emetine dihydrochloride	Active	5.66	Active	3.6	Active	22.5
50-76-0	Actinomycin D*	Active	8.23	Active	34.09	Inactive	–
82657-04-3	Bifenthrin	Active	4.96	Active	34.63	Active	52.58
77-09-8	Phenolphthalein	Active	4.495	Active	55.11	Active	43.15
77-40-7	Bisphenol B	Active	4.26	Active	11.91	Active	3.87
3691-35-8	Chlorphocinone[1]	Active	NA	Active	8.11	Active	5.45
70288-86-7	Ivermectin[1]	Active	NA	Active	2.35	Active	11.23
113507-06-5	Moxidectin[1]	Active/Inactive	NA	Active	12.54	Active	6.34
64-86-8	Colchicine	Inactive	–	Inactive	–	Inactive	–
538-23-8	Tricaprylin*	Inactive	–	Inactive	–	Active	1.24
330-54-1	Diuron*	Inactive	–	Active	28.33	Active	17.83

[1] Chlorphocinone, Ivermectin, Moxidectin were reported to be antagonists in Hsu et al. 2016. Moxidectin was reported as inactive in the Tox21 qHTS data. * Indicates difference between Tox21 reported activity and observed transactivation activity in this study.

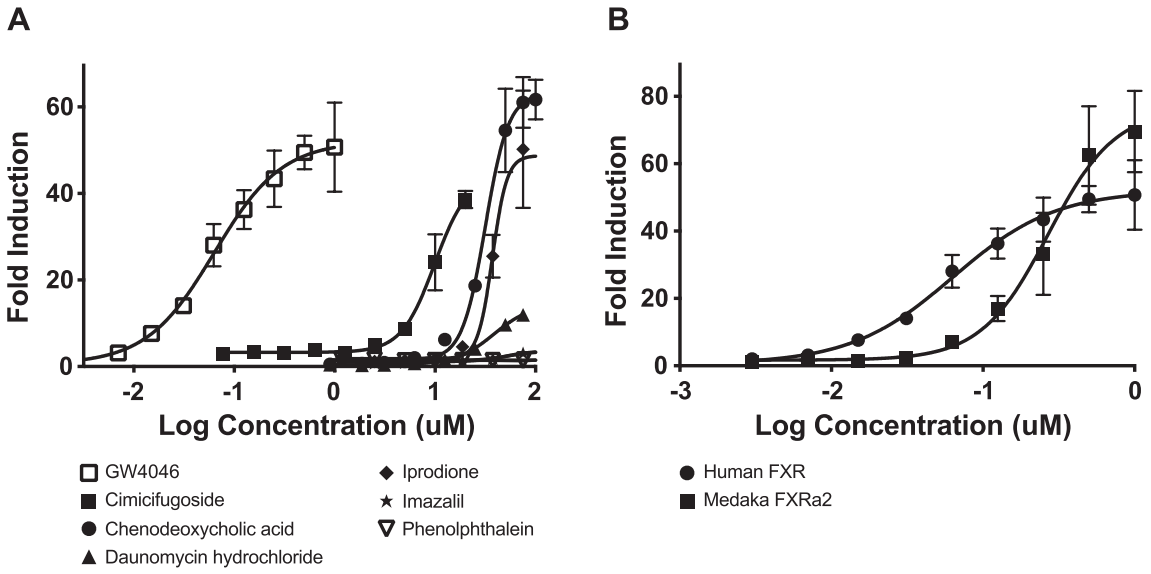


Fig. 1. Representative concentration response curves of select FXR agonists identified by transient transactivation assay in HEK293T cells with the (hsp27EcRE)2-tk-Luciferase reporter. A. human FXRα agonists. Data represents fold induction of human FXRα with GW4046 (open square, EC50 0.0632), Cimicifugoside (Closed square, EC50 9.8uM), Chenodeoxycholic acid (circle, EC50 40.1uM), Daunomycin hydrochloride (triangle, EC50 38.3uM), Iprodione (diamond, EC50 37.3), Imazalil (star, EC50 78.8uM), Phenolphthalein (open diamond EC50 > 100uM). Data expressed as mean ± SEM (n = 3). B. Representative concentration response curves of GW4064 as a positive control agonist for demonstrating functional transactivation of both human FXRα (circle) (EC50 0.0632uM) and medaka FXRα2 (square (EC50 0.2688uM). Assays were run in HEK293T cells with the (hsp27EcRE)2-tk-Luciferase reporter and data are expressed as mean ± SEM (n = 3).

podofilox was inactive in medaka FXRα2 transactivation assays contrary to Tox21 where podofilox exhibited FXRα activity. Three inactive compounds were also tested as negative controls. Interesting we were able to confirm only colchicine with medaka FXRα2. The other two compounds, tricaprylin and diuron, exhibited antagonist activity with medaka FXRα2. Tricaprylin proved to be rather potent with an AC50 of 1.24 μM. Activity of diuron as a functional medaka FXRα2 antagonist is consistent with human FXRα further illustrating some inconsistency with reported activities within the Tox21 dataset.

