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Differential modulation of FXR activity by chlorophacinone and ivermectin analogs

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Abstract

Chemicals that alter normal function of farnesoid X receptor (FXR) have been shown to affect the homeostasis of bile acids, glucose, and lipids. Several structural classes of environmental chemicals and drugs that modulated FXR transactivation were previously identified by quantitative high-throughput screening (qHTS) of the Tox21 10K chemical collection. In the present study, we validated the FXR antagonist activity of selected structural classes, including avermectin anthelmintics, dihydropyridine calcium channel blockers, 1,3-indandione rodenticides, and pyrethroid pesticides, using *in vitro* assay and quantitative structural-activity relationship (QSAR) analysis approaches. (Z)-Guggulsterone, chlorophacinone, ivermectin, and their analogs were profiled for their ability to alter CDCA-mediated FXR binding using a panel of 154 coregulator motifs and to induce or inhibit transactivation and coactivator recruitment activities of constitutive androstane receptor (CAR), liver X receptor alpha (LXR α), or pregnane X receptor (PXR). Our results showed that chlorophacinone and ivermectin had distinct modes of action (MOA) in modulating FXR-coregulator interactions and compound selectivity against the four aforementioned functionally-relevant nuclear receptors. These findings collectively provide mechanistic insights regarding compound activities against FXR and possible explanations for *in vivo* toxicological observations of chlorophacinone, ivermectin, and their analogs.

Keywords

Farnesoid X receptor; nuclear receptor; ivermetin; moxidectin; chlorophacinone; diphacinone

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INTRODUCTION

The farnesoid X receptor (FXR) (NR1H4) is a multi-functional nuclear receptor that governs the equilibrium of endogenous bile acids, glucose, and lipids (Kemper, 2011). In the cytoplasm, FXR activity can be stimulated by endogenous ligands such as chenodeoxycholic acid (CDCA) through its ligand-binding domain (LBD). Upon activation, agonist-bound FXR forms a heterodimer with retinoid X receptor (RXR) in the nucleus, where the DNA-binding domain (DBD) of FXR can bind to FXR response elements (FXREs) of target genes for transactivation. FXR transcription is tightly regulated by coactivator and corepressor proteins. These coactivators and corepressors alter gene expression by modulation of FXR-RXR interactions or post-translational modifications (PTMs) of the FXR target gene-regulating histones. Examples of FXR-interacting coregulators include cAMP-response-element-binding (CREB)-binding protein (CBP or CREBBP), E1A binding protein p300 (EP300 or p300), mediator of RNA polymerase II transcription subunit 1 (MED1), nuclear receptor co-repressor 2 (NCOR2/SMRT), peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator 1-alpha (PGC-1 α), receptor-interacting protein 140 (RIP140), and steroid receptor coactivators (e.g., SRC1, SRC2, and SRC3) (Lien *et al.*, 2014).

Reducing FXR activity by small molecules, gene silencing, or gene knockout methods showed either beneficial or adverse effects in various *in vivo* models, depending on health states, age and diets (Lamers *et al.*, 2014). Guggulsterone, an FXR antagonist, reduced the hepatic cholesterol levels in an FXR specific manner when mice were fed a high-cholesterol diet (Urizar *et al.*, 2002). Ivermectin, a widely used antiparasitic drug, recently identified as an FXR modulator, was also shown to decrease cholesterol and serum glucose levels. As a result, insulin sensitivity improved in mice through interactions with FXR, suggesting that ivermectin or its analogues as possible treatments for diabetes (Jin *et al.*, 2013; Hsu *et al.*, 2014). Studies using FXR deficient mouse models showed both beneficial and adverse effects of FXR deficiency. Zhang *et al.* reported that compared to wild type controls, female Fxr $-/-$ mice had increased energy expenditure and were resistant to diet-induced obesity (Zhang *et al.*, 2012). On the other hand, several studies have demonstrated that Fxr $-/-$ mice were more susceptible to liver injury induced by chemicals including bile acids, and developed severe non-alcoholic steatohepatitis (NASH)-like liver pathology, especially on a high-fat diet (Yang *et al.*, 2007; Kong *et al.*, 2009; Bjursell *et al.*, 2013). Studies also indicated that FXR deficiency deregulated intestinal innate immunity, altered neurobehavior and promoted tumorigenesis in mice (Maran *et al.*, 2009; Wang *et al.*, 2010). In addition to FXR, constitutive androstane receptor (CAR), liver X receptor (LXR), and pregnane X receptor (PXR) also heterodimerize with the RXR and regulate transcription of genes involved in bile acid (Guo *et al.*, 2003) and lipid (Handschin and Meyer, 2005) homeostasis, complicating interpretation of the results generated with FXR.

Unlike effects mediated through other endocrine- or xenobiotic metabolism-regulating nuclear receptors such as estrogen receptors (ERs) and PPARs, effects of environmental chemicals on FXR have not been well characterized. Due to the lack of characterization and important role in homeostasis, FXR was selected as one of the targets for toxicological investigation by the Toxicology in the 21st Century (Tox21) program (Huang *et al.*, 2011; Hsu *et al.*, 2014). We previously profiled the chemicals present in the Tox21 10K chemical

collection for their ability to modulate FXR agonism and antagonism on a quantitative high-throughput screening (qHTS) platform and identified over 3,000 environmental chemicals and drugs that potentially stimulate and/or inhibit CDCA-induced FXR transactivation (Hsu *et al.*, 2014). The study identified several FXR-active structural classes, including anthracycline-based chemotherapeutic agents, avermectin and milbemyacin-based anthelmintics, benzimidazole-based anti-fungal agents and fungicides, dihydropyridine-based calcium channel blockers, pyrethroid-based pesticides, and vinca alkaloid-based chemotherapeutic agents as FXR antagonists. In the present report, we confirm the FXR antagonist activity of the aforementioned compounds and focus on the characterization of chlorophacinone, ivermectin and their analogs in modulating FXR, CAR, LXR α , and PXR activities using *in vitro* and computational approaches.

MATERIALS AND METHODS

Chemicals and cell culture reagents

FXR-bla HEK293 cells, components of individual thaw media, growth media, assay media, beta-lactamase assay reagents, and coactivator assays for CAR, FXR, and LXR α were purchased from Life Technologies (Carlsbad, CA, USA). CAR-luc cells were generated as described previously (Lynch *et al.*, 2015). LXR α -luc and PXR-luc assays were acquired from Indigo Biosciences (State College, PA, USA) and Puracyp (Carlsbad, CA, USA), respectively. Compound solutions were prepared from NCATS' in-house compound collections (e.g., Tox21 10K library). Powder compounds were purchased from SelleckChem (Houston, TX, USA) and Sigma-Aldrich (St. Louise, MO, USA) and sample quality control (QC) analysis was measured on Agilent LC/UV/MS system (Santa Clara, CA, USA) using a standard gradient of 4-100% acetonitrile in the presence of 0.05% trifluoroacetic acid over seven minutes on a 3 micron Luna C18 (3 $\times$ 75 mm) column. All 1536-well compound storage and assay plates were obtained from Greiner Bio One USA (Monroe, NC, USA). CellTiter-Fluor cell viability, CellTiter-Glo cell viability, and One-Glo luciferase assay reagents were acquired from Promega (Madison, WI, USA).

QSAR analysis

To identify important structural features of FXR antagonists that are active in both FXR-bla transactivation assay and FXR-SRC2 coactivator assay, QSAR analysis was conducted. To increase confidence of the actives, we used a >30% efficacy cutoff. The FXR antagonists identified in the FXR-bla transactivation assay with efficacy >30% were categorized into Group 1 and Group 2 depending on their activity in the FXR-SRC2 coactivator assay. Group 1 included compounds that had no activity in the FXR coactivator assay. Group 2 included compounds that had significant activity in the FXR coactivator assay with >30% efficacy and <3-fold EC₅₀ change between the transactivation assay and coactivator assay. The chemical structures of compounds in both groups were curated to remove inorganic and organometallic salts. The curated structures were fed into CASE Ultra software (MultiCase Inc., Cleveland, OH) for QSAR analysis. CASE Ultra generates dataset-dependent chemical fragments and performs statistical analysis for identification of fragments that are differentiable between Group 1 and 2. The model was validated based on repeated 10-fold

external cross validation, yielding area under the curve (AUC) values of receiver operating characteristic (ROC) curve, which estimates the prediction power of QSAR model.

