Quantitative High-Throughput Profiling of Environmental Chemicals and Drugs that Modulate Farnesoid X Receptor

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The farnesoid X receptor (FXR) is a bile acid-activated nuclear receptor that plays a crucial role in maintaining the homeostasis of bile acids, lipids, and glucose. Because endogenous chemicals bind and activate FXR, it is important to examine which xenobiotic compounds would disrupt normal receptor function. We used a cell-based human FXR β-lactamase (Bla) reporter gene assay to profile the Tox21 10K compound collection of environmental chemicals and drugs. Structure-activity relationships of FXR-active compounds revealed by this screening were then compared against the androgen receptor, estrogen receptor α, peroxisome proliferator-activated receptors δ and γ, and the vitamin D receptor. We identified several FXR-active structural classes including anthracyclines, benzimidazoles, dihydropyridines, pyrethroids, retinoic acids, and vinca alkaloids. Microtubule inhibitors potently decreased FXR reporter gene activity. Pyrethroids specifically antagonized FXR transactivation. Anthracyclines affected reporter activity in all tested assays, suggesting non-specific activity. These results provide important information to prioritize chemicals for further investigation, and suggest possible modes of action of compounds in FXR signaling.

The farnesoid X receptor (FXR), a bile acid-activated nuclear receptor, plays a crucial role in maintaining the homeostasis of bile acids, lipids, and glucose.4,5,25,26 FXR contains a DNA-binding domain for docking to target genes with an FXR response element (FXRE) and a ligand-binding domain (LBD) used by intracellular ligands. Binding of FXR agonists to FXR-LBD induces conformational changes in FXR and promotes expression of target genes including small heterodimer partner (SHP) and bile salt export pump (BSEP).27 Activation of SHP results in repression of two key cytochrome P450 enzymes in bile acid biosynthesis, cholesterol 7-alpha-monooxygenase (CYP7A1) and 12-alpha-hydroxylase (CYP8B1), as well as several important regulators of glucose metabolism including glucose 6-phosphatase (G6Pase), fructose-1,6-bisphosphatase 1 (FBP1), and phosphoenolpyruvate carboxykinase (PEPCK).28 The well-characterized endogenous ligands of FXR are bile acids including chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA).29,30 Guggulsterone, a plant steroid, was the first natural FXR antagonist identified.31 Other FXR ligands include natural products and investigational drugs. Ivermectin, an avermectin antiparasitic, has been recently identified to bind FXR-LBD and decrease serum glucose and cholesterol levels in mice.32 Synthetic FXR agonists including GW4064, INT-747, PX-102, FXR-450, and their analogs are under development for treating dyslipidemia, diabetes, primary biliary cirrhosis (PBC), and nonalcoholic steatohepatitis (NASH).33-35 GW4064, an isoxazole derivative that selectively activates FXR at submicromolar potencies, has been shown to lower serum triglyceride levels in rats but to have limited clinical uses caused by poor bioavailability, fast metabolism, and toxicity at high doses.36,37 INT-747, so called obeticholic acid (OCA) or 6-alpha-ethylchenodeoxycholic acid (6-ECDCA), is a 6-alpha-alkyl-substituted analog of CDCA selectively inducing FXR transactivation at 1 μM.38,39 INT-747 is being tested as a monotherapy in a Phase 3 clinical trial and as a combination therapy with UDCA in a Phase 2 clinical trial for treating patients with PBC (ClinicalTrials.gov identifier: NCT01473524 and NCT00550862). PX-102 or PX20606, a non-steroidal FXR agonist developed to treat NASH, showed safety and good tolerance in a Phase 1 trial (ClinicalTrials.gov identifier: NCT1998659 and NCT1998672). FXR-450, or so called WAY-362450, is an azepino[4,5-b]indole-based FXR agonist capable of lowering plasma triglycerides and total cholesterol levels in a dyslipidemia model.39,40
Despite the growing interest in FXR ligands in drug discovery, little is known with regard to the roles of FXR in mediating or protecting xenobiotic-induced toxicity. Abnormal FXR function leads to numerous disorders such as cholestasis, diabetes, and cancer, and plays a role in liver regeneration. Depending on the dose of FXR ligands, distinct outcomes have been observed across different species. Exposure of GW4064 has been reported to cause hepatobiliary injury to medaka eleutheroembryos. CA and GW4064 have been found to protect mice from acetaminophen-induced hepatotoxicity yet a CDCA-rich diet has been reported to induce liver hyper trophy in mice. Treatment with the FXR antagonist tempol or intestine-specific deletion of FXR led to similar anti-obesity effects in mice. Theonellasterol, a recently discovered FXR antagonist from marine sponge, protected mice susceptible to cholestasis from numerous disorders such as cholestasis, diabetes, and cancer, and city yet a CDCA-rich diet has been reported to induce liver hyper trophy in mice. These studies suggest that agonists, antagonists, and modulators of FXR could exert protective or adverse effects depending on health states and exposure doses.