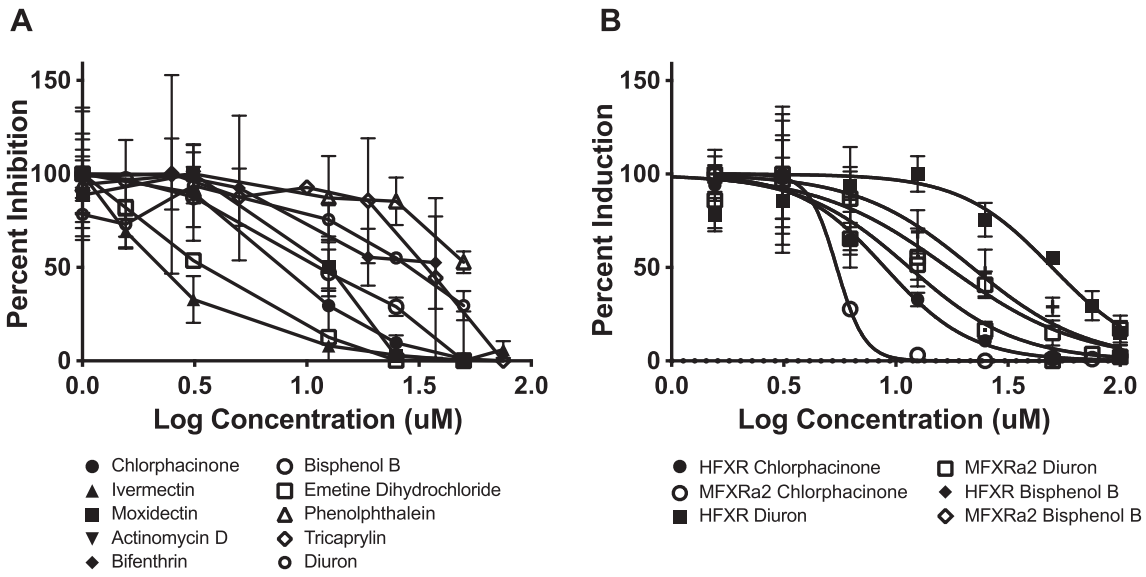


Fig. 2. A. Representative concentration response curves of select FXR antagonists identified by transient transactivation assay in HEK cells with the (hsp27EcRE)2-tk-Luciferase reporter. Assays we conducted in the presence of 100 nM GW4064 as a potent agonist. A. Human FXRα with Chlorphacinone (open circles EC50 8.1uM), Ivermectin (closed triangle EC50 2.4 uM), Moxidectin (closed square EC50 12.5 uM), Bifenthrin (closed diamond EC50 34.6uM), Bisphenol B (closed circle 11.9uM), Emetine Dihydrochloride (open square EC50 3.6 uM), Phenolphthalein (open triangle EC50 55.1uM), Tricaprylin (open diamond EC50 nd), Diuron (star EC50 28.3uM). All data has been normalized to set max transactivation to (1 0 0) and bottom (0) in order to compare activities across chemicals. B. Comparison of human FXRα (closed symbols) and medaka FXRα2 (open symbols) activities. Chlorphacinone (circles, hFXRα EC50 8.1uM, mFXRα2 EC50 5.45 uM), Diuron (Squares, hFXRα EC50 28.3, mFXRα2 EC50 17.83 uM), Bisphenol B (diamonds, hFXRα EC50 11.9, mFXRα2 EC 50 3.9). Data expressed as mean ± SEM (n = 3). Note data were normalized to set max transactivation to (1 0 0) and bottom (0) in order to compare activities across species.

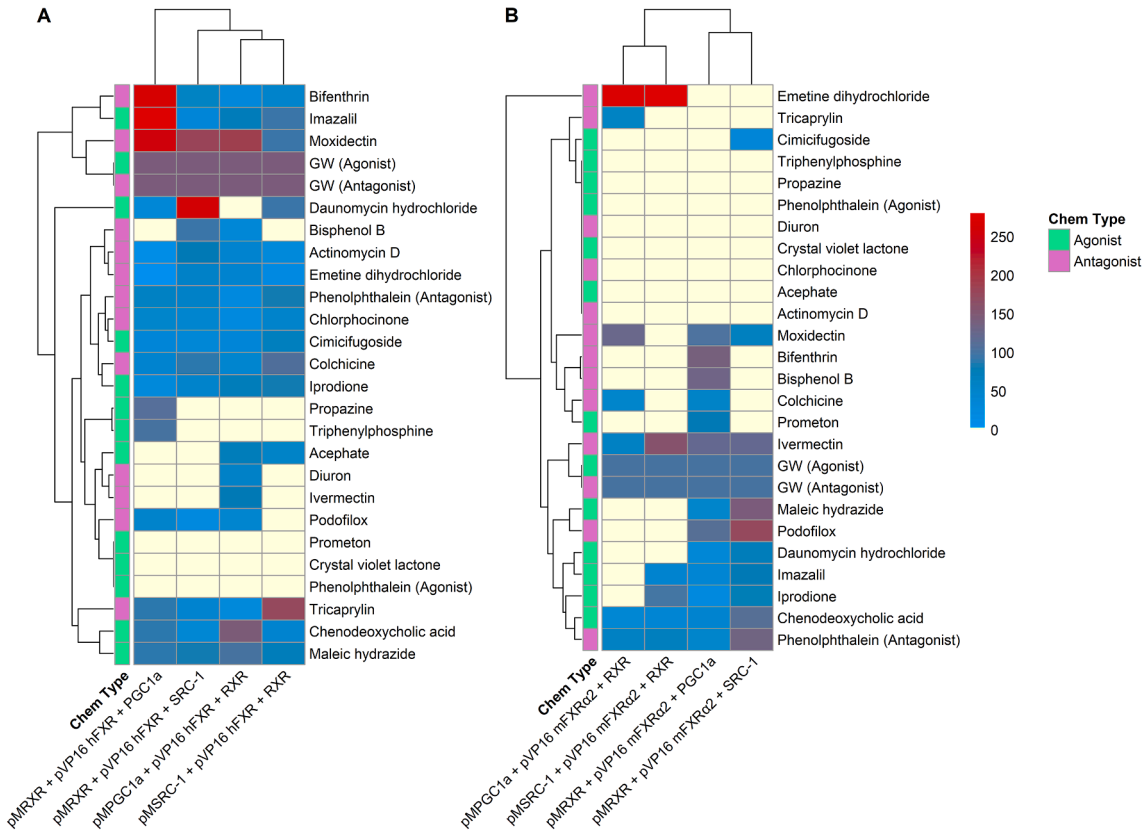


Fig. 3. Heat map showing the variability in the selective preference of chemicals to enhance or inhibit the ability of FXR to recruit coregulator (RXRα) and coactivators SRC-1 or PGC1α. Higher recruitment values are indicated in red while lower values are in blue. A Human FXRα. B Medaka FXRα2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Protein-protein interactions

While transient transfection assays provide a global context of chemical receptor transactivation, we next conducted further functional analysis of ligand induced receptor:coregulator interactions to gain mechanistic insights into FXRα-chemical partnerships. The mammalian two-hybrid (M2H) assay is a robust tool for studying protein–protein interactions between structural domains or full-length nuclear receptors and other proteins associated with transactivation. We thus conducted M2H assays to examine protein:protein interactions between FXRα and its obligate heterodimerization partner RXR. We also examined interactions between FXRα and selected nuclear receptor coregulators including SRC-1 and PGC1α.