FXR-bla reporter gene assay

FXR-bla HEK293 cells were seeded at 5000 cells per 5 μ L per well in 1536-well black clear-bottom assay plates using a BioRAPTR flying reagent dispenser (FRD) (Beckman Coulter, Indianapolis, IN, USA), followed by incubation for 5 h in a humidified incubator at 37°C, 5% CO₂. Added to the plates were 23 nL per well of compound solution prepared by cherry picking on a compound transfer workstation (Kalypsys, San Diego, CA, USA) and 1 μ L of CDCA (50 μ M final concentration)-containing medium for the FXR-bla antagonist assay using BioRAPTR FRD. After 16 h incubation in a humidified incubator set at 37°C, 5% CO₂, 1 μ L of CCF₄-AM substrate was added to each well using a BioRAPTR and incubated at room temperature in the dark for additional 2 h. An excitation wavelength of 405 nm and two emission wavelengths of 530 nm (Ch1) and 460 nm (Ch2) were used to obtain FXR-bla reporter signals on an Envision plate reader (Perkin Elmer, Shelton, CT, USA), yielding beta-lactamase activity as fluorescence emission intensity ratios (Ch2/Ch1). Ratios were normalized to CDCA control wells as 100% FXR activity and DMSO-only wells (0.4% v/v DMSO) as 0% FXR activity. The concentration-response curves of the cherry-pick confirmation and powder confirmation plates were generated using an in-house data analysis protocol (Hsu *et al.*, 2014) and GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) to determine IC₅₀ and efficacy values, respectively.

CAR-luc, LXR α -luc, and PXR-luc reporter gene assays

The CAR-luc and PXR-luc assays, generated by stably transfected parent cell lines with a nuclear receptor-driven luciferase reporter gene, were conducted as described previously (Shukla *et al.*, 2011; Lynch *et al.*, 2015). The LXR α -luc assay was optimized into a 1536-well format and conducted according to the manufacturer's instructions. Briefly, CAR-luc, LXR α -luc, or PXR-luc cells were seeded into 1536-well white solid-bottom plates at 2000 to 2500 cells/well using BioRAPTR FRD, 23 nL of compound solution were added in each well, and the plates were then incubated for an indicated period in a humidified incubator at 37°C, 5% CO₂. For antagonist mode, the CAR-luc, LXR α -luc, and PXR-luc cells had 1 μ L of agonist-containing medium using BioRAPTR FRD added to every well, yielding final concentrations of 50 nM CAR agonist (CITCO), 0.4 μ M LXR α agonist (T0901317), and 5 μ M PXR agonist (rifampicin), respectively. The CAR-luc and PXR-luc cells were incubated with 4 μ L per well of One-Glo luciferase reagent at room temperature for 20 min. The LXR α -luc cells were incubated with 5 μ L per well of Luciferase Detection Reagent at room temperature for 10 min. The luciferase signals were acquired as luminescence intensity values on a ViewLux plate reader and normalized to DMSO-only wells (0.4% v/v DMSO) as 0% nuclear receptor activity and agonist control wells as 100% nuclear receptor activity. Concentration-response curves of each compound were generated using GraphPad Prism software to determine EC₅₀, IC₅₀, and efficacy values.

Cell viability assays

Assay plates of FXR-bla or LXR α -luc cells were added with 3 μ L of CellTiter-Glo cell viability reagent per well using BioRAPTR FRD after fluorescence reading for FXR-bla

cells and after compound treatment for LXR α -luc cells, followed by a 30-minute incubation in the dark at room temperature. Assay plates of CAR-luc or PXR-luc cells were added with 1 μ L of CellTiter-Fluor cell viability reagent per well using BioRAPTR FRD before luminescence reading, followed by 30-minute incubation in a humidified incubator at 37°C, 5%CO₂. The luminescence intensity values of CellTiter-Glo reagent and the fluorescence intensity values of CellTiter-Fluor reagent were acquired on a ViewLux plate reader and normalized to DMSO-only wells (0.4% v/v DMSO) as 100% cell viability and tetraoctylammonium bromide (TOAB) control wells as 0% cell viability. Concentration-response curves of each compound were generated using GraphPad Prism software to determine IC₅₀ and efficacy values.

Nuclear receptor coactivator assays for CAR, FXR, and LXR α

The coactivator assays of CAR, FXR, and LXR α were miniaturized into 1536-well formats and conducted according to manufacturer's instructions. Compound solutions of 23 nL prepared by cherry picking were added to a 6 μ L mixture of the nuclear receptor of interest's ligand binding domain (LBD) with detection reagents at indicated concentrations in 1536-well black solid bottom assay plates using a compound transfer workstation. The plates were centrifuged at 1000 rpm for 1 min, and incubated at room temperature in the dark for 30 min. The plates were then measured using an Envision plate reader with an excitation wavelength of 340 nm and two emission wavelengths of 495 nm and 520 nm. The CAR assay used 10 nM GST-tagged CAR-LBD protein, 5 nM Tb-labeled anti-GST antibody (goat), and 125 nM fluorescein-labeled PGC1 α coactivator-derived peptide in the absence (agonist mode) and presence (antagonist mode) of 0.1 μ M CITCO. The FXR assay used 5 nM GST-tagged FXR-LBD protein, 5 nM Tb-labeled anti-GST antibody (goat), and 500 nM fluorescein-labeled SRC2-2 coactivator-derived peptide in the absence (agonist mode) and presence (antagonist mode) of 50 μ M CDCA. The LXR α assay used 5 nM GST-tagged LXR α -LBD protein, 10 nM Tb-labeled anti-GST antibody (goat), and 250 nM fluorescein-labeled TRAP220/DRIP-2 coactivator-derived peptide in the absence (agonist mode) and presence (antagonist mode) of 0.4 μ M T0901317. Fluorescence ratios (520 nm/495 nm) were normalized to DMSO-only wells (0.4% v/v DMSO) as 0% nuclear receptor activity and agonist control wells as 100% nuclear receptor activity. Concentration-response curves of each compound were generated using GraphPad Prism software to determine EC₅₀/IC₅₀ and efficacy values.

Microarray assay for real-time coregulator-nuclear receptor interaction (MARCoNI)

Ligand-modulated interaction of FXR with coregulators was assessed using a PamChip[®] plate (PamGene International B.V.'s-Hertogenbosch, The Netherlands), which allows for analysis of 96 identical peptide microarrays in parallel (Koppen *et al.*, 2009; Houtman *et al.*, 2012; Aarts *et al.*, 2013). Each array of 154 coregulator-derived motifs (PamChip #88101, PamGene, The Netherlands) was incubated with the assay mixture containing the receptor and detection antibody in the absence or presence of compound(s). In short, compounds were prediluted in DMSO. Next, assay mixtures (Coregulator Buffer G, PV4553, Invitrogen, USA) were prepared on ice in a master 96-well plate and contained 5 nM GST-tagged FXR ligand-binding domain (FXR LBD-GST, PV4834, Invitrogen, USA), 25 nM of Alexa488-conjugated GST-antibody (A11131, Invitrogen, USA), 60 μ M CDCA (antagonist mode) and

10 or 20 μM test compound (final DMSO concentration was 2% v/v). Each condition was analyzed using four technical replicates (arrays). Incubation was performed in a fully automated microarray processing platform, PamStation®96, PamGene, The Netherlands) at 20 °C for 80 cycles (two cycles per min). After removal of the unbound receptor by washing each array with 25 μL of Tris-buffered saline, tiff images were obtained by the CCD camera which is part of the PamStation®96. Tiff images were analyzed for quantification of FXR binding (in arbitrary units of fluorescence) using Bionavigator software (PamGene, The Netherlands) and data analysis and visualization were performed by R software v2.15.3. The significance level of data ($p < 0.05$; $p < 0.01$; $p < 0.001$) were determined by Student's t-Test.