One challenge in defining the role of FXR in mediating xenobiotic-induced toxicity is the depth of data on the structural classes of chemicals that act on FXR. Here, we report the profiling of 10,766 substances (8599 unique compounds) in modifying FXR signaling and associated cytotoxicity as part of the Tox21 Phase II program. We utilized the quantitative high-throughput screening (qHTS) data combined with computational methods to identify biological activity patterns of the Tox21 10K compound collection in order to prioritize chemicals for more extensive follow-up studies. Compounds identified as FXR-actives were grouped into several clusters based on similarities in chemical structure, drug class, or known biological target. The representative FXR-active clusters were further compared for their selectivity against other tested human nuclear receptors including the androgen receptor (AR), estrogen receptor alpha (ERα), peroxisome proliferator-activated receptor delta (PPARδ), peroxisome proliferator-activated receptor gamma (PPARγ), and the vitamin D receptor (VDR) to identify FXR-specific chemical scaffolds.

Results
qHTS performance of FXR-bla and viability assays. To identify environmental chemicals and drugs that modulate FXR signaling, we screened the Tox21 10K compound library against the FXR-bla assay in both agonist and antagonist modes. To rule out FXR antagonist response caused by compound cytotoxicity, a cell viability assay was conducted in the same well as the FXR-bla assay. The ratiometric readouts of FXR-bla assay for measuring FXR activity are based on the β-lactamase-coupled fluorescence resonance energy transfer (FRET) technology, CDCA and (Z)-guggulsterone, positive controls for agonist and antagonist screening, respectively, yielded an EC50 value [i.e. concentration calculated to induce a half maximal response with standard deviation (SD)] of 29 ± 6 μM and an IC50 value (i.e. concentration calculated to inhibit a half maximal response with SD) of 50 ± 12 μM. The agonist and antagonist screening worked well as evaluated by average signal-to-background (S/B) ratios of 4.4 for both assays, and average coefficients of variation (CV) of 7.0% and 3.5%, respectively. The average Z’ factors of the agonist and antagonist screening were 0.35 and 0.7%, respectively. Cytotoxicity screening in the FXR agonist and antagonist screening also showed consistent responses with average S/B ratios of 67.1 and 67.7, average CV values of 13.0% and 12.0%, and average Z' factors of 0.60 and 0.69, respectively. Data reproducibility of a given compound was assigned as active agonist/antagonist match, inactive match, inconclusive, or mismatch based on average curve rank and percentage of inactive outcomes of the three independent measurements. The triplicate runs of the Tox21 10K compound collection as well as the 88 compounds duplicated in each plate showed low mismatch rates of <1% in the FXR-bla screening (Figure 1). The agonist and agonist screening identified 8% (861 substances) and 2% (215 substances) active matches, respectively, containing FXR-active compounds and positives resulting from assay artifacts.