In general, agonists and antagonists that exhibited receptor transactivation with human FXRα also exhibited significant coregulator recruitment. Of the active agonists a total of 4/6 compounds tested resulted in coregulator recruitment. For example, the well described low affinity agonist chenodeoxycholic acid (CDCA) exhibited recruitment of RXR, PGC-1α and SRC-1 in the presence of 100 μM agonist (Fig. 3**A**, **Supplementary** Table 1). Similarly, treatment with the agonists cimicifugoside, and imazalil, facilitated recruitment of RXR, PGC-1α and SRC-1 with human FXRα. Daunomycin hydrochloride, facilitated interactions between hFXRα and RXR and induced a preferential interaction with SRC-1 over PCG1α. Interestingly, 5/6 inactive (Tox21 and our orthogonal assays) compounds tested exhibited recruitment of coregulators. Iprodione, which is inactive in the Tox21 FXR dataset but active in our assays demonstrated recruitment of RXR, PGC-1α and SRC-1 with human FXRα. Acephate, malaic hydrazide, propazine, triphenylphosphine, each facilitated recruitment between hFXRα, PGC-1α /SRC-1 and or RXR. Notably, two compounds, crystal violet lactone and phenolphthalein did not facilitate recruitment between human FXRα and any coregulators in concert with their low transactivation activity. This observation suggests that these chemicals are likely capable of binding hFXRα, altering receptor protein conformation from an apo to holo form, but are insufficient to facilitate transactivation.

Consistent with human FXRα, medaka FXR2α:RXR interactions occurred across a broad spectrum of active receptor agonists and inactive compound controls in the presence of over expressed PGC1α and SRC1 (Fig. 3**B**, **Supplementary** Table 2). Conversely, medaka FXRα2 exhibited a markedly different coregulator (SRC-1, PGC-1α) interaction profile from that of human FXRα. Limited interactions were observed between mFXRα2 and PGC-1α with only GW4064 and CDCA facilitating PGC-1α recruitment. Similarly, only two active agonists including CDCA, imazalil, facilitated recruitment between mFXRα2 and SRC-1. By comparison, a few inactive compound controls including prometon and maleic hydrazide facilitated mFXRα2 and SRC-1 interactions. However, we also observed significant recruitment between mFXRα2 and SRC1 with iprodione which is considered in active in the Tox21 dataset but is active in our transactivation assays.

As with agonists, FXRα antagonists exhibited diverse and complex ligand induced protein:protein interactions between human FXRα and coregulators. In general antagonists attenuated GW4064 induced protein:protein recruitment between human FXRα:RXR, human FXRα:PGC1α and human FXRα:SRC1 (Fig. 3**A**, **Supplementary** Table 1). The magnitude of attenuation was most significant with direct FXRα:PGC1α interactions and with indirect interactions between human FXRα:RXR and either PGC1α or SRC1. In several instances, attenuation of protein:protein interactions appeared to be coregulator specific. For example, ivermectin selectively attenuated human FXRα:PGC1α interactions, while no loss in recruitment was observed between human FXRα:SRC1 or human FXRα:RXR. Several antagonists additionally facilitated human FXRα:coregulator interactions, demonstrating enhanced recruitment in the presence of GW4064. For example, moxidectin enhanced recruitment of FXRα:PGC1α interactions (132 %) and FXRα:RXR interactions in the presence of PGC1α (179 %) and SRC1 (127 %) above that observed with GW4064 alone. Yet, moxidectin proved to be a potent inhibitor of human FXRα transactivation in our assays.

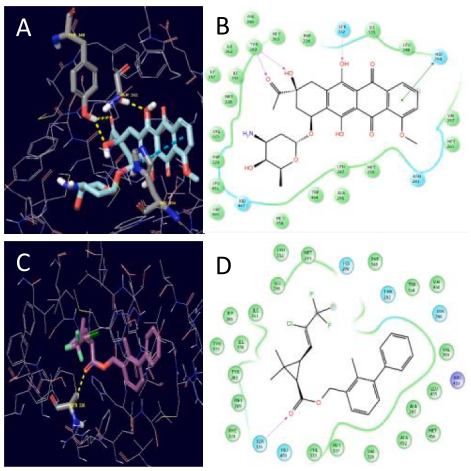
Medaka FXRα2 additionally exhibited selective interactions with NR coregulators in the presence of antagonists (**Supplementary** Table 2). Two of the nine active antagonists (phenolphthalein, ivermectin) attenuated mFXRα2:PGC1α interactions, while a single compound, phenolphthalein was effective towards attenuating mFXRα2:SRC1 interactions. Attenuation of mFXRα2:RXR interactions proved marginal however, with only two compounds phenolphthalein and moxidectin exhibiting activity. Interestingly, phenolphthalein attenuated mFXRα2:RXR interactions only in the presence of PGC1α and moxidectin only in the presence of SRC1 expression. Like human FXRα, select antagonists were able to directly facilitate enhanced recruitment of mFXRα2 coregulator interactions contrary to expected outcomes. For instance, moxidectin facilitated recruitment of mFXRα2:PGC1α interactions 126 %. Similarly, select antagonists facilitated mFXRα2:RXR recruitment however this interaction appeared to be coregulator dependent. For instance, active antagonists, bifenthrin and ivermectin, facilitated mFXRα2:RXR only in the presence of PGC1α. Podofilox, which is inactive in our assays, behaved similarly. Conversely, phenolphthalein and podofilox facilitated recruitment of mFXRα2:RXR only in the presence of SRC1. As with human FXRα, select compounds (colchicine, podofilox) that do not exhibit transcriptional activity do facilitate protein:protein interactions. Overall protein interaction results are consistent with the observation that the holo conformation of FXRα and mFXRα2 is ligand-specific and is pivotal for revealing receptor:coregulator interaction domains associated with RXR heterodimerization and recruitment on coactivators and corepressors.

