Evaluation of FXR-Active Chemicals Identified from Tox21 Screening

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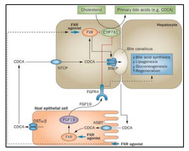
Introduction

- The Tox21 in vitro high throughput screening (HTS) program uses human cell line-based assays to rapidly screen chemicals for effects on toxicity pathways thought to be relevant to human disease.
- Some of these assays evaluate disruption of nuclear receptor signaling, including disruption of farnesoid X receptor alpha (FXR). FXR is associated with bile acid homeostasis, glucose metabolism, lipid homeostasis, and hepatic regeneration.
- In this study, we used in vitro and in vivo models to further evaluate chemicals identified through Tox21 in vitro screens as FXR agonists and antagonists.



Farnesoid X receptor alpha pathway

Materials and Methods

- Chemicals (**Tables 1** and **2**) were selected based on Tox21 AC50 and Emax values in FXR agonist and antagonist assays.
  - Selected chemicals included eight putative active antagonists and antagonists along with four inactive, negative controls.
  - Actives were selected to represent a range of activities and include chemicals with high potential for human exposure (Gangwal et al. 2012).
- Chemicals were evaluated for:
  - Ligand-induced transactivation of FXR in HepG2 cells including full-length human and medaka receptors (HEPG2-Luc assays) (Kollitz et al. 2016)
  - Interaction with FXR coregulator proteins using mammalian two-hybrid assays (Howarth et al. 2010)
  - Interaction with human FXR using 3D molecular docking studies with X-ray structures (Howarth et al. 2010)
  - Quantitative RT-PCR to assess in vivo effects on hepatic gene expression medaka larvae (Howarth et al. 2010)

Transactivation Studies

- Chemicals exhibited a wide spectrum of FXR potencies in the HEPG2-Luc transactivation assays.
- Most Tox21 active agonists (7/8) exhibited FXR transactivation activity with human FXR (hFXR), while all four chemicals that were inactive in the Tox21 agonist assay were confirmed as inactive (**Table 1**).
- Results using medaka FXR alpha-2 (mFXR) were mostly consistent with hFXR activities. Cimicifugoside, imazalil, and iprodione exhibited strong to weak activity with hFXR but were inactive in the medaka assay (**Table 1**).
- Before testing of antagonists was initiated, structure-activity relationship studies (Hsu et al. 2016) identified additional chemicals of interest. As a result, three inactive chemicals were replaced by three Tox21 active antagonists.
- Most Tox21 active antagonists (8/11) exhibited antagonist activity in the hFXR transactivation assay (**Table 2**). Results of the mFXR assay were concordant with human FXR results except for tricaprylin (**Table 2**).
- Diuron, inactive in Tox21, was a potent antagonist in both hFXR and mFXR assays.

Table 1. Activity of Tox21 FXR Agonists in Human and Medaka HEPG2-Luc Assays

Chemical Name	Activity in Tox21[a]	AC50 (μM) in Tox21	Activity in human HEPG2-Luc	AC50 (μM) in human HEPG2-Luc	Activity in medaka HEPG2-Luc	AC50 (μM) in medaka HEPG2-Luc
Acephate	Inactive	-	Inactive	-	Inactive	-
Chenodeoxycholic acid	Active	4.61	Active	40.08	Active	41.44
Cimicifugoside	Active	5.52	Active	9.80	Inactive[b]	-
Crystal violet lactone	Active	4.29	Active	104.80	Active	50.76
Daunomycin hydrochloride	Active	6.19	Active	38.31	Active	pmf
Imazalil	Active	4.31	Active	78.81	Inactive	-
Iprodione	Active	4.23	Active	63.29	Inactive	-
Maleic hydrazide	Inactive	-	Inactive	-	Inactive	-
Phenolphthalein	Active	4.91	Active	pmf	Active	pmf
Prometon	Inactive	-	Inactive	-	Inactive	-
Propazine	Inactive	-	Inactive	-	Inactive	-
Triphenylphosphine	Active	4.91	Inactive	-	Inactive	-

pmf = poor model fit – no AC50 value determined

[a] Assay used: fxrant_hek293_bla_ratio_ac50_4toxpi human FXR assay.

[b] Activities indicated in RED are inconsistent from reported Tox21 values.