Identification of FXR agonists and antagonists. After the primary screening, the test compounds were categorized as active agonists/antagonists, inconclusive, or inactive compounds based on the activities observed in both ratiometric and 460 nm readings. There were 1141 and 2172 compounds that showed activities in the FXR-bla agonist and antagonist mode assays, respectively. Four known FXR agonists, CDCA (EC50 = 28.62 μM), DCA (EC50 = 47.31 μM), GW4064 (EC50 = 0.003 μM), and UDCA (EC50 = 120.70 μM) as well as two well-characterized FXR antagonists, (E)-guggulsterone (IC50 = 24.06 μM) and (Z)-guggulsterone (IC50 = 39.05 μM), were identified from the screening (Table S1, Figure 2a, Figure 2b). Some positive compounds were further verified and re-assigned as inconclusive compounds by additional criteria to exclude potential false positives as a result of compound autofluorescence (efficacyFXR-bla, 460 nm/efficacyFXR-bla ratio or efficacyFXR-bla > 2, PubChem assay identifier: 720681 and 720682) or cytotoxicity (IC50, viability/IC50, FXR-bla ratio < 3, p < 0.05). For example, benzo(k)fluoranthene and triamterene are highly fluorescent at 460 nm in the assay medium, producing a concentration-dependent increase in all β-lactamase-based assays (data not shown). Digoxin and bortezomib were inconclusive antagonists of FXR because the two compounds showed FXR antagonist activity at or near cytotoxic concentrations (Table S1). Two hundred and sixty-six unique compounds including potent FXR-active compounds (IC50 or IC50 values < 10 μM) identified from the primary screening and selected structural analogs were re-tested in the same FXR-bla and viability assays, yielding confirmation rates of 67% (73 of 109) and 90% (144 of 160) in the agonist and antagonist screening, respectively. Twenty-five novel and representative compounds with agonist or antagonist activities confirmed in the FXR-bla assay were shown in (Table 1) detailing compound efficacy, potency, curve class, and data reproducibility in the primary and confirmatory screening. The 25 compounds were further tested in a FXR coactivator recruitment assay.

Figure 1 | Reproducibility of FXR qHTS data. Data reproducibility of the triplicate run of the Tox21 10K compounds and the 88 replicated compounds in the primary screening of the FXR-bla assay. Data reproducibility is measured by the fraction of active match, inactive, mismatch and inconclusive cases.
assay to determine whether a given FXR-active compounds is an FXR ligand or a potential FXR signaling modulators (Table 1). The agonist control CDCA showed an EC$_{50}$ value of 29.90 µM in binding of FXR-LBD and inducing coactivator recruitment, and the known FXR ligand ivermectin fully inhibited CDCA-induced coactivator recruitment, and the known coactivator recruitment with an IC$_{50}$ value of 0.91 µM. Cyclopinamide (EC$_{50}$ = 10.57 µM, efficacy = 94%) and 9-aminoacridine (EC$_{50}$ = 11.17 µM, efficacy = 152%) showed full agonist activity, and both compounds were unable to induce coactivator recruitment to FXR-LBD (Table 1). Several partial FXR agonists including daunorubicin (EC$_{50}$ = 1.02 µM, efficacy = 48%), doxorubicin (EC$_{50}$ = 1.35 µM, efficacy = 68%) and epirubicin (EC$_{50}$ = 5.78 µM, efficacy = 44%) also showed antagonist effects in the FXR-bla assay with IC$_{50}$ values of 5.53 µM, 2.80 µM and 17.80 µM, respectively (Table 1). These FXR-active anthracyclines were able to inhibit CDCA-induced coactivator recruitment at potencies similar to their antagonist activity in the FXR-bla assay (Table 1). Among the confirmed compounds that completely inhibited CDCA-induced FXR-bla activity, actinomycin D (IC$_{50}$ = 0.02 µM) was the most potent, followed by flavopiridol (IC$_{50}$ = 0.02 µM), nemorubicin (IC$_{50}$ = 0.13 µM), gimatecan (IC$_{50}$ = 2.69 µM), and emetine (IC$_{50}$ = 4.23 µM). Colchicine (IC$_{50}$ = 0.03 µM, efficacy = 54%), nocodazole (IC$_{50}$ = 0.29 µM, efficacy = 68%), picrotoxophyllin (IC$_{50}$ = 0.02 µM, efficacy = 55%), and vinorelbine (IC$_{50}$ = 0.03 µM, efficacy = 62%) caused partial inhibition of CDCA-induced FXR transactivation in both primary screening and confirmation studies (Table 1). Colchicine was also identified as an FXR antagonist in the coactivator recruitment assay with an IC$_{50}$ value of 0.03 µM and 52% efficacy (Table 1).

**Structural clusters of FXR agonists and antagonists.** The Tox21 10K compound collection was first grouped into 1,014 clusters of structural classes using the self-organizing map (SOM) algorithm$. As shown in Figure 3, each cluster containing unique and replicated compounds was colored according to the significance of enrichment (negative logarithmic scale of a p-value) in FXR-active agonists or antagonists to assess the probability of a given chemical scaffold to activate FXR-bla or inhibit CDCA-induced FXR-bla transactivation. The FXR-active compounds yielded 189 structural classes in which 56 and 152 clusters were significantly enriched with compounds that activate or inhibit FXR, respectively. The representative FXR-active clusters are described in Table S2.