Cheminformatics modeling of FXR-ligand interactions

We next conducted 3D molecular docking studies for each active compound identified in our screening assays so that we could evaluate and better understand their respective binding modes in the human FXRα active site. Since the first co-crystalized structure of the FXRα receptor was reported in 2003/2004, multiple X-ray structures of the FXRα receptor in complex with different small molecule ligands have been published and deposited in the online Protein Data Bank (https://www.wwpdb.org/). In this initial analysis, we compared FXRα agonists and antagonists to select X-ray structures for the human FXRα co-crystalized with CDCA (PBD 4QE6) as a prototypic agonist and NDB as a selective antagonist (PBD 4OIV). The computational screening tool Glide (Schrödinger, New York, NY, USA) was used to dock Structure Data File (SDF) input of both agonists and antagonists of human FXRα. Fig. 4**A-D** illustrate representative docking images of daunomycin hydrochloride (active in both human and medaka FXRα transactivation assays) as the top scoring agonist and bifenthrin (active in human and weakly active in medaka transactivation assays) as the top scoring antagonist. In each image, the interactions between the chemical structure and FXRα ligand binding domain amino acid residues are indicated with yellow dotted lines representing H-bonds and dotted cyan line representing pi-pi interactions. Structure depictions are also included illustrating specific ligand interactions. Green residues surrounding the ligand indicate hydrophobic interactions while pale blue indicates polarity. Fig. 4**E** lists the "Glide scores" (Halgren et al., 2004) for all evaluated chemicals, with smaller values representing a more efficient docking within the crystal structure. Ligands not listed fell above the cutoff energy that will allow Glide to dock a compound. These data support a direct compound:receptor interaction but do not distinguish between receptor transactivation or receptor transrepression.

In vivo assessment of FXR ligands

In these assays we utilized a small aquarium fish model to validate FXRα agonists/antagonist activity. Based on data previously established (Howarth et al., 2010b), we utilized the FXRα agonists GW4064 as a model ligand to induce FXRα hepatic gene targets including Shp and



E

Agonists docked into PBD 4qe6	Glide score	Antagonists docked into PBD 4OIV	Glide score
Chenodeoxycholic acid	-12.70	Bifenthrin	-9.90
Daunomycin hydrochloride	-11.70	Chlorphocinone	-8.50
Phenolphthalein	-8.80	Phenolphthalein	-7.90
Triphenylphosphine	-8.00	Emetine dihydrochloride	-6.70
Iprodione	-8.00	Bisphenol B	-6.40
Imazalil	-7.00	Diuron	-4.50
Prometon	-5.40	Tricaprylin	-3.50
Propazine	-5.00		
Acephate	-4.30		
Maleic hydrazide	-3.60		

Fig. 4. Structure-based molecular docking using Glide and the human FXR structure PDB code 4Qe6 for agonists and PDB code 4OIV for antagonists. A. Binding modes of Daunomycin hydrochloride as a prototypic agonist (A, B) and Bifenthrin as a prototypic antagonist (C, D) superimposed in the binding site. Yellow dotted lines represent H-bonds and dotted cyan line represent pi-pi interactions. B. Structure depiction of daunomycin hydrochloride illustrating ligand interactions with designated amino acid residues. Green residues surrounding the ligand indicate hydrophobic interactions while pale blue indicates polarity. C. interactions between the bifenthrin chemical structure and FXR ligand binding domain amino acid residues. Interactions as above. D. interactions between the bifenthrin chemical structure and FXR ligand binding domain amino acid residues. Interactions depicted as above. E. Docking results for all chemicals with their associated XP docking and eModel scores. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bsep. Gene expression data for nine of the 12 Tox21 FXRα agonists (active and inactive) are provided in Fig. 5 and reported as percent induction in relation to our solvent control DMSO**.** The remaining Tox21 agonists (active and inactive) maleic hydrazide, phenolphthalein, and triphenylphosphine exhibited significant cytotoxicity between 0.5 and 10 μM and thus could not be evaluated. Exposure to GW4064, a potent agonist for both human and medaka FXRα *in vitro*, resulted in a robust induction of both Bsep and Shp as anticipated. Likewise, individual FXRα agonists produced robust to moderate induction of Bsep (108–18.5 %) and Shp (73.8–10.3 %) relative to the maximal induction of GW4064 positive control. **Supplemental Table 3** additionally reports FXR agonists data in relation to GW4064 as a positive control with cimicifugoside exhibiting the highest level of Bsep and Shp induction of all agonists examined.

All FXR antagonists (active and inactive), were evaluated in the presence of 125 nM GW4064 and 0.25 μM selected antagonists. These conditions were optimized to minimize larval toxicity and enabled the assessment of modulation in GW4064 induced gene targets. Select antagonists (active and inactive) proved to be highly effective at attenuating induction of Bsep and Shp by GW4064 (Fig. 6). Actinomycin D, bisphenol B, diuron, emetine dyhydrochloride, phenolphthalein, chlorophacinone (all active in transactivation assays) and tricaprylin (inactive in transactivation assays) attenuated *in vivo* induction by GW4064 95.7–76.9 % for Bsep and 95.6–74.6 % for Shp (**Supplementary Table 3**). Conversely, several compounds including colchicine, podofilox, bifenthrin, ivermectin and moxidectin could not be evaluated due to continued overt larval toxicity during the assay.