Table 2. Activity of Tox21 FXR Antagonists in Human and Medaka HEPG2-Luc Assays[a]

Chemical Name	Activity in Tox21[b]	AC50 (μM) in Tox21	Activity in human HEPG2-Luc	AC50 (μM) in HEPG2-Luc	Activity in medaka HEPG2-Luc	AC50 (μM) in medaka HEPG2-Luc
Actinomycin D	Active	8.23	Active	pmf	Active	pmf
Bifenthrin	Active	4.96	Active	52.16	Weak Active	52.58
Bisphenol B	Active	4.26	Active	20.40	Active	pmf
Chlorphocinone[d]	Active	6.27	Active	8.11	Active	4.64
Colchicine	Active	7.97	Inactive[c]	-	Inactive	-
Diuron	Inactive	-	Active	60.39	Active	17.09
Emetine dihydrochloride	Active	5.66	Active	1.67	Active	22.50
Ivermectin[d]	Active	NA	Active	1.79	Active	11.23
Moxidectin[d]	Active	NA	Active	13.20	Active	6.34
Phenolphthalein	Active	4.50	Active	83.79	Active	43.15
Podofilox	Active	7.52	Inactive	-	Inactive	-
Tricaprylin	Active	4.31	Inactive	-	Weak Active	pmf

pmf = poor model fit – no AC50 value determined; NA = not available.

[a] Antagonists tested in the presence of 10 μM GW4064.

[b] Assay used: fxrant_hek293_bla_ratio_ac50_4toxpi human FXR assay.

[c] Activities indicated in RED are inconsistent from reported Tox21 values.

[d] Chlorphocinone, ivermectin, and moxidectin replaced three inactive FXR antagonists and were added based on structure-activity studies completed with FXR active chemicals in the Tox21 library (Hsu et al. 2016). AC50 values were not available (NA) for ivermectin and moxidectin.

Protein-protein Interactions

- Mammalian two-hybrid assays were used to assess test chemicals' ability to facilitate or inhibit recruitment of FXR cofactor proteins SRC-1 and PGC1-alpha.
- In general, agonists and antagonists that exhibited receptor transactivation with hFXR also exhibited significant coregulatory recruitment (**Tables 3** and **4**).
- Acephate and malaic hydrazide were inactive in Tox21 transactivation assays. Both facilitated significant recruitment activity between hFXR and cofactor proteins. This suggests that they are capable of binding hFXR but have insufficient activity to facilitate transactivation.
- In general, antagonists attenuated protein:protein recruitment between hFXR and cofactor proteins (**Table 4**).
- In contrast to interactions with hFXR, most agonists did not facilitate interaction between mFXR and PGC1-alpha (**Table 5**). Some chemicals (daunomycin hydrochloride, imazalil, iprodione, and malaic hydrazide) facilitated interaction between mFXR and SRC-1 similar to that observed for hFXR. However, only daunomycin hydrochloride exhibited significant transactivational activity with mFXR. Receptor binding may facilitate coregulatory recruitment but not receptor transactivation.
- In the presence of antagonists, mFXR exhibited diminished interaction between FXR and nuclear receptor coregulators as compared to hFXR. Medaka FXR additionally exhibited a preferential interaction with PGC1-alpha (**Table 6**).

Summary of Mammalian Two-hybrid Data

Table 3. Human FXR Agonists

	pMPGC1a + pVP16 hFXR		pMSRC-1 + pVP16 hFXR		pMRXR + pVP16 hFXR			
Co-regulator addition	+RXR		+RXR		+PGC1a		+SRC-1	
Chemical Name	**Outcome**	**Value[a]**	**Outcome**	**Value**	**Outcome**	**Value**	**Outcome**	**Value**
GW4064	FR	100.0	FR	100.0	FR	100.0	FR	100.0
Acephate	FR	51.9	FR	37.9	NS[b]	-	NS	-
Chenodeoxycholic acid	FR	102.6	FR	30.1	FR	63.5	FR	29.0
Cimicifugoside	FR	24.9	FR	45.7	FR	24.8	FR	23.2
Crystal violet lactone	NS	-	NS	-	NS	-	NS	-
Daunomycin hydrochloride	NS	-	FR	67.8	FR	23.5	FR	184.0
Imazalil	FR	49.6	FR	65.5	FR	198.0	FR	24.6
Iprodione	FR	51.5	FR	58.2	FR	18.5	FR	35.3
Maleic hydrazide	FR	72.2	FR	52.9	FR	63.7	FR	59.1
Phenolphthalein	NS	-	NS	-	NS	-	NS	-
Prometon	NS	-	NS	-	FR	77.1	NS	-
Propazine	NS	-	NS	-	FR	71.7	NS	-
Triphenylphosphine	NS	-	NS	-	NS	-	NS	-