The clusters of FXR agonists include cholic acids (k39.24), avermectins (k3.3), and retinoic acids (k22.22 and k20.7). The cholic acid cluster (k39.24) contains three known FXR agonists, CDCA, DCA, and LCA. CDCA was two-fold and six-fold more efficacious than DCA and LCA in activating FXR (Figure 2a and Table S1), respectively. Avermectins (k3.3), which include abamectin, doramectin, milbemectin, and ivermectin, were identified as partial FXR agonists with EC$_{50}$ values from 0.44 µM to 37.71 µM and maximum efficacy values of 27–90% (Table S1). Ivermectin also antagonized CDCA-induced FXR transactivation with an IC$_{50}$ value of 2.50 µM. The retinoic acids (k22.22)-13-cis retinoic acid, tretinoin as well as the benzoic analogs AM80 and AM580 (k20.7) acted as FXR agonists and mixed agonists/antagonists, with EC$_{50}$ values in the agonist mode that ranged from 24.61 µM to 51.11 µM (Table S1). However, moxidectin, a milbemycin derivative structurally similar to avermectins, did not show agonist activity in the FXR-bla assay (data not shown).

The anthracycline (k1.10) and the dihydropyridine (k10.12 and k11.12) clusters are enriched with both partial FXR agonists and FXR antagonists. All compounds in the anthracycline cluster except nemorubicin showed mixed FXR agonist/antagonist responses (Table 1 and Table S1). For example, nemorubicin, doxorubicin, and epirubicin inhibited CDCA-induced FXR transactivation at potencies ranging from 0.21 µM to 13.10 µM (Figure 2c). Ten of 19 unique dihydropyridine compounds (e.g., felodipine, lemlidipine, nicardipine) were found to be partial FXR agonists (EC$_{50}$ values of

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**Figure 2 | Concentration response curves of selective compounds identified by the FXR-bla assay.** Concentration response curves of (a) cholic acids: CDCA (EC$_{50}$ = 28.62 µM), DCA (EC$_{50}$ = 47.31 µM), LCA (EC$_{50}$ = 34.90 µM), and UDCA (EC$_{50}$ = 120.70 µM) in agonist mode; (b) GW4064 (EC$_{50}$ = 0.003 µM) and cyclopinamide (EC$_{50}$ = 10.57 µM) in agonist mode; (c) anthracyclines: nemorubicin (IC$_{50}$ = 0.21 µM), doxorubicin (IC$_{50}$ = 2.74 µM), and epirubicin (IC$_{50}$ = 13.10 µM) in antagonist mode; (d) benzimidazoles: nocodazole (IC$_{50}$ = 0.32 µM), cyclobendazole (IC$_{50}$ = 3.00 µM), and benomyl (IC$_{50}$ = 18.28 µM) in antagonist mode. Data were collected from primary screening and expressed as mean ± SD from 3 experiments.
### Table 1 | Compound potency (µM, EC<sub>50</sub>/IC<sub>50</sub>) and efficacy (% in parenthesis) in FXR-<i>bla</i>, viability, and TR-FRET co-activator assays