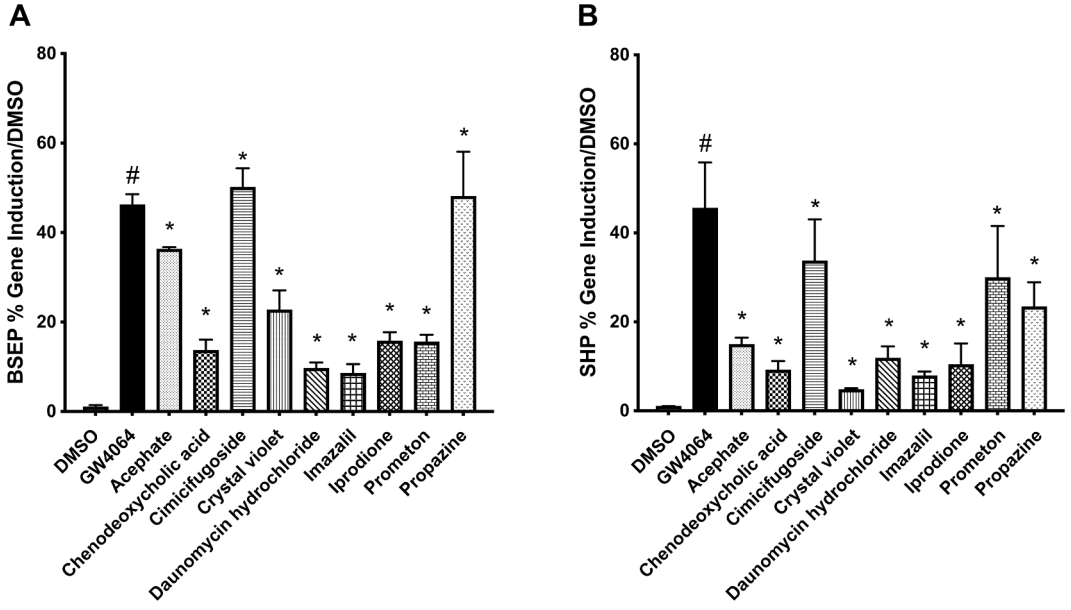


Fig. 5. Medaka *in vivo* exposures to Tox21 FXR agonists. qPCR results of *Bsep* (A) and *Shp* (B). GW4064 a potent FXR agonist is used as a positive control. All values are for FXR agonists have been normalized to DMSO and are presented as a percentage induction compared to the solvent control. All assays were conducted as an N of three biological replicates. * Indicates significance of at least $p < 0.05$ between DMSO and GW4064, or DMSO and FXR agonists.

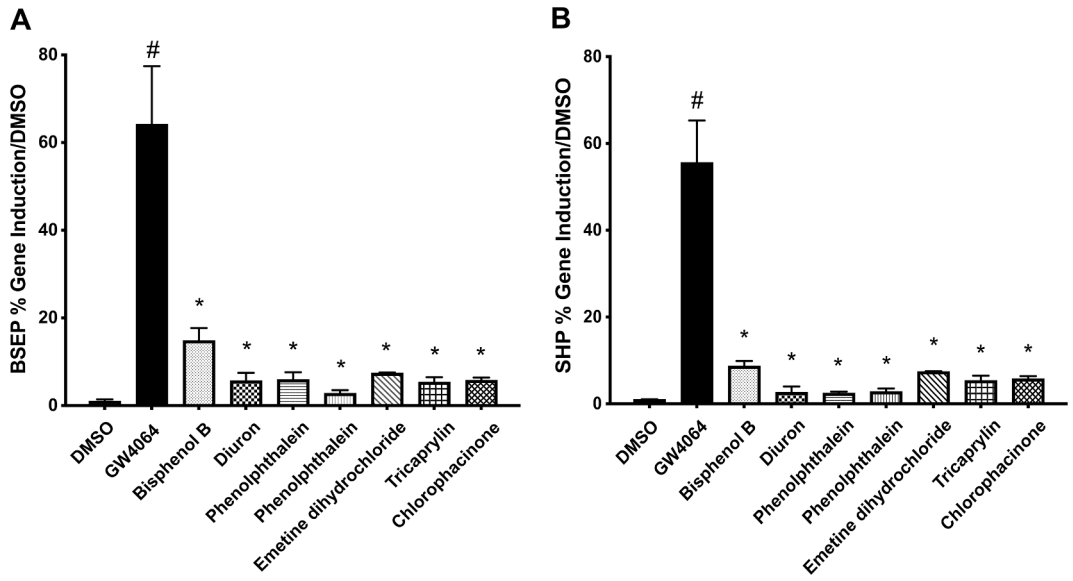


Fig. 6. Medaka *in vivo* exposures to FXR antagonists. qPCR results of Bsep (A) and Shp (B). All assays were conducted in the presence of 1uM GW4064; a potent FXR agonist. All values are normalized to GW4064 and are presented as a percentage of 0.125uM GW4064 induction. All assays were conducted as an N of three biological replicates. # Indicates significance of at least $p < 0.05$ between DMSO and GW4064, * Indicates significance of at least $p < 0.05$ between GW4064 and FXR antagonists.

Discussion

FXRα is a member of the nuclear receptor superfamily and plays an important role in a number of biological processes including bile acid homeostasis, glucose metabolism, lipid synthesis and metabolism and hepatic regeneration (Fang et al., 2021). In this study, we sought to confirm Tox21 screening results for FXRα agonists and antagonists and expand our understanding of FXRα functional modalities using orthogonal assays. Using select cell-based assays including receptor transactivation, coregulator recruitment, and *in silico* modeling we demonstrated that FXRα undergoes complex ligand -receptor interactions that facilitate its role as a transcriptional regulator. We also utilized a small aquarium *in vivo* fish model to illustrate how translational models may facilitate a greater biological understanding of receptor activation/repression and further our ability to interpret results obtained from *in vitro* qHTS assays.

Mechanisms associated with toxicity of environmental chemicals often involve interaction with components of the transcriptional machinery at the molecular level. In this regard nuclear receptors present themselves as attractive targets for exogenous molecules (Delfosse et al., 2015). Nuclear receptors are ligand-dependent transcription factors that bind to lipophilic signaling molecules resulting in the control and expression of target genes. They facilitate the cellular response to endogenous and exogenous ligands by coordinating complex transcriptional responses (Mangelsdorf and Evans, 1995). The nuclear receptor superfamily includes receptors for multiple endobiotics including steroids hormones, retinoids, thyroid hormone, vitamin D, prostaglandins, in addition to exogenous ligands including dietary components and xenobiotics. Most nuclear receptors share a common structure with a conserved *N*-terminal DNA-binding domain and a C-terminal ligand-binding domain. The ligand-binding domain confers ligand specificity and contains a ligand-inducible transactivation function (AF2) essential for transcriptional activation. In the absence of a ligand, nuclear receptors are associated with a nuclear receptor corepressor complex, resulting in inhibition of basal transcription activity of the associated promoter (Mangelsdorf and Evans, 1995; Ordentlich, 2001). Corepressor proteins (SMART, NCoR) couple non-liganded, DNA-bound nuclear receptors to enzymes with histone deacetylase activity resulting in chromatin condensation and a subsequent repression of gene expression (Polly et al., 2000). Ligand binding causes a conformational change within the carboxy-terminal ligand-binding domain resulting in release of corepressors and facilitating interaction with additional nuclear proteins, including RXR, coactivators, and mediator proteins (Glass and Rosenfeld, 2000). These coactivators couple the ligand-activated NRs to enzymes displaying histone acetyltransferase activity facilitating chromatin remodeling.