Table 4. Human FXR Antagonists

	pMPGC1a + pVP16 hFXR		pMSRC-1 + pVP16 hFXR		pMRXR + pVP16 hFXR			
Co-regulator addition	+RXR		+RXR		+PGC1a		+SRC-1	
Chemical Name	**Outcome**	**Value[a]**	**Outcome**	**Value**	**Outcome**	**Value**	**Outcome**	**Value**
GW4064	FR	100.0	FR	100.0	FR	100.0	FR	100.0
Actinomycin D	IR	30.1	IR	18.2	IR	8.7	IR	56.0
Bifenthrin	IR	21.3	IR	36.7	FR	187.0	IR	44.0
Bisphenol B	IR	21.8	NS[b]	-	NS	-	IR	68.3
Chlorphocinone	IR	16.2	IR	35.5	IR	35.2	IR	32.7
Colchicine	IR	31.8	IR	75	IR	29.7	IR	63.8
Diuron	IR	39.4	NS	-	NS	-	NS	-
Emetine dihydrochloride	IR	30.3	IR	10.9	IR	2.96	IR	39.8
Ivermectin	IR	55.7	NS	-	NS	-	NS	-
Moxidectin	FR	132.0	IR	66.3	FR	179	FR	127.0
Phenolphthalein	IR	16.5	IR	60.0	IR	41.6	IR	39.5
Podofilox	IR	31.8	NS	-	IR	36.9	IR	16.1
Tricaprylin	IR	19.1	FR	122	IR	62.3	IR	30.0

FR = facilitates recruitment; IR = inhibits recruitment; NS = not significant; PGC1a = PPAR-gamma coactivator-1; RXR = retinoic acid receptor, an FXR co-factor; SRC-1 = steroid receptor co-activator 1; pM and pVP16 are plasmids.

[a] Cofactor recruitment activity is expressed as a percentage of positive control (GW4064) activity. Both agonists and antagonists facilitate or inhibit recruitment in the presence or absence of co-transfected coregulators/coactivators.

[b] NS indicates nonsignificant induction/inhibition values for coregulator/coactivator recruitment.

Summary of Mammalian Two-hybrid Data (cont'd)

Table 5. Medaka FXR Agonists

	pMPGC1a + pVP16 mFXR		pMSRC-1 + pVP16 mFXR		pMRXR + pVP16 mFXR			
Co-regulator addition	+RXR		+RXR		+PGC1a		+SRC-1	
Chemical Name	**Outcome**	**Value**	**Outcome**	**Value**	**Outcome**	**Value**	**Outcome**	**Value**
GW4064	FR	100.0[a]	FR	100.0	FR	100.0	FR	100.0
Acephate	NS[b]	-	NS	-	NS	-	NS	-
Chenodeoxycholic acid	FR	39.9	FR	36.5	FR	42.6	FR	113.4
Cimicifugoside	NS	-	NS	-	NS	-	FR	34.2
Crystal violet lactone	NS	-	NS	-	NS	-	NS	-
Daunomycin hydrochloride	NS	-	NS	-	FR	30.52	FR	73.1
Imazalil	NS	-	FR	43.4	FR	36.4	FR	80.3
Iprodione	NS	-	FR	99.6	FR	23.2	FR	75.1
Maleic hydrazide	NS	-	NS	-	FR	48.9	FR	142.0
Phenolphthalein	NS	-	NS	-	NS	-	NS	-
Prometon	NS	-	NS	-	FR	79.5	NS	-
Propazine	NS	-	NS	-	NS	-	NS	-
Triphenylphosphine	NS	-	NS	-	NS	-	NS	-

Table 6. Medaka FXR Antagonists

	pMPGC1a + pVP16 mFXR		pMSRC-1 + pVP16 mFXR		pMRXR + pVP16 mFXR			
Co-regulator addition	+RXR		+RXR		+PGC1a		+SRC-1	
Chemical Name	**Outcome**	**Value**	**Outcome**	**Value**	**Outcome**	**Value**	**Outcome**	**Value**
GW4064	FR	100.0	FR	100.0	FR	100.0	FR	100.0
Actinomycin D	NS[b]	-	NS	-	NS	-	NS	-
Bifenthrin	NS	-	NS	-	FR	139.0	NS	-
Bisphenol B	NS	-	NS	-	FR	133.0	NS	-
Chlorphocinone	NS	-	NS	-	NS	-	NS	-
Colchicine	IR	48.9	NS	-	IR	53.7	NS	-
Diuron	NS	-	NS	-	NS	-	NS	-
Emetine dihydrochloride	FR	269.0	FR	280.0	NS	-	NS	-
Ivermectin	IR	60.3	FR	158.0	FR	119.4	FR	120.0
Moxidectin	FR	126.0	NS	-	FR	105.0	IR	67.8
Phenolphthalein	IR	58.3	IR	66.0	IR	49.3	FR	133.8
Podofilox	NS	-	NS	-	FR	111.6	FR	174.1
Tricaprylin	IR	63.0	NS	-	NS	-	NS	-