RESULTS

Confirmation of FXR Antagonist Activity in Receptor Transactivation and Coactivator Recruitment Assays

A selection of FXR-active compounds identified from the primary screening of the Tox21 10K chemical collection (Hsu *et al.*, 2014) were prepared by cherry picking and re-tested for their ability to inhibit chenodeoxycholic acid (CDCA)-induced FXR transactivation, cell viability of FXR-bla cells, and CDCA-induced recruitment of a SRC2 coactivator. In addition to the positive controls—(E)-guggulsterone and (Z)-guggulsterone, the selected compounds consists of structural classes including: anthracycline chemotherapeutics (e.g., daunorubicin and doxorubicin), avermectin and milbemycin anthelmintics (e.g., ivermectin and moxidectin), benzimidazole anti-fungal agent and fungicides (e.g., albendazole and nocosazole), conazole fungicides (e.g., econazole and sulconazole), dihydropyridine calcium channel blockers (e.g., nicardipine and nimodipine), 1,3-indandione rodenticides (e.g., chlorphacinone and diphacinone), organochloride pesticides (e.g., chlordane and dieldrin), pyrethroid pesticides (e.g., bifenthrin and cypermethrin), and vinca alkaloid chemotherapeutics (e.g., vinblastine and vincristine). Despite the fact that conazoles displayed apparent cytotoxicity, the majority of compounds in the aforementioned structural classes reduced CDCA-induced FXR transactivation at non-cytotoxic or slightly cytotoxic concentrations in the FXR-bla reporter gene assay measuring FXR-driven expression of the beta-lactamase reporter gene (Table S1). Seven out of ten of FXR-bla-active structural classes seemed to disrupt CDCA-induced recruitment of an SRC2 coactivator-derived peptide to the ligand binding domain (LBD) of FXR in the time resolved fluorescence resonance energy transfer (TR-FRET)-based biochemical assay (Table S2). All of the avermectin- and milbemectin-based anthelmintic drugs were identified by the coactivator assay as FXR antagonists with a wide range of IC_{50} values from 1.74 to 34.74 μM (Fig. 1A). Nicardipine, one of the dihydropyridine calcium channel blockers, was the most potent and efficacious with an IC_{50} of $1.59 \pm 0.57 \mu\text{M}$ in the coactivator assay (Fig. 1B). 1,3-Indandione rodenticides (Fig. 1C) displayed weak FXR antagonist activity (efficacy <50%) in inhibiting CDCA-induced recruitment of SRC2-2 coactivator peptide to FXR-LBD. Most benzimide-based microtubule inhibitors and anthracycline-based chemotherapeutic agents such as albendazole and daunorubicin did not show CDCA-dependent reduction of FXR activity in the SRC2-2 coactivator assay (Table S2).

Using QSAR Analysis to Identify Structural Features of FXR Antagonists

Not all actives identified in the confirmatory FXR-bla transactivation assay were active in the FXR-SRC2 coactivation assay; therefore the validated results of the selected compounds in the two FXR antagonist assays were further analyzed to identify functional groups important for FXR-SRC2 binding interactions using quantitative structural activity relationship (QSAR) analysis. Compounds with >30% efficacy in the FXR assays were assigned as active compounds. We first categorized the identified FXR antagonists into Group 1 of compounds that were only active in the FXR-bla assay and Group 2 of compounds that were active in both the FXR-bla and the FXR-SRC2 coactivator assays with a <3-fold difference in EC_{50} values. In total, there were 53 and 62 compounds in Group 1 and Group 2, respectively. After chemical structure curation and data collapsing, in total, there were 43 and 60 compounds in Group 1 and Group 2 amenable for QSAR analysis, respectively. To identify the structural features crucial for modulating FXR-SRC2 interactions, SAR analysis were conducted based on the structural fragments of the test compounds and compound activities in the two FXR antagonist assays (Table 1). The area under the receiver operating characteristic (AUC-ROC) curve was over 0.78, indicating a high accuracy of the model (i.e., AUC = 0.5 for random data). Fifteen avermectin anthelmintics (e.g., ivermectin) and cyclodiene pesticides (e.g., aldrin), with a common cyclodiene-containing fragment, were classified to Group 2. Several Group 2 compounds were environmental chemicals that contain a phenol group (e.g., 2,4-bis(2-methylbutan-2-yl)phenol and 2,2'-methylenebis(4-methyl-6-tert-butylphenol)) or a bisphenol group (e.g., bisphenol B and oxyphenisatin). Five phenylacetone-containing compounds such as dihydropyridine-based calcium channel blockers (e.g., cilnidipine and nimodipine) and 1,3-indandione-based rodenticides (e.g., chlorophacinone and diphacinone) were also Group 2 compounds.

Among the Group 2 compounds, avermectin and milbemectin-based anthelmintics, 1,3-indandione-based rodenticides, and pyrethroid-based pesticides might cause FXR-dependent toxicities at their exposure levels and thus require more in-depth testing to confirm their FXR antagonist activity as shown by the primary screening and the cherry-pick confirmation data. New batches of representative compounds for each structural class were obtained in powder form from alternative sources and tested in the FXR-bla antagonist assay to further confirm their FXR antagonist activity. While avermectins/milbemectins and 1,3-indanediones had similar potencies in antagonizing CDCA-induced FXR transactivation in the cherry-pick confirmation and powder confirmation experiments (Table 2), the re-procured samples of all tested pyrethroids were inactive in the FXR-bla assay (data not shown). Next, new samples of (E)-guggulsterone, (Z)-guggulsterone, ivermectin, moxidectin (a milbemectin derivative), the other three FXR-active ivermectin analogs (abamectin, doramectin and selamectin), chlorophacinone, diphacinone, and additional four chlorophacinone analogs (2-acetyl-1,3-indanedione, 1,1-diphenylacetone, phenindione and pindone) (Fig. 2A) were first tested for their FXR antagonist activity in the FXR-bla, viability, and FXR coactivator assays. The FXR antagonist activities of (E)-guggulsterone and (Z)-guggulsterone were confirmed in the FXR-bla and the FXR coactivator assays (Table 2). Despite the fact that several avermectins and moxidectin were cytotoxic to FXR-bla cells, the IC_{50} values of the five compounds yielded a good correlation coefficient ($R^2 =$

0.94) in inhibiting CDCA-induced FXR transactivation and recruitment of SRC2-2 coactivator-derived peptide. The three FXR-active 1,3-indandiones identified from the primary screening, chlorophacinone, diphacinone and pindone, reduced CDCA-induced FXR-bla expression and the first two compounds also antagonized CDCA-induced coactivator recruitment. Two structural analogs of chlorophacinone—2-acetyl-1,3-indanedione and phenindione— had no FXR antagonist activity in either FXR-bla or coactivator assays. 1,1-Diphenylacetone, a structural fragment present in chlorophacinone and diphacinone, showed weak FXR antagonist activity in the FXR-bla and FXR coactivator assays with an efficacy of <30%.