Tox 21 and ToxCast programs serve as publicly available sources of data that can be used for application of computational and predictive principles for hazard identification and risk assessment and generating hypotheses on toxicity mechanisms (Dix et al., 2007). Previous studies using qHTS data have demonstrated diverse ligand interactions with human FXRα. A pilot screen of Tox21 ligands illustrated that select environmental chemicals and drugs acted as human FXRα agonists capable of transactivation of a beta lactamase reporter system (Huang et al., 2011). Interestingly the authors demonstrated high concordance between receptor activities and nuclear receptor ligand-binding domain sequence phylogeny. Conversely, FXRα antagonists did not demonstrate this relationship, most likely due to differences in ligand-binding characteristics with the ligand-binding domain or allosteric or noncompetitive binding interactions within other regions of the nuclear receptor. A second study conducted across the entire Tox21 10 K compound collection of environmental chemicals and drugs demonstrated that human FXRα exhibits a wide range and diverse ligand interaction profile encompassing several structural classes including anthracyclines, benzimidazoles, dihydropyridines, pyrethroids, retinoic acids, and vinca alkaloids.

Results presented in this study are consistent with previous findings, indicating that FXR exhibits a notable breadth in ligand interactions. We confirm that that 5/5 designated Tox21 FXRα agonists exhibit functional receptor activities in our orthogonal assays with the notable caveats that crystal violet lactone and phenolphthalein exhibited weak AC50 values (AC50 > 100uM), and that iprodione proved functional active in our transactivation assays compared to Tox21 data where it is designated inactive.

Assays conducted in antagonist mode demonstrated that eight of the nine active Tox21 FXRα antagonists exhibited significant activity with human FXR transactivation assays. Activity of chemicals ranged with AC50 values between 2.4 μM for ivermectin up to 55.1 μM for

phenolphthalein. Consistent with previous studies examining Tox21 compounds (Hsu et al., 2014), we demonstrate a broad structural diversity in FXRα antagonists with chlorophacinone and ivermectin demonstrating potent antagonist activities. We additionally observed that Diuron, an aryl urea herbicide functioned as a potent FXR antagonist in both human FXRα and medaka FXRα2 assays. Conversely, we found that the tubulin binder podofilox was not an efficacious human FXRα antagonist as reported in Tox21. Additionally, we confirmed that colchicine and tricaprylin were inactive with human FXRα.

Data outcomes from M2H experiments in this study suggest significantly diverse and complex ligand induced protein:protein interactions with FXRα and selected NR coregulators. In general, FXRα agonists tested induced significant recruitment between human FXRα, RXR and NR coregulators PGC1α and SRC1. We also observed that in general FXRα antagonists attenuated GW4064 mediated RXR heterodimerization and recruitment of PGC1α or SRC-1. Interestingly, select chemicals including bifenthrin and moxidectin enhanced select human FXRα:coregulator interactions. Hsu et al. (2016) observed as similar phenomena where select FXRα antagonists enhanced humanFXRα:coregulator interactions in the presence of CDCA. These results are also similar to that observed with vitamin D receptor antagonists (Mahapatra et al., 2018) suggesting that this process is not unique to human or medaka FXRα. Rather, this finding suggests that alternate mechanisms may be associated with receptor inhibition in lieu of coactivator recruitment. There are many possibilities for this, including alterations within other regions of the receptor essential for DNA binding and or subsequent coregulator recruitment.

The use of secondary orthogonal assays such as the M2H assay can assist in providing greater mechanistic insight to the functionality of these FXRα agonists and antagonists. While transactivation assays provide a global context of agonists and antagonist function, the M2H assay provides further evidence that chemicals may have a direct effect on critical receptor functionalities. Results are consistent with the observation that the holo conformation of FXRα is ligand-specific and is pivotal for revealing receptor:coregulator interaction domains associated with RXR heterodimerization and recruitment on coactivators and corepressors. Ligand binding of FXRα induces an allosteric shift in receptor conformation, where helix 12 (H12) within the receptor AF2 domain rotates and packs tightly over other helices creating a hydrophobic ligand-binding pocket. The repositioning of H12 creates a "charge clamp" between the negatively charged residues of the AF2 region of H12, and positively charged of helix 3. The charge clamp is responsible for coactivator interaction by directly binding with the NR coregulator binding domain (LXXLL amino acid motif) (Savkur et al., 2005). Small changes in ligand structure appear to affect receptor configurations impacting coactivator binding interface and ultimately varying efficacy and potency of NR transactivation. One caveat to an approach with alternate species is, however, the uncertainty of cross reactivity between human coregulators and nonmammalian NR's. This remains to be investigated.

Based on transactivation data with medaka FXRα2 we anticipated *in vivo* activity (Bsep, Shp induction) with select FXRα agonists which was confirmed except for phenolphthalein which proved to be toxic to medaka larvae at concentrations examined. These results support an *in vitro* to *in vivo* translation of receptor agonists and provides an initial mechanism to establish some predictive value of high-throughput assays that may result in *in vivo* activities. This observation however is complicated by the fact that we additionally observed induction of both Bsep and Shp with cimicifugoside, imazalil and iprodione which proved to be weaker mFXRα2 agonists but positive in our human FXRα transactivation assay and/or inactive in the Tox21 FXRα dataset (iprodione). This observation may be explained by the fact that medaka possess a second FXR isoform (FXRα1). Previously we demonstrated that the mFXRα1 isoform was inactive with GW4064 (Howarth et al., 2010b) however recent studies investigating receptor paralogs in teleosts demonstrated differential activities with varying chemical profiles (Goodale et al., 2015). Given that these ligands were active with human FXRα, suggests the possibility that there may be species selective interactions that have not been identified with all forms of medaka FXR. This hypothesis would be consistent with that observed for PXR which demonstrates highly species specific ligand:receptor interactions (Sinz et al., 2007). Alternatively, there may be multiple mechanisms for induction of bile acid transporters including Bsep and associated orphan nuclear receptors including Shp (Cheng et al., 2007). Supporting this notion is the observation that three compounds chosen as inactive negative controls for our assays including acephate, prometon and propazine exhibited significant induction of Bsep and Shp *in vivo* but proved to be negative in the Tox21 assay and with our human and medaka FXR transient transactivation assays. These data suggested the selected gene expression markers may not be as specific to FXR activation as desired and such *in vivo* assays may be enhanced by selection of additional gene targets that show greater degrees of specificity for select NR targets. This observation speaks to potential complexities of *in vitro* to *in vivo* data translations for definitive assessments of ligand/compound activity. This fact additionally supports the utility of screening protocols that apply a multiplicative approach utilizing a combination of orthogonal assays with a "weight of the evidence" approach for assignment of compound activity.