FR = facilitates recruitment; IR = inhibits recruitment; NS = not significant; PGC1a = PPAR-gamma coactivator-1; RXR = retinoic acid receptor, an FXR co-factor; SRC-1 = steroid receptor co-activator 1; pM and pVP16 are plasmids.

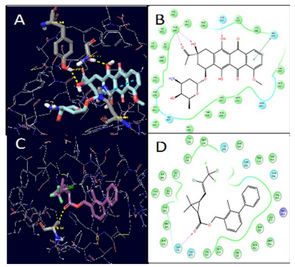
[a] Cofactor recruitment activity is expressed as a percentage of positive control (GW4064) activity. Both agonists and antagonists facilitate or inhibit recruitment in the presence or absence of co-transfected coregulators/coactivators.

[b] NS indicates nonsignificant induction/inhibition values for coregulator/coactivator recruitment.

Modeling of FXR-ligand Interactions

- Tox21 FXR agonists and antagonists were docked in X-ray structures for the human FXR co-crystallized with chenodeoxycholic acid as a prototypic agonist and N-benzyl-N-(3-(tert-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) benzamide as a selective antagonist.
- The molecular docking studies (representative image in **Figure 1**) produce "glide scores" that approximate the ligand binding free energy. Smaller glide scores (**Tables 7** and **8**) represent more efficient docking within the crystal structure (Friesner et al. 2004, 2006).
- Poor scoring agonist compounds (**Table 7**) correlate with in vitro inactive molecules (**Table 1**).

Figure 1. Molecular Docking of FXR Agonist and Antagonist



A and B. Interaction between the agonist daunorubicin and the FXR ligand binding domain. Yellow dotted lines in **A** represent H-bonds, and dotted cyan line represents pi-pi interactions. Green residues (**B**) surrounding the ligand indicate hydrophobic interactions; pale blue indicates polarity.

C and D. Interactions between the antagonist bifenthrin and the FXR ligand binding domain. Interactions are represented by color-coding as described above.

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Molecular Docking Glide Scores

Table 7. Agonists Docked into PBD 4qe6

Entry Name	Glide Score
Chenodeoxycholic acid	-12.765
Daunorubicin	-11.773
Phenolphthalein	-8.829
Triphenylphosphine	-9.076
Iprodione	-8.040
Imazalil	-7.049
Prometon	-5.412
Propazine	-5.026
Acephate	-4.336
Maleic hydrazide	-3.693

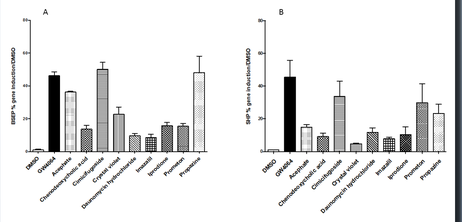
Table 8. Antagonists Docked into PBD 4oiv

Entry Name	Glide Score
Bifenthrin	-9.958
Chlorphocinone	-8.510
Phenolpthalein	-7.905
Colchicine	-6.762
Emetine dihydrochloride	-6.459
Bisphenol B	-6.402
Diuron	-4.553
Tricaprylin	-3.549

In Vivo Assessment of Gene Expression

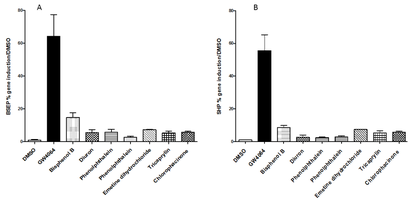
- Quantitative RT-PCR was used to assess altered expression of the bile salt export pump (BSEP) and small heterodimer partner (SHP) genes in medaka larvae (48 hrs post-exposure).
- Individual FXR agonists exhibited moderate to robust induction of both BSEP and SHP compared to the DMSO negative control (**Figure 2, Tables 9** and **10**).
  - Cimicifugoside, imazalil, and iprodione, which were negative in vitro for agonist activity in medaka but positive in humans, induced both BSEP and SHP in vivo. This could be explained by interactions with a second medaka FXR isoform, FXR alpha-1, in the in vivo assay.
- Most chemicals that exhibited antagonist activity in the Tox21 chemicals also significantly reduced expression of FXR-responsive genes (**Figure 3**).