| Compound Name (CAS No.) | Chemical Structure | Cluster No. | EC<sub>50</sub>, µM (Efficacy, %) | IC<sub>50</sub>, µM (Efficacy, %) | Cell viability IC<sub>50</sub>, µM (Efficacy, %) | FXR-<i>bla</i>, agonist IC<sub>50</sub>, µM (Efficacy, %) | FXR-<i>bla</i>, antagonist IC<sub>50</sub>, µM (Efficacy, %) | FXR TR-FRET, agonist EC<sub>50</sub>, µM (Efficacy, %) | FXR TR-FRET, antagonist IC<sub>50</sub>, µM (Efficacy, %) |
|-------------------------|-------------------|-------------|-------------------------------|-------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Albendazole (54965-21-8) | ![Albendazole](image1) | k7.9 | Inactive | 0.37 ± 0.07 (52 ± 2) | Inactive | Inactive | Inactive | Inactive | Inactive |
| 9-Aminoacridine (52417-2208) | ![9-Aminoacridine](image2) | k3.18 | 11.17 ± 2.09 (152 ± 22) | Inactive | Inactive | Inactive | Inactive | Inactive | Inactive |
| Actinomycin D (50-76-0) | ![Actinomycin D](image3) | k10.3 | Inactive | 0.02 ± 0.01 (77 ± 27) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Colchicine (64-86-8) | ![Colchicine](image4) | k2.20 | Inactive | 0.03 ± 0.01 (54 ± 6) | Inactive | Inactive | Inactive | 0.03 ± 0.01 (52 ± 16) | Inactive |
| Dipyridamole (58-32-2) | ![Dipyridamole](image5) | k6.5 | Inactive | 3.50 ± 0.40 (81 ± 5) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Doxorubicin (25316-40-9) | ![Doxorubicin](image6) | k1.10 | 1.35 ± 0.00 (68 ± 8) | 2.08 ± 0.19 (114 ± 9) | Inactive | Inactive | 4.39 ± 2.32 (185 ± 54) | Inactive | Inactive |
| Emetine (316-42-7) | ![Emetine](image7) | k2.24 | Inactive | 4.23 ± 0.28 (120 ± 19) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Enzastaurin (170364-57-5) | ![Enzastaurin](image8) | k38.3 | Inactive | 10.23 ± 0.69 (110 ± 9) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Epirubicin (56390-09-1) | ![Epirubicin](image9) | k1.10 | 5.78 ± 0.74 (44 ± 7) | 17.80 ± 4.07 (108 ± 1) | Inactive | Inactive | 7.91 ± 1.48 (233 ± 15) | Inactive | Inactive |
| Flavopiridol (146426-40-6) | ![Flavopiridol](image10) | k1.12 | Inactive | 0.02 ± 0.00 (123 ± 7) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Gimatecan (292618-32-7) | ![Gimatecan](image11) | k1.7 | Inactive | 2.69 ± 1.07 (87 ± 9) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Idarubicin (57852-57-0) | ![Idarubicin](image12) | k1.10 | 1.37 ± 0.38 (42 ± 16) | 4.07 ± 0.28 (107 ± 3) | Inactive | Inactive | 6.29 ± 1.32 (162 ± 41) | Inactive | Inactive |
| Mebendazole (31431-39-7) | ![Mebendazole](image13) | k7.10 | Inactive | 2.19 ± 1.51 (70 ± 9) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Nemorubicin (108852-90-0) | ![Nemorubicin](image14) | k1.10 | Inactive | 0.13 ± 0.01 (99 ± 6) | Inactive | Inactive | 5.75 ± 0.39 (191 ± 23) | Inactive | Inactive |
| Nocodazole (31430-18-9) | ![Nocodazole](image15) | k7.10 | Inactive | 0.29 ± 0.19 (68 ± 20) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Oxibendazole (20559-55-1) | ![Oxibendazole](image16) | k5.20 | Inactive | 0.58 ± 0.07 (55 ± 5) | Inactive | Inactive | Inactive | Inactive | Inactive |
| Picropodophyllin (518-28-5) | ![Picropodophyllin](image17) | k3.21 | Inactive | 0.02 ± 0.00 (55 ± 3) | 10.72 ± 14.88 (42 ± 14) | Inactive | Inactive | Inactive | Inactive |
| Proflavin hemisulfate (181128-5) | ![Proflavin hemisulfate](image18) | k30.7 | Inactive | 9.39 ± 3.90 (142 ± 12) | Inactive | 2.21 ± 0.25 (182 ± 5) | Inactive | 0.20 ± 0.03 (73 ± 1) | Inactive |
| Surinabant (288104-79-0) | ![Surinabant](image19) | k24.3 | Inactive | 1.84 ± 0.77 (86 ± 25) | 17.14 ± 1.12 (71 ± 5) | Inactive | Inactive | Inactive | Inactive |

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3.64 μM to 28.90 μM). In contrast, benidipine (k10.12), nifedipine (k11.12), and nimodipine (k11.12) in the same structural classes showed FXR antagonist activities with IC50 values of 24.78 μM, 34.02 μM, and 31.06 μM, respectively.