Assessment of FXRα antagonists *in vivo* is based on the premise that FXR antagonists will attenuate observed gene expression changes induced with GW4064, a prototypic FXRα agonist. Initially all assays were conducted with 1 μM GW4064 and 10 μM antagonists. However, under these conditions we observed significant larval toxicity and thus repeated the assay using 0.125 μM GW4064 and 0.25 μM selected antagonists (Fig. 6, **Supplementary Table 3**). Lowering the concentration of FXRα antagonists greatly alleviated medaka larval toxicity, however several chemicals including colchicine, podofilox, bifenthrin, ivermectin and moxidectin still could not be evaluated due to continued adverse developmental effects which could skew gene expression outcomes. For compounds not exhibiting larval toxicity, *in vivo* assays exhibited high concordance with *in vitro* transactivation assays for actinomycin D, bisphenol B, emetine dihydrochloride, and phenolphthalein. Interestingly, tricaprylin, exhibited significant attenuation of GW4064 mediated gene expression *in vivo* consistent with our medaka FXRα2 transactivation assay but proved to be inactive in our human FXRα transactivation assay and is reported as inactive in the Tox21 FXRα dataset. Conversely, diuron was considered inactive in the Tox21 FXRα dataset proved to be significantly active as an FXR antagonist in all *in vitro* and *in vivo* assays tested. Given that diuron is a current use pesticide/herbicide this observation may merit further investigation.

Results of orthogonal assays combined with Tox21 receptor studies *in vitro* specifically denotes ligand-receptor interactions and provides a means to discern putative chemical mechanisms. Conversely, it is well established that *in vitro* methods lack organismal complexity and raise concerns about the ability to extrapolate to *in vivo* toxicity. Thus, there remains a need to further investigate the activity/toxicity of high-priority chemicals *in vivo*. This aspect has been recognized by many of the existing testing agencies and a priority has been established to identify and further develop translational methods for extrapolation of *in vitro* toxicity data (Dix et al., 2007).

Overall, we observed significant concordance between Tox21 data outcomes and demonstrated receptor activities with human FXR which may serve to further validate Tox21 predications. There were a few exceptions however that may reflect low false positive and or false negative rates within the Tox21 screening program. For example, relatively weak transactivation was observed with iprodione with both human FXRα and medaka FXRα2 transactivation assays but this compound is reported inactive with the Tox21 dataset (**Supplementary Table 4**). Conversely, diuron may represent a false negative in the Tox21 dataset as this compound consistently resulted in a significant antagonist activity with both human and medaka FXRα. Lastly, there is the possibility of potential source difference in the condition and purity of the

chemical standard itself utilized in this study compared to Tox21. Given that validation of chemical purities was not established, discrepancies in AC50 between this current study and Tox21 data may be a result of differences in chemical purities.

Quantitative high-throughput chemical screens have been instrumental in identifying chemicals that are active toward a variety of nuclear receptors. Those experimental bioprofiles have provided a convenient method of gaining novel information on hundreds of chemicals that are potentially toxic and provide global assessment of ligand interactions with nuclear receptors. In line with the continued surge in scientific interest in dissecting the roles played by NR's specifically FXRα in mechanisms associated with toxicity, we explored the utility of confirming high-throughput analysis with orthogonal assays and the potential of FXRα as a target of select xenobiotics. Through application of *in vitro* cell-based assays and *in silico* modeling approaches, we demonstrate the molecular complexity of FXRα:ligand interactions and confirm the ability of diverse ligands to modulate FXRα, facilitate differential coregulator recruitment and activate/repress receptor-mediated transcription. Further, we illustrate that in addition to assessment of *in vitro* receptor activities, *in vivo* assessments may facilitate a greater understanding of the biological and mechanistic complexities of NR ligands and further our ability to interpret broad HTS outcomes in a more complex translational model.

CRediT authorship contribution statement

Jon Hamm: Conceptualization, Writing – original draft, Writing – review & editing. **Debabrata Mahapatra:** Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Megan M. Knuth:** Investigation, Writing – review & editing. **Jaleh Abedini:** Visualization, Formal analysis, Writing – review & editing. **Mary Lingerfelt:** Investigation, Visualization, Formal analysis, Writing – review & editing. **Sean Ekins:** Writing – original draft, Writing – review & editing. **Seth W. Kullman:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number P30ES025128, and T32ES007046. ILS staff provided support under NIEHS contract no. HHSN273201500010C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health." The authors would like to thank Kim To and Lois Wyrick for assistance with the manuscript figures. Special thanks to Dr. Jim Boyer, Yale Liver Center (New Haven, CT, USA) for providing the pSG5-Human FXRα and the (hsp27EcRE)2-tk constructs and Dr. Donald McDonnell, Duke University (Durham, NC, USA) for the mammalian 2-hybrid constructs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2022.100092.

References

Cai, P., Mao, X., Zhao, J., Nie, L., Jiang, Y., Yang, Q., Ni, R., He, J., Luo, L., 2021. Farnesoid X receptor is required for the redifferentiation of bipotential progenitor cells during biliary-mediated zebrafish liver regeneration. Hepatology 74 (6), 3345–3361. PMID: 34320243.

Cheng, X., Buckley, D., Klaassen, C.D., 2007. Regulation of hepatic bile acid transporters Ntcp and Bsep expression. Biochem. Pharmacol. 74 (11), 1665–1676. PMID: 17897632.

Delfosse, V., Maire, A.L., Balaguer, P., Bourguet, W., 2015. A structural perspective on nuclear receptors as targets of environmental compounds. Acta Pharmacol. Sin. 36 (1), 88–101. PMID: 25500867.

Dix, D.J., Houck, K.A., Martin, M.T., Richard, A.M., Setzer, R.W., Kavlock, R.J., 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol. Sci. 95 (1), 5–12. PMID: 16963515.