Figure 2. Hepatic Gene Expression Effects of Tox21 FXR Agonists



Hepatic expression of BSEP (**A**) and SHP (**B**) genes was measured by qPCR in medaka larvae 48 hrs post-exposure. GW4064, a potent FXR agonist, was the positive control and DMSO was the negative control. Expression was normalized to GW4064 in-plate values to control for plate-to-plate variation. Maleic hydrazide, phenolphthalein, and triphenylphosphine could not be evaluated due to adverse developmental effects.

Figure 3. Inhibition of FXR Responsive Gene Expression in Medaka Exposed to GW4064 and Tox21 FXR Antagonists



Hepatic expression of BSEP (**A**) and SHP (**B**) genes was measured by qPCR in medaka larvae 48 hrs post-exposure. Assays measured inhibition by the test chemical of expression induced by GW4064. GW4064 was the positive control and DMSO was the negative control. Expression was normalized to GW4064 in-plate values to control for plate-to-plate variation. Colchicine, podofilox, bifenthrin, ivermectin and moxidectin could not be evaluated due to adverse developmental effects.

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In Vivo Gene Induction of BSEP and SHP

Table 9. Gene Induction in Response to FXR Agonists

	% BSEP induction		% SHP induction	
Chemical Name[a]	AVE	SEM	AVE	SEM
GW4064[b]	100.00	4.23	99.65	22.61
Acephate	78.52	0.86	32.60	2.80
Chenodeoxycholic acid	29.56	5.25	20.01	4.50
Cimicifugoside	108.49	9.27	73.75	20.48
Crystal violet lactone	49.11	7.80	10.34	0.62
Daunomycin hydrochloride	20.84	2.95	25.85	5.89
Imazalil	18.48	4.47	17.14	2.19
Iprodione	34.04	3.54	22.69	8.51
Prometon	33.58	2.95	65.48	25.46
Propazine	104.11	21.66	51.15	12.14

AVE = average; BSEP = bile salt export pump; SEM = standard error of the mean; SHP = small heterodimer partner.

[a] Agonists maleic hydrazide, phenolphthalein, and triphenylphosphine exhibited significant cytotoxicity between 0.5-10 μM and thus could not be evaluated.

[b] Activity was normalized to GW4064.

Table 10. Gene Induction in Response to FXR Antagonists

	% BSEP induction		% SHP induction	
Chemical Name[a]	AVE	SEM	AVE	SEM
GW4064[b]	100.00	16.84	99.97	14.21
Actinomycin D	4.33	0.93	24.80	7.21
Bisphenol B	23.06	3.67	15.56	1.74
Chlorphocinone	8.94	1.02	3.60	0.83
Diuron	8.78	2.32	4.73	2.00
Emetine dihydrochloride	11.52	0.21	22.88	5.46
Phenolphthalein	9.19	2.65	4.38	0.66
Tricaprylin	8.33	1.78	25.40	5.05

AVE = average; BSEP = bile salt export pump; SEM = standard error of the mean; SHP = small heterodimer partner.

[a] Even after lowering the concentration of antagonists colchicine, podofilox, bifenthrin, ivermectin and moxidectin still could not be evaluated due to adverse developmental effects.

[b] Activity was normalized to GW4064.

Conclusions

- The majority of Tox21 results were confirmed in the transactivation assays. Discordant results could be due to false positives or false negatives in the Tox21 assays.
  - For example, transactivation was not observed with triphenylphosphine in either the human or medaka FXR transactivation assays even though this chemical was positive in Tox21.
  - Conversely, diuron consistently inhibited FXR with both human and medaka FXR, but was negative in Tox21.
- Results from mammalian two-hybrid assays suggest these chemicals exhibit diverse and complex interactions with FXR and coregulators SRC-1 and PGC1-alpha.
- In vivo qPCR data indicated that FXR agonists induce selected gene targets. However, each of the selected "inactive" transactivation chemicals also exhibited some in vivo expression of gene induction.
- Expression of target genes in qPCR studies of FXR antagonists was consistent with in vitro results for assayed chemicals, but some chemicals could not be evaluated due to adverse developmental effects.
- Chemicals that were positive in humans, were negative in vitro in medaka, and induced FXR-responsive gene expression in the medaka in vivo assay should be tested with medaka FXR alpha-1 to better understand the species-specific response of the in vivo assay.

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