Other clusters of potential FXR antagonists identified from the screening were vinca alkaloids (k2.26), benzimidazoles (k7.9 and k7.10), flavonoids (k1.13 and k2.12), estradiols (k20.11), and pyrethroids (k28.23 and k28.24) (Table S1). The vinca alkaloids (k2.26) including vincristine, vinblastine, and vinorelbine inhibited CDCA-induced FXR activation with IC50 values of 0.03 μM to 0.12 μM (Table 1). In the benzimidazole clusters (k7.9 and k7.10), 9 of 13 unique compounds including nodocazole, cyclobendazole, and benomyl acted as FXR antagonists (Figure 2d, Table 1 and Table S1). Several flavonoids including genistin (k1.13), biochanin A (k1.13), apigenin (k2.12), and chrysir (k2.12) showed partial to complete antagonistic response in the FXR-bla assay with IC50 values ranging from 12.07 μM to 46.19 μM (Table S1). Four of eight unique estradiol analogs (k20.11) including 17β-estradiol (IC50 = 33.15 μM) and 17α-ethinylestradiol (IC50 = 16.65 μM) exhibited antagonistic activity against FXR (Table S1). Twelve out of 17 unique pyrethroids (e.g., bifenthrin in k28.23, cyhalothrin in k28.24) were able to inhibit CDCA-induced FXR activation with IC50 values ranging from 9.36 μM to 33.90 μM (Table S1).

Mechanism of action and targets in FXR signaling. Compounds which share similar biological functions despite distinct chemical scaffolds may be used to identify potential interactions between their biological target and FXR. Using the known primary targets and mechanism of action of approved and investigational drugs, 35 FXR-active drugs more potent than the natural FXR ligands [e.g., CDCA for agonists; (Z)-guggulsterone for antagonists] were FXR-active drugs more potent than the natural FXR ligands [e.g., (Z)-guggulsterone for antagonists] were able to inhibit activity against FXR (Table S1). Twelve out of 17 unique pyrethroids (e.g., bifenthrin in k28.23, cyhalothrin in k28.24) were able to inhibit CDCA-induced FXR activation with IC50 values ranging from 9.36 μM to 33.90 μM (Table S1).

Selectivity of FXR-active compounds against a family of nuclear receptors. To detect potential assay artifacts and study compound selectivity, activity patterns of the top twenty-seven FXR-active clusters against AR, ERα, PPARδ, PPARγ, and VDR, which used the same β-lactamase reporter gene technology (Table S4 and Table S5), were evaluated. Retinoic acids (k20.7) and alpha-cyano 3-hydroxysteroids (type I) pyrethroids (k28.24) were two-fold more responsive to the natural FXR ligands (ethinylestradiol) and the synthetic steroids are used as an ER agonist (ethinylestradiol) or as an AR agonist (ethylestrenol). Other FXR-active drugs include two synthetic steroids (ethinylestradiol and ethylestrenol), an anticoagulant pyrimidine (dipyridamole), an antimetabolodalis isoxquinoline (emetine), an anti-infective aminoacidine (ethacridine lactate), and an anticoagulant pyrimidine (dipyridamole), and an anticoagulant pyrimidine (dipyridamole), and a pyrazole drug to treat nicotine addiction (surinabant).

There are six target classes of these FXR-active drugs targeting DNA, tubulin, calcium channel, enzymes, nuclear receptors, and other miscellaneous targets (Figure 4b). The compounds directly binding tubulin are four benzimidazoles (the three anthelmintics plus nodocazole)53, threevinca alkaloids (vinblastine, vincristine, and vinorelbine)54, and three anticoagulant pyrimidine (dipyridamole), an anticoagulant pyrimidine (dipyridamole), and an anticoagulant pyrimidine (dipyridamole), and a pyrazole drug to treat nicotine addiction (surinabant).

Other clusters against AR, ERα, PPARδ, PPARγ, and VDR, which used the same β-lactamase reporter gene technology (Table S4 and Table S5), were evaluated. Retinoic acids (k20.7) and alpha-cyano 3-hydroxysteroids (type I) pyrethroids (k28.24) were two-fold more responsive to the natural FXR ligands (ethinylestradiol) and the synthetic steroids are used as an ER agonist (ethinylestradiol) or as an AR agonist (ethylestrenol). Other FXR-active drugs include two synthetic steroids (ethinylestradiol and ethylestrenol), an anticoagulant pyrimidine (dipyridamole), an anticoagulant pyrimidine (dipyridamole), and a pyrazole drug to treat nicotine addiction (surinabant).
(k7.9) and the nocodazole-like benzimidazoles (k7.10) shared similar selectivity patterns in the AR-bla antagonist and the FXR-bla antagonist screening (Figure 5). The nicardipine-like dihydropyridine cluster (k10.12) showed antagonistic or cytotoxic response in all tested β-lactamase assays for nuclear receptors (Figure S2). Benidipine, manidipine, and nicardipine exhibited weak FXR agonist activity. The nifedipine-like dihydropyridine cluster (k11.12) yielded several distinct selectivity profiles. Felodipine, lacidipine, and lemlidipine activated AR, ER, FXR, and PPARδ. Cilnidipine and nitrendipine were found to be FXR agonists, and nitrendipine was also an AR antagonist. Nimodipine, nifedipine, and nisoldipine were identified as antagonists of FXR and AR. The

Figure 3 | Heat maps of structural classes versus FXR activity. Each hexagon represents a cluster of structurally similar compounds. Clusters are annotated with a (x,y) coordinate and colored according to the enrichment of FXR actives \(-\log(p\text{-value})\) in the cluster. Warmer colors indicate higher enrichment of FXR actives and colder colors indicates less significant enrichment of FXR actives. Empty clusters are colored in white.

Figure 4 | Distribution of biological functions of selective potent FXR-actives. Thirty-five FXR-active drugs with submicromolar potency and known biological targets were chosen to form clusters based on biological functions. (a) Distribution of drug classes. (b) Distribution of target classes. (c) Concentration-dependent inhibition curves of nocodazole (IC_{50} = 320 nM), colchicine (IC_{50} = 11 nM), docetaxel (IC_{50} = 8 nM), and vincristine (IC_{50} = 9 nM) measured from the primary screen. (d) Chemical structures of nocodazole, colchicine, docetaxel, and vincristine.
cholic acids (CDCA, DCA, and LCA; Figure 5) and the FXR-active
benzoic retinoic acids (AM80 and AM580; Figure S1) selectively
induced transactivation of FXR. Guggulsterones exhibited
antagonist activity to AR and FXR (Figure 5). Some FXR-active
pyrethroid insecticides such as cyfluthrin and bifenthrin were
highly selective FXR antagonists (Figure 5). The anthracycline
chemotherapeutics, including doxorubicin, daunorubicin, idarubi-
cin, and pirarubicin, displayed mixed agonist/antagonist response
against AR, ER\textsubscript{a}, and FXR, and antagonist response against
PPAR\textsubscript{\textgamma}, PPAR\textsubscript{\textdelta}, and VDR (Figure S3). The six structural classes of
tubulin binders showed distinct selectivity patterns (Figure 5).
Griseofulvin, colchicine, podofilox, and the majority of FXR-active
benzimidazoles (i.e., nocodazole, carbendazol, and mebendazol)
shared similar activity patterns in inhibiting β-lactamase expres-
sion driven by FXR, AR, PPAR\textsubscript{\textgamma}, and VDR. Griseofulvin and
colchicine also antagonized estradiol-induced ER transactivation.
Three FXR-active vinca alkaloid-based tubulin binders acted as
mixed agonists/antagonists of ER and antagonists of AR and FXR
(Figure 5).

**Discussion**

In the present study, we used a qHTS platform in combination with
chemoinformatics to profile 8,599 unique environmental chemicals
and drugs for their potential to modulate FXR signaling. False posi-
tive and false negative rates were minimized by using a format con-
sisting of three replicate concentration-response curves with 15
concentrations for each test compound in primary screening.
Additionally, the FXR results were compared with Tox21 qHTS
screening results against several additional targets to identify non-
specific effects such as compound autofluorescence, cytotoxicity,
reporter gene-dependent response, or other artifacts. Overall, the
high data reproducibility of replicate compounds indicated that the
screening was robust. The mismatch rates of the 10K triplicate runs
and the 88 replicates were both less than 1%, indicating good assay
reproducibility. From the primary screening, more FXR antagonists
than FXR agonists were identified. Interestingly, many of the FXR-
active drugs identified (e.g., k1.10 for anthracycline chemotherapeu-
tics and k7.9/k7.10 for dihydropyridine anti-hypertensive drugs)
showed a range