Profiling of FXR-coregulator interactions of avermectins and 1,3-indandiones

An allosteric conformational change of nuclear receptors upon ligand alters their affinity for coregulators, and the receptor-coregulator complex forms a physical and functional bridge with the gene transcription machinery. Depending on the nature of the ligand, coactivators and corepressors are recruited or displaced thus altering gene transcription levels. In this study we applied MARCoNI (Microarray Assay for Real-time Coregulator Nuclear receptor Interaction) in which a set of 154 immobilized peptides represents a variety of NR-interaction motifs from coregulators. Modulation of CDCA-activated FXR binding (antagonist mode) to these peptides by 12 compounds including (Z)-guggulsterone, ivermectin, chlorophacinone, and their analogs was analyzed. Coregulators display a variety of functions and are differentially expressed in a cell specific manner. Compounds dictate receptor conformation and subsequent affinity for coregulators. Compounds which display similar modulation are expected to act similarly while differential modulation is linked with an alternate mode of action. Fig. 2B shows the modulation of CDCA-activated FXR-coregulator interaction by each of the 12 compounds that were tested in powder confirmation studies. Modulation of each individual interaction is indicated by the modulation index (MI), or compound-induced log-fold change of binding vs. CDCA alone. To visualize similarity and dissimilarity in mode of action, the 154-point modulation signatures of the compounds, were subjected to R-based hierarchical clustering, which was used to classify coregulator interactions. (Z)-Guggulsterone, chlorophacinone, and ivermectin altered CDCA-induced binding between FXR and several known FXR-interacting coregulator motifs (Table 4). The three compounds reduced binding interactions between FXR and the coactivator binding motif of SRC1, SRC2, and SRC3 (NCOA1_677_700, NCOA2_677_700, and NCOA3_673_695). Additionally, ivermectin uniquely strengthened the binding interactions between FXR and one of the corepressor domains of N-CoR1 and SMRT (NCOR1_2251_2273 and NCOR2_2330_2352).

A large cluster of interactions at the right side of the heat map (Fig. 2B) displays decreased FXR binding (indicated by blue) upon addition of compound. This cluster contains a number of motifs derived from coactivators. A particular example of this cluster, NCOA2_677_700 (SRC2-2), is shown in more detail in Fig. 3A. Binding of FXR, compared to the DMSO control, was significantly increased by addition of the natural FXR agonist, CDCA. This effect was significantly but not fully reversed by the FXR antagonist, (Z)-guggulsterone. All the FXR antagonists tested in the study displayed inhibitory effect on CDCA-induced FXR activity expressed as coactivator binding, to various extents. All members in the avermectin

class displayed antagonism that was similar or superior to the positive control, (Z)-guggulsterone. Chloro- and di-phacinone also displayed strong antagonism. Chlorophacinone even showed the highest efficacy of up to full antagonism of CDCA-induced binding compared to all other tested compounds. Other 1,3-indanediones displayed much weaker antagonism than (Z)-guggulsterone. The selected motif is found in NCOA2, a histone acetyl transferase. This enzymatic activity enhances target gene availability and thus elevates gene transcription levels. A large number of motifs in this cluster are derived from coactivator proteins. Compound-induced antagonism of FXR function, i.e., expression of target genes, can be explained by decreased affinity and subsequent release of transcription facilitating coregulators.

While all avermectins, chlorophacinone and diphacinone displayed strong passive antagonism, clustering revealed an additional class of interactions with different mechanisms of action at the far left of the heatmap (Fig. 2B), which is illustrated in detail in Fig. 3B. At basal level (DMSO), FXR interacted with this coregulator motif, but CDCA treatment released this binding. (Z)-Guggulsterone, and many FXR antagonists did not affect this binding; however, avermectins, except for moxidectin, reversed the CDCA effect. Selamectin, abamectin and ivermectin even induced an FXR binding level to this motif that exceeded that of the apo receptor. The selected motif and the other motif in this class of interactions in the heatmap are derived from NCORs. These corepressor proteins are well characterized for histone deacetylation and chromatin condensation, which disfavors gene transcription. Enhanced recruitment of these proteins to FXR by some of the compounds reveals a different mode of action which we refer to as active antagonism (i.e. an active way to reduce car speed is to press a foot to the brake). In addition to classification of the compound by weak and strong passive antagonism, the latter group can be further classified by absence or presence of displayed active antagonism.

The FXR-coregulator binding interactions were tested with two concentrations of chlorophacinone (Fig. S1) or three concentrations of ivermectin (Fig. S2) in the presence of CDCA. Ivermectin showed concentration-dependent enhancement in CDCA-mediated FXR binding to several coregulators including CBP_2055_2007, EP300_2039_2061, NCOR1_2251_2273, NCOR2_2330_2352, and NRIP1_120_142 as well as reduction in CDCA-mediated FXR binding to several coregulators including NCOA6_875_897. Chlorophacinone enhanced FXR binding interactions to CNOT1_557_579 and RBL2_875_897_C879S/C894S at higher concentrations, while significantly attenuating FXR binding interactions to several coregulators such as EP300_69_91, LCOR_40_62, JHD2C_2054_2076, TIF1A_747_769, and UBE3A_649_671. Both compounds effectively reduced binding interactions between FXR and several coregulators including BRD8_254_276, CBP_57_80, NCOA1_677_700, NCOA2_677_700, NCOA2_733_755, NCOA3_673_695, NCOA3_725_747, MED1_591_614, MED1_632_655, NR0B2_106_128, PRGC1_130_155, PRGC_134_154, PRGC2_146_166, PRGC2_388_358, and WIP1_119_141.

Receptor Selectivity of avermectins and indandiones

To better translate the compound activity of (Z)-guggulsterone, chlorophacinone, ivermectin, and their analogs from receptor assays to functional outcomes, these compounds were also tested for their ability to modulate other functionally relevant nuclear receptors, which coordinate with FXR to regulate downstream signaling, such as the constitutive androstane receptor (CAR), the liver X receptor alpha (LXR α), and pregnane X receptor (PXR) using cell-based luciferase reporter gene assays to measure luciferase expression under the control of the corresponding nuclear receptors and TR-FRET coactivator recruitment assays to measure the receptor-coactivator interactions.

In the CAR-luc assay, guggulsterones and several indandiones were identified as potential CAR agonists and avermectins as potential CAR antagonists (Table 2). (E)-guggulsterone, (Z)-guggulsterone, chlorophacinone, diphacinone, and phenindione partially induced CAR transactivation but exerted no agonist effects on recruiting PGC-1 α coactivator-derived peptide to CAR-LBD (Table 3). Avermectins were at least 5-fold more potent in inhibiting CITCO-induced coactivator recruitment than CITCO-induced CAR-luc expression with comparable cytotoxicity to CAR-luc cells.

In the LXR α -luc assay, avermectins and several indandiones were identified as potential LXR α antagonists (Table 2). Avermectins completely suppressed T0901317-induced LXR α transactivation at slightly cytotoxic concentrations ($IC_{50_Viability}/IC_{50_LXR\alpha-luc} > 3$), with doramectin as the most potent LXR α antagonist among the tested compounds. Chlorophacinone had similar IC_{50} values in reducing T0901317-induced LXR α activity in both cell-based and biochemical assays, while diphacinone and phenindione were active only in the LXR α -luc assay (Table 2 and Table 3). Guggulsterones showed neither agonistic nor antagonistic effects on LXR α within the tested concentration range.

In the PXR-luc assay, guggulsterones showed partial activities on both PXR agonist and antagonist modes. Moxidectin, diphacinone and phenidione had PXR agonist activity, while other avermectins, chlorophacinone and pindone had PXR antagonist activity (Table 2). Both isoforms of guggulsterones were more potent and less efficacious in inducing PXR-luc transactivation than inhibiting rifampicin-induced PXR activation. Doramectin and selamectin potently reduced rifampicin-induced PXR-luc expression with submicromolar IC_{50} values. Abamectin and pindone also inhibited rifampicin-induced PXR activity at low micromolar concentrations. Due to lack of available PXR coactivator recruitment assays, agonist and antagonist activities of the identified compounds at biochemical level were not explored in this study.

DISCUSSION

In this study, we combined *in vitro* and computational approaches to investigate the mechanisms of action of FXR-active chemicals and their analogs identified from a previous Tox21 screening (Hsu *et al.*, 2014). A battery of *in vitro* cell-based reporter gene assays and biochemical coregulator assays in 1536-well plate or 154-plex microarray formats enabled high-throughput generation of data regarding compound activity and selectivity. Quantitative structure-activity relationship (QSAR) modeling method was employed to identify key

structural features correlated to inhibited recruitment of an SRC2 coactivator-derived peptide to FXR.

Reporter gene assays are useful experimental methods to assess compounds' ability to potentially interfere with the physiological functions of targets of interest in a cellular environment, and many reporter gene assays can be adapted to high-throughput screening platforms to rapidly profile large numbers of chemicals. Our previous study (Hsu *et al.*, 2014) used a cell-based FXR-responsive beta-lactamase reporter gene FXR-bla assay to profile the Tox21 10K chemical collection of environmental chemicals and drugs for FXR activity and also identified some potential assay artifacts including compound autofluorescence and compound cytotoxicity by comparing compound activities in available Tox21 10K qHTS data sets generated by a panel of beta-lactamase assays. In the follow-up studies, we utilized the same FXR-bla assay to test compounds prepared from same sources used for primary screening by cherry-picking and from re-procured alternatives. The weak concordance of pyrethroids in FXR activity tested in both cherry-picked and re-procured forms is likely due to the unknown breakdown products of pyrethroids in stocks used for primary screening. In addition, luciferase reporter gene assays were employed to explore compound selectivity against nuclear receptors (e.g., CAR, LXR α , and PXR) that also heterodimerize with the RXR and coordinate with FXR to regulate subsets of downstream effectors and signaling pathways involved in energy and xenobiotic metabolism (Cave *et al.*, 2016). Avermectin- and milbemycin-based drugs (e.g., ivermectin and moxidectin) not only interacted with FXR but also CAR, LXR α , and PXR. The antiparasitic drug ivermectin was previously shown to stimulate FXR activity and to inhibit CDCA-induced FXR activity in cell-based and biochemical assays (Jin *et al.*, 2013; Yu *et al.*, 2013; Hsu *et al.*, 2014). Ivermectin was also shown to decrease cholesterol and serum glucose levels, and improve insulin sensitivity in mice by stimulating FXR activity, suggesting the potential use of ivermectin or its analogs for diabetes treatment (Jin *et al.*, 2013). However, the potential *in vivo* outcomes of ivermectin's FXR antagonist activity have not been well characterized. All avermectins tested in our experiments showed antagonistic activity to FXR, CAR, LXR α , and PXR. Moxidectin, a milbemycin analog, antagonized transactivation of FXR, CAR and LXR α , but partially increased the expression of the PXR-luc reporter gene. Our results showed that the macrolactam moiety and the disaccharide moiety of avermectins and milbemycins are crucial for FXR antagonist activity and compound selectivity, respectively. It will be interesting to study how the antagonist activities of these compounds on FXR, CAR, LXR α , and PXR affect their *in vivo* outcomes.

The 1,3-indandione-based rodenticides such as chlorophacinone and diphacinone are identified as FXR antagonists in our study. The two rodenticides are anti-coagulants that act through antagonizing the action of vitamin K. Exposures of chlorophacinone and diphacinone through oral, inhalation, or dermal routes cause toxic effects including hemorrhage, liver necrosis, developmental toxicities, and death. Our results showed that chlorophacinone and diphacinone had FXR antagonist, CAR agonist, and LXR α antagonist activities. The two compounds had distinct activity on PXR: Chlorophacinone partially decreased the SR 12813-induced PXR-luc expression and diphacinone partially stimulated PXR transactivation at high micromolar concentrations. 1,1-Diphenylacetone, which contains the common structural feature in both chlorophacinone and diphacinone, weakly

inhibited CDCA-induced FXR transactivation and showed no activity to CAR, LXR α , and PXR. These results indicate the importance of the diphenyl indane moiety in antagonizing FXR activity. Since chlorophacinone and diphacinone are potent rodenticides that might cause severe lethality at concentrations lower than those exerting FXR-mediated toxicities, it will be valuable to use non-rodent species to study their *in vivo* FXR antagonist activities.

The distinct functional profile of nuclear receptors in response to binding of ligands is largely determined by the ligand-specific recruitment of coregulators, which ultimately controls specific transcription of target genes and affects energy and xenobiotic metabolism. Upon ligand binding, FXR undergoes conformational changes to release corepressors such as the nuclear corepressor (NCoR) and recruits coactivators including steroid receptor coactivator-1 (SRC-1). Cholesterol 7 α -hydroxylase (CYP7A1) is the rate-limiting enzyme in the conversion of cholesterol to bile acids. It is known that bile acids regulate the transcription of CYP7A1 by feedback repression through FXR binding. Studies have shown that the ability of various bile acids to recruit transcriptional coactivator protein (e.g., SRC-1) to the ligand binding domain of FXR is consistent with their ability to repress Cyp7A1 mRNA levels (Bramlett *et al.*, 2000). On the other hand, *in vitro* pharmacological studies on liver cells demonstrated that theonellasterol antagonized the expression of FXR target genes including *ABCC4* for the multidrug resistance-associated protein 4 (MRP-4) by abolishing the release of nuclear corepressor NCoR from the promoter of genes (Renga *et al.*, 2012), and the *in vivo* studies using mice with obstructive cholestasis showed that theonellasterol increased MRP-4 expression in the liver and protected mice from liver injury. To determine whether a compound is a ligand of the nuclear receptor, and to assist the prediction of potential outcomes of modulating specific receptor-coregulator interactions, biochemical assays that measure binding interactions between a nuclear receptor and its corresponding coregulator(s) can be used. The two types of coregulator assays used in the present study generated rich information regarding FXR-coregulator interactions. The first coregulator assay is a homogeneous nuclear receptor binding assay that relies on time-resolved fluorescence resonance energy transfer (TR-FRET) between a donor dye for FXR and an acceptor dye for an SRC2 coactivator-derived peptide. Because the terbium-based donor dye used in the TR-FRET assay has a long fluorescence lifetime that can be detected at a later time point, the TR-FRET assay generates less false positives coming from compounds with a short fluorescence lifetime or precipitated compounds that scattered light. Unlike the TR-FRET assay, which measures a single FXR-coactivator interaction at a time, the second coregulator assay, MARCoNI, enables simultaneous measurement of 154 multiplex FXR-coregulator assays by fluorescence imaging of coregulator-bound FXR complexes on a microarray of immobilized peptides derived from various coregulator motifs. Our results showed that (Z)-guggulsterone, chlorophacinone, ivermectin, and some of their analogs antagonized CDCA-induced recruitment of a SRC2-derived peptide to FXR in the TR-FRET assay, but the FXR MARCoNI profiling study revealed that the three structural classes differentially modulated binding interactions between FXR and subsets of coregulator-derived peptides. Our results with ivermectin agree with previous findings, in which ivermectin was shown to partially promote the SRC-1,-2,-3 recruitment by FXR and also induced the recruitment of corepressor NCoR-2 and SMRT-2 by FXR (Jin *et al.*, 2013).

Computational approaches are powerful in identifying potential structural features important for modulating FXR activity. The HTS assays conducted in the current study, including the FXR-bla assay, the FXR TR-FRET coactivator assay, and the FXR MARCoNI profiling experiment generated rich information about the FXR activities of compounds and enabled us to develop and apply computational approaches such as QSAR and hierarchical clustering to rapidly analyze and interpret the HTS assay data. Despite the fact that some compounds identified from the FXR-bla antagonist screen were cytotoxic, many of the cytotoxic FXR antagonists also disrupted FXR-SRC2 interactions in the TR-FRET biochemical assay. By grouping compounds according to their FXR-bla and FXR TR-FRET assay results, our QSAR analysis show that not every compound modulates FXR signaling through SRC2; this identifies several structural features unique to SRC2-interacting compounds. Additionally, by hierarchical clustering of the FXR MARCoNI profiling data, we found that chlorophacinone, ivermectin, (Z)-guggulsterone, and their analogs differentially modulated the binding interactions between FXR and subsets of coactivator- or corepressor-derived peptides.

In addition to using FXR antagonists, reducing FXR activity by gene knockout is another popular method to study FXR functions. FXR deficiency has been associated with a number of beneficial effects including decreased cholesterol levels. In general, *Fxr*^{-/-} mice have normal phenotypes that are externally indistinguishable from their wild type littermates: FXR deficient mice are similar to wild type mice in terms of body weight, body composition, fertility, energy intake and expenditure as well as behaviors at a young age (Lamers *et al.*, 2014). However, depending on diet, age and health status, distinguished phenotypes have been observed in *Fxr*^{-/-} mice; some of which seem to have beneficial health effects. For example, female *Fxr*^{-/-} mice had increased energy expenditure and were resistant to diet-induced obesity (Zhang *et al.*, 2012). Aging *Fxr*^{-/-} mice display late onset leanness associated with elevated energy expenditure and improved glucose control (Bjursell *et al.*, 2013). Interestingly, depending on the cancer type, FXR deficiency resulted in either beneficial or adverse effects on tumor growth. Over-expression of FXR was previously found to be associated with larger tumor size and grade as well as lymph node metastasis in esophageal adenocarcinoma, while loss of FXR suppressed tumor growth (Guan *et al.*, 2013). However, some studies showed that sustained high levels of bile acids in *Fxr*^{-/-} mice may contribute to liver tumorigenesis (Yang *et al.*, 2007) and FXR deficiency increased the size of small intestine adenocarcinomas in adenomatous polyposis coli mutant mice and colon carcinogen-treated C57BL/6 mice (Maran *et al.*, 2009). Taken together, these *in vivo* findings further illustrate the complex nature of FXR biology.

In summary, the current study provides mechanistic insights of *in vitro* FXR antagonist activity of guggulsterones, avermectin-based drugs, and 1,3-indandione-based rodenticides as well their ability to alter signaling activity of other functionally relevant nuclear receptors, including CAR, LXR α , and PXR. The *in vitro* results generated from the present study and the *in vivo* evidence of FXR deficiency from the literature warrant continuing exploration of the identified FXR antagonists in appropriate *in vivo* models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Aarts JM, Wang S, Houtman R, van Beuningen RM, Westerink WM, Van De Waart BJ, Rietjens IM, Bovee TF. Robust array-based coregulator binding assay predicting ERalpha-agonist potency and generating binding profiles reflecting ligand structure. *Chem Res Toxicol*. 2013; 26:336–346. [PubMed: 23383871]
- Bjursell M, Wedin M, Admyre T, Hermansson M, Bottcher G, Goransson M, Linden D, Bamberg K, Oscarsson J, Bohlooly YM. Ageing Fxr deficient mice develop increased energy expenditure, improved glucose control and liver damage resembling NASH. *PLoS One*. 2013; 8:e64721. [PubMed: 23700488]
- Bramlett KS, Yao S, Burris TP. Correlation of farnesoid X receptor coactivator recruitment and cholesterol 7alpha-hydroxylase gene repression by bile acids. *Mol Genet Metab*. 2000; 71:609–615. [PubMed: 11136553]
- Cave MC, Clair HB, Hardesty JE, Falkner KC, Feng W, Clark BJ, Sidey J, Shi H, Aqel BA, McClain CJ, Prough RA. Nuclear receptors and nonalcoholic fatty liver disease. *Biochim Biophys Acta*. 2016; 1859:1083–1099. [PubMed: 26962021]
- Guan B, Li H, Yang Z, Hoque A, Xu X. Inhibition of farnesoid X receptor controls esophageal cancer cell growth in vitro and in nude mouse xenografts. *Cancer*. 2013; 119:1321–1329. [PubMed: 23280144]
- Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB Jr, Kliewer SA, Gonzalez FJ, Sinal CJ. Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem*. 2003; 278:45062–45071. [PubMed: 12923173]
- Handschin C, Meyer UA. Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR. *Arch Biochem Biophys*. 2005; 433:387–396. [PubMed: 15581595]
- Houtman R, de Leeuw R, Rondaij M, Melchers D, Verwoerd D, Ruijtenbeek R, Martens JW, Neeffjes J, Michalides R. Serine-305 phosphorylation modulates estrogen receptor alpha binding to a coregulator peptide array, with potential application in predicting responses to tamoxifen. *Mol Cancer Ther*. 2012; 11:805–816. [PubMed: 22319200]
- Hsu CW, Zhao J, Huang R, Hsieh JH, Hamm J, Chang X, Houck K, Xia M. Quantitative high-throughput profiling of environmental chemicals and drugs that modulate farnesoid X receptor. *Sci Rep*. 2014; 4:6437. [PubMed: 25257666]
- Huang R, Xia M, Cho MH, Sakamuru S, Shinn P, Houck KA, Dix DJ, Judson RS, Witt KL, Kavlock RJ, Tice RR, Austin CP. Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. *Environ Health Perspect*. 2011; 119:1142–1148. [PubMed: 21543282]
- Jin L, Feng X, Rong H, Pan Z, Inaba Y, Qiu L, Zheng W, Lin S, Wang R, Wang Z, Wang S, Liu H, Li S, Xie W, Li Y. The antiparasitic drug ivermectin is a novel FXR ligand that regulates metabolism. *Nat Commun*. 2013; 4:1937. [PubMed: 23728580]

- Kemper JK. Regulation of FXR transcriptional activity in health and disease: Emerging roles of FXR cofactors and post-translational modifications. *Biochim Biophys Acta*. 2011; 1812:842–850. [PubMed: 21130162]
- Kong B, Luyendyk JP, Tawfik O, Guo GL. Farnesoid X receptor deficiency induces nonalcoholic steatohepatitis in low-density lipoprotein receptor-knockout mice fed a high-fat diet. *J Pharmacol Exp Ther*. 2009; 328:116–122. [PubMed: 18948497]
- Koppen A, Houtman R, Pijnenburg D, Jenning EH, Ruijtenbeek R, Kalkhoven E. Nuclear receptor-coregulator interaction profiling identifies TRIP3 as a novel peroxisome proliferator-activated receptor gamma cofactor. *Mol Cell Proteomics*. 2009; 8:2212–2226. [PubMed: 19596656]
- Lamers C, Schubert-Zsilavecz M, Merk D. Medicinal chemistry and pharmacological effects of Farnesoid X Receptor (FXR) antagonists. *Curr Top Med Chem*. 2014; 14:2188–2205. [PubMed: 25388533]
- Lien F, Berthier A, Bouchaert E, Gheeraert C, Alexandre J, Porez G, Prawitt J, Dehondt H, Ploton M, Colin S, Lucas A, Patrice A, Pattou F, Diemer H, Van Dorsselaer A, Rachez C, Kamilic J, Groen AK, Staels B, Lefebvre P. Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. *J Clin Invest*. 2014; 124:1037–1051. [PubMed: 24531544]
- Lynch C, Zhao J, Huang R, Xiao J, Li L, Heyward S, Xia M, Wang H. Quantitative high-throughput identification of drugs as modulators of human constitutive androstane receptor. *Sci Rep*. 2015; 5:10405. [PubMed: 25993555]
- Maran RR, Thomas A, Roth M, Sheng Z, Esterly N, Pinson D, Gao X, Zhang Y, Ganapathy V, Gonzalez FJ, Guo GL. Farnesoid X receptor deficiency in mice leads to increased intestinal epithelial cell proliferation and tumor development. *J Pharmacol Exp Ther*. 2009; 328:469–477. [PubMed: 18981289]
- Renga B, Mencarelli A, D'Amore C, Cipriani S, D'Auria MV, Sepe V, Chini MG, Monti MC, Bifulco G, Zampella A, Fiorucci S. Discovery that theonellasterol a marine sponge sterol is a highly selective FXR antagonist that protects against liver injury in cholestasis. *PLoS One*. 2012; 7:e30443. [PubMed: 22291955]
- Shukla SJ, Sakamuru S, Huang R, Moeller TA, Shinn P, Vanleer D, Auld DS, Austin CP, Xia M. Identification of clinically used drugs that activate pregnane X receptors. *Drug Metab Dispos*. 2011; 39:151–159. [PubMed: 20966043]
- Urizar NL, Liverman AB, Dodds DT, Silva FV, Ordentlich P, Yan Y, Gonzalez FJ, Heyman RA, Mangelsdorf DJ, Moore DD. A natural product that lowers cholesterol as an antagonist ligand for FXR. *Science*. 2002; 296:1703–1706. [PubMed: 11988537]
- Wang XX, Jiang T, Shen Y, Caldas Y, Miyazaki-Anzai S, Santamaria H, Urbanek C, Solis N, Scherzer P, Lewis L, Gonzalez FJ, Adorini L, Pruzanski M, Kopp JB, Verlander JW, Levi M. Diabetic nephropathy is accelerated by farnesoid X receptor deficiency and inhibited by farnesoid X receptor activation in a type 1 diabetes model. *Diabetes*. 2010; 59:2916–2927. [PubMed: 20699418]
- Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. *Cancer Res*. 2007; 67:863–867. [PubMed: 17283114]
- Yu DD, Lin W, Chen T, Forman BM. Development of time resolved fluorescence resonance energy transfer-based assay for FXR antagonist discovery. *Bioorg Med Chem*. 2013; 21:4266–4278. [PubMed: 23688559]
- Zhang Y, Ge X, Heemstra LA, Chen WD, Xu J, Smith JL, Ma H, Kasim N, Edwards PA, Novak CM. Loss of FXR protects against diet-induced obesity and accelerates liver carcinogenesis in ob/ob mice. *Mol Endocrinol*. 2012; 26:272–280. [PubMed: 22261820]

Highlights

- A subset of Tox21 chemicals was investigated for FXR antagonism
- In vitro and computational approaches were used to evaluate FXR antagonists
- Chlorophacinone and ivermectin had distinct patterns in modulating FXR activity

Fig. 1. Selected FXR-active structural classes inhibited CDCA-induced association between FXR and a SRC2-derived coactivator peptide

a. Concentration-response curves of avermectin and milbemycin-based anthelmintic drugs. **b.** Concentration-response curves of dihydropyridine-based calcium channel blockers. **c.** Concentration-response curves of 1,3-indandione-based rodenticides. All data points were collected in the presence of 50 μ M CDCA and shown as mean $\pm$ SEM from three experiments.

Fig. 2. FXR-coregulator interaction profiling of (Z)-guggulsterone, chlorophacinone, ivermectin, and their analogs

a. Chemical structures of compounds tested in the FXR MARCoNI assays. **b.** Compounds and coregulators were clustered (hierarchical clustering, Euclidean distance, average linkage) based on modulation index (MI). Significance of the binding modulation is indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 3. Differential modulation of coactivators and corepressors by (Z)-guggulsterone, chlorophacinone, ivermectin, and their analogs

a. Negative modulation of binding between FXR and NCOA2_677_700 (SRC2-2) by all test compounds. **b.** Positive modulation of binding between FXR and NCOR1_2251_2273 by ivermectin and its analogs. Each data point was presented as mean receptor binding $\pm$ SEM from three experiments. Bars are colored by MI standing for modulation index, i.e., compound-induced log-fold change of (CDCA-induced) FXR-coregulator interaction.

Significance of the binding modulation (vs. CDCA) is indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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Table 1

Structure-activity relationship (SAR) results of representative FXR-active Tox21 chemicals

Structural fragment	# in Group1/ # in Group2	Representative compound groups	Example chemical structures
0/15	Avermectin-based anthelmintic and organochlorine pesticides	Mibemectin Aldrin
0/6	Environmental chemicals	2,2'-Methylenebis(4-methyl-6-tert-butylphenol)
0/4	Environmental chemicals and laxatives	4,4'-(9H-Fluorene-9,9-diyldiphenol) Oxyphenisatin
0/5	Dihydropyridine-based calcium channel blockers and 1,3-indandione-based rodenticides	Nicardipine Chlorophacinone
4/12	Imidazole antifungals	Econazole
8/0	Anthracycline-based chemotherapeutic	Daunorubicin
8/2	Calcium sensitizers & dyes	Levosimendan Toluidine Blue

Table 2

Selectivity of guggulsterones, avermectins, and 1,3-indandiones against FXR, CAR, LXRo, and PXR in cell-based assays.

Compound Name (CASRN)	FXR-bla (antagonist) IC ₅₀ , μM (Efficacy, %)	Viability (FXR) IC ₅₀ , μM (Efficacy, %)	CAR-luc (agonist) EC ₅₀ , μM (Efficacy, %)	CAR-luc (antagonist) IC ₅₀ , μM (Efficacy, %)	Viability (CAR) IC ₅₀ , μM (Efficacy, %)	LXR-luc (antagonist) IC ₅₀ , μM (Efficacy, %)	Viability (LXR) IC ₅₀ , μM (Efficacy, %)	PXR-luc (agonist) EC ₅₀ , μM (Efficacy, %)	PXR (antagonist) IC ₅₀ , μM (Efficacy, %)
(E)-Guggulsterone (39025-24-6)	15.88 ± 3.79 (65 ± 10)	Inactive	(35 ± 4 at 38 μM)	Inactive	Inactive	(48 ± 12 at 38 μM)	Inactive	(26 ± 2 at 38 μM)	(35 ± 11 at 38 μM)
(Z)-Guggulsterone (39025-23-5)	20.03 ± 2.76 (71 ± 5)	Inactive	(44 ± 1 at 38 μM)	Inactive	Inactive	11.62 ± 6.02 (58 ± 11)	Inactive	(24 ± 4 at 38 μM)	(43 ± 15 at 38 μM)
Abamectin (71751-41-2)	3.25 ± 0.88 (122 ± 8)	8.32 ± 0.25 (98 ± 0)	Inactive	31.39 ± 19.00 (132 ± 9)	Inactive	6.22 ± 7.17 (73 ± 40)	(37 ± 24 at 38 μM)	Inactive	(41 ± 14 at 38 μM)
Doramectin (117704-25-3)	3.07 ± 1.01 (125 ± 6)	9.96 ± 0.08 (98 ± 1)	Inactive	43.23 ± 23.00 (134 ± 8)	12.47 ± 0.34 (103 ± 2)	2.18 ± 1.68 (95 ± 3)	29.87 ± 4.68 (61 ± 5)	Inactive	(45 ± 6 at 38 μM)
Ivermectin (70288-86-7)	1.18 ± 0.98 (110 ± 31)	38.16 ± 3.90 (90 ± 5)	Inactive	243.10 ± 143.39 (131 ± 9)	37.74 ± 17.86 (104 ± 1)	6.03 ± 4.39 (86 ± 4)	(45 ± 10 at 38 μM)	Inactive	(45 ± 8 at 38 μM)
Moxidectin (113507-06-5)	9.56 ± 3.08 (105 ± 6)	16.67 ± 7.70 (97 ± 1)	Inactive	44.05 ± 0.91 (135 ± 8)	22.28 ± 2.05 (100 ± 2)	5.31 ± 3.45 (98 ± 1)	31.13 ± 2.60 (64 ± 2)	6.21 ± 2.59 (51 ± 10)	Inactive
Selamectin (220119-17-5)	3.33 ± 0.73 (116 ± 6)	17.90 ± 0.60 (75 ± 5)	Inactive	29.04 ± 17.03 (58 ± 5)	21.63 ± 1.30 (54 ± 2)	10.84 ± 3.75 (69 ± 4)	(49 ± 13 at 38 μM)	Inactive	0.90 ± 0.31 (52 ± 11)
2-Acetyl-1,3-indandione (1133-72-8)	Inactive	Inactive	Inactive	Inactive	Inactive	(49 ± 13 at 77 μM)	Inactive	Inactive	Inactive
Chlorophacinone (3691-35-8)	6.07 ± 0.89 (107 ± 2)	33.60 ± 0.46 (88 ± 4)	45.46 ± 0.58 (62 ± 2)	Inactive	Inactive	24.26 ± 12.46 (89 ± 11)	Inactive	Inactive	(48 ± 18 at 77 μM)
Diphacinone (82-66-6)	11.33 ± 2.61 (107 ± 8)	Inactive	(32 ± 8 at 77 μM)	Inactive	Inactive	35.73 ± 35.51 (67 ± 14)	Inactive	60.67 ± 16.90 (29 ± 5)	Inactive
1,1-Diphenylacetone (781-35-1)	(28 ± 6 at 77 μM)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Phenindione (83-12-5)	Inactive	Inactive	525.33 ± 148.42 (54 ± 5)	Inactive	Inactive	25.01 ± 7.08 (73 ± 11)	Inactive	(44 ± 7 at 77 μM)	Inactive

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Compound Name (CASRN)	FXR-bla (antagonist) IC ₅₀ , μM (Efficacy, %)	Viability (FXR) IC ₅₀ , μM (Efficacy, %)	CAR-luc (agonist) EC ₅₀ , μM (Efficacy, %)	CAR-luc (antagonist) IC ₅₀ , μM (Efficacy, %)	Viability (CAR) IC ₅₀ , μM (Efficacy, %)	LXR-luc (antagonist) IC ₅₀ , μM (Efficacy, %)	Viability (LXR) IC ₅₀ , μM (Efficacy, %)	PXR-luc (agonist) EC ₅₀ , μM (Efficacy, %)	PXR (antagonist) IC ₅₀ , μM (Efficacy, %)
Pindone (83-26-1)	(42 ± 8 at 77 μM)	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	(39 ± 6 at 77 μM)

Data are presented as mean ± SEM from three experiments. Compounds with efficacy values smaller than 20% were considered as inactive.

Table 3

Selectivity of guggulsterones, avermectins, and 1,3-indandiones against FXR, CAR, LXRA, and PXR in the TR-FRET coactivator antagonist mode assays.

Compound Name	FXR Coactivator IC ₅₀ , μ M (Efficacy, %)	CAR Coactivator IC ₅₀ , μ M (Efficacy, %)	LXRA Coactivator IC ₅₀ , μ M (Efficacy, %)
(E)-Guggulsterone	(40 $\pm$ 4 at 38 μ M)	Inactive	Inactive
(Z)-Guggulsterone	(34 $\pm$ 2 at 38 μ M)	Inactive	Inactive
Abamectin	2.14 $\pm$ 0.40 (116 $\pm$ 3)	(49 $\pm$ 13 at 38 μ M)	2.03 $\pm$ 1.62 (88 $\pm$ 47)
Doramectin	1.15 $\pm$ 0.11 (98 $\pm$ 2)	1.84 $\pm$ 0.54 (55 $\pm$ 6)	2.47 $\pm$ 2.13 (98 $\pm$ 5)
Ivermectin	0.76 $\pm$ 0.09 (124 $\pm$ 2)	(40 $\pm$ 6 at 38 μ M)	3.57 $\pm$ 2.67 (97 $\pm$ 2)
Moxidectin	13.89 $\pm$ 0.92 (59 $\pm$ 1)	8.79 $\pm$ 1.35 (67 $\pm$ 8)	11.11 $\pm$ 5.30 (67 $\pm$ 3)
Selamectin	0.94 $\pm$ 0.11 (117 $\pm$ 3)	2.76 $\pm$ 0.99 (61 $\pm$ 8)	29.13 $\pm$ 11.22 (57 $\pm$ 5)
2-Acetyl-1,3-indanedione	Inactive	Inactive	Inactive
Chlorophacinone	53.79 $\pm$ 11.66 (64 $\pm$ 12)	Inactive	35.25 $\pm$ 10.58 (52 $\pm$ 15)
Diphacinone	(34 $\pm$ 5 at 77 μ M)	Inactive	Inactive
1, 1'-Diphenylacetone	(26 $\pm$ 5 at 77 μ M)	Inactive	Inactive
Phenindione	Inactive	Inactive	Inactive
Pindone	Inactive	Inactive	Inactive

Data are presented as mean $\pm$ SEM from three experiments. Compounds with efficacy values smaller than 20% were considered as inactive.

Table 4

FXR MARCoNI data of known FXR coregulators.

MARCoNI Assay ID	Target	CDCA + DMSO	CDCA + Guggulsterone	CDCA + Ivermectin	CDCA + Chlorophacinone
CBP_2055_2077	CBP	50 ± 0	1508 ± 254	184 ± 36	60 ± 13
EP300_2039_2061	p300	74 ± 22	82 ± 18	199 ± 99	88 ± 16
MED1_591_614	DRIP205	13670 ± 209	4221 ± 363	1100 ± 321	1319 ± 32
MED1_632_655	DRIP205	2056 ± 182	1078 ± 228	273 ± 273	50 ± 0
NCOA1_1421_1441	SRC1	9945 ± 208	3398 ± 334	1381 ± 216	680 ± 48
NCOA1_620_643	SRC1	50 ± 0	50 ± 0	50 ± 0	50 ± 0
NCOA1_677_700	SRC1	51347 ± 1459	18599 ± 2630	13835 ± 1149	5001 ± 276
NCOA2_628_651	SRC2	50 ± 0	50 ± 0	63 ± 11	50 ± 0
NCOA2_677_700	SRC2	39425 ± 928	13472 ± 1900	7761 ± 515	3781 ± 192
NCOA3_104_123_N-KKK	SRC3	11268 ± 252	157 ± 63	141 ± 63	199 ± 233
NCOA3_673_695	SRC3	27836 ± 971	8847 ± 926	3919 ± 405	1809 ± 223
NCOA3_725_747	SRC3	6033 ± 282	2367 ± 288	626 ± 30	561 ± 30
NCOA6_1479_1501	ASC2	185 ± 44	279 ± 143	50 ± 0	50 ± 0
NCOR1_2251_2273	N-CoR1	50 ± 0	82 ± 39	2346 ± 288	61 ± 12
NCOR1_2376_2398	N-CoR1	50 ± 0	50 ± 0	50 ± 0	50 ± 0
NCOR2_2123_2145	SMRT	344 ± 26	238 ± 19	414 ± 45	261 ± 18
NCOR2_2330_2352	SMRT	75 ± 20	265 ± 65	4133 ± 419	167 ± 22
NR0B2_106_128	SHP	264 ± 40	337 ± 162	64 ± 17	50 ± 0
NR0B2_237_257	SHP	58 ± 6	51 ± 1	222 ± 175	69 ± 23
NRIP1_253_275_C263S	RIP140	2060 ± 142	1045 ± 224	334 ± 108	337 ± 43
NRIP1_368_390	RIP140	6435 ± 412	3171 ± 217	1187 ± 139	1070 ± 76
NRIP1_488_510	RIP140	4184 ± 262	1614 ± 217	835 ± 105	524 ± 41
NRIP1_701_723	RIP140	51 ± 1	107 ± 58	194 ± 92	50 ± 0
NRIP1_805_831	RIP140	30916 ± 1213	10978 ± 1112	6980 ± 1034	2428 ± 172
NRIP1_924_946	RIP140	2257 ± 161	1595 ± 196	898 ± 161	657 ± 35
PCAF_178_200	PCAF	69 ± 16	127 ± 14	159 ± 74	116 ± 21
PRGC1_130_155	PGC1 α	22951 ± 488	9079 ± 820	2971 ± 247	2025 ± 224
PRGC1_134_154	PGC1 α	24330 ± 183	8668 ± 717	3649 ± 475	2425 ± 114
PRGC2_146_166	PGC1 β	1106 ± 136	666 ± 111	483 ± 525	183 ± 39
PRGC2_338_358	PGC1 β	482 ± 98	305 ± 60	109 ± 72	50 ± 0

Data are presented as mean ± SEM from three experiments.