Fang, Y., Hegazy, L., Finck, B.N., Elgendy, B., 2021. Dec 10.. Recent advances in the medicinal chemistry of farnesoid X receptor. J. Med. Chem. PMID: 34889100.

Filer, D.L., Kothiya, P., Woodrow Setzer, R., Judson, R.S., Martin, T.S., 2016. tcpl: the ToxCast pipeline for high-throughput screening data. Bioinformatics. 33, 618–620. PMID: 27797781.

Friesner, R.A., Murphy, R.B., Repasky, M.P., Frye, L.L., Greenwood, J.R., Halgren, T.A., Sanschagrin, P.C., Mainz, D.T., 2006. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. J. Med. Chem. 49 (21), 6177–6196. PMID: 17034125.

Gangwal S, Reif DM, Mosher S, Egeghy PP, Wambaugh JF, Judson RS. Cohen Hubal EA. Incorporating exposure information into the toxicological prioritization index decision support framework. Sci Total Environ. 2012. 435-436:316-25. PMID: 2286380.

Glass, C.K., Rosenfeld, M.G., 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 14, 121–141. PMID: 10652267.

Goodale, B.C., La Du, J., Tilton, S.C., Sullivan, C.M., Bisson, W.H., Waters, K.M., Tanguay, R.L., 2015. Ligand-specific transcriptional mechanisms underlie aryl hydrocarbon receptor-mediated developmental toxicity of oxygenated PAHs. Toxicol. Sci. 2, 397–411. PMID: 26141390.

Halgren, T.A., Murphy, R.B., Friesner, R.A., Beard, H.S., Frye, L.L., Pollard, W.T., Banks, J.L., 2004. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. J. Med. Chem. 47 (7), 1750–1759. PMID: 15027866.

Howarth, D.L., Law, S.H., Law, J.M., Mondon, J.A., Kullman, S.W., Hinton, D.E., 2010a. Exposure to the synthetic FXR agonist GW4064 causes alterations in gene expression and sublethal hepatotoxicity in eleutheroembryo medaka (Oryzias latipes). Toxicol. Appl. Pharmacol. 243 (1), 111–121. PMID: 19963001.

Howarth, D.L., Hagey, L.R., Law, S.H.W., Ai, N., Krasowski, M.D., Ekins, S., Moore, J.T., Kollitz, E.M., Hinton, D.E., Kullman, S.W., 2010b. Two farnesoid X receptor alpha isoforms in Japanese medaka (Oryzias latipes) are differentially activated in vitro. Aquat. Toxicol. 98 (3), 245–255. PMID: 20430454.

Hsu, C.W., Zhao, J., Huang, R., Hsieh, J.H., Hamm, J., Chang, X., Houck, K., Xia, M., 2014. Quantitative high-throughput profiling of environmental chemicals and drugs that modulate farnesoid X receptor. Sci. Rep. 4, 6437. PMID: 25257666.

Hsu, C.W., Zhao, J., Xia, M., 2016. Transactivation and Coactivator Recruitment Assays for Measuring Farnesoid X Receptor Activity. Methods Mol. Biol. 1473, 43–53. PMID: 27518622.

Huang, R., Xia, M., Cho, M.H., Sakamuru, S., Shinn, P., Houck, K.A., Dix, D.J., Judson, R. S., Witt, K.L., Kavlock, R.J., Tice, R.R., Austin, C.P., 2011. Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. Environ. Health Perspect. 119 (8), 1142–1148. PMID: 21543282.

Iwamatsu, T., 2004. Stages of normal development in the medaka Oryzias latipes. Mech. Dev. 121, 605–618. PMID: 15210170.

Kavlock, R.J., Austin, C.P., Tice, R.R., 2009. Toxicity testing in the 21st century: implications for human health risk assessment. Risk Anal. 29 (4), 485–487. PMID: 19076321.

Krewski, D., Acosta Jr, D., Andersen, M., Anderson, H., Bailar 3rd, J.C., Boekelheide, K., Brent, R., Charnley, G., Cheung, V.G., Green Jr, S., Kelsey, K.T., Kerkvliet, N.I., Li, A. A., McCray, L., Meyer, O., Patterson, R.D., Pennie, W., Scala, R.A., Solomon, G.M., Stephens, M., Yager, J., Zeise, L.J., 2010. Toxicity testing in the 21st century: a vision and a strategy. Toxicol Environ Health B Crit Rev. 2–4, 51–138. PMID: 20574894.

Mahapatra, D., Franzosa, J.A., Roell, K., Kuenemann, M.A., Houck, K.A., Reif, D.M., Fourches, D., Kullman, S.W., 2018. Confirmation of high-throughput screening data and novel mechanistic insights into VDR-xenobiotic interactions by orthogonal assays. Sci. Rep. 8 (1), 8883. PMID: 29891985.

Mangelsdorf, D.J., Evans, R.M., 1995. The RXR heterodimers and orphan receptors. Cell 83 (6), 841–850. PMID: 8521508.

National Research Council. "Toxicity testing in the 21st century: a vision and a strategy. Washington, DC: The National Academies Press. 2007. Htts//doi.org.10.17226/11970.

Ordentlich, P., Downes, M., Evans, R.M., 2001. Corepressors and nuclear hormone receptor function. Curr. Top. Microbiol. Immunol.. 254, 1–116. PMID: 11190569.

Pfaffi, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nuclei Acids Res. 29, 2002–2007. PMID: 11328886.

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., Carlberg, C., 2000. VDR-Alien: a novel, DNA-selective vitamin D(3) receptor corepressor partnership. FASEB J. 14, 1455–1463. PMID: 10877839.

Savkur, R.S., Bramlett, K.S., Clawson, D., Burris, T.P., 2004. In: Pharmacology of Nuclear Receptor- Coregulator Recognition. (Elsevier Academic Press, pp. 145–183.
Sinz, M.W., Pray, D., Raucy, J., 2007. The Utility of stable cell lines to assess species differences in PXR transactivation. Drug Metab. Lett. 2, 147–152. PMID: 19356035.
Wang, D., 2018. Infer the in vivo point of departure with ToxCast in vitro assay data using a robust learning approach. Arch. Toxicol. 9, 2913–2922. PMID: 29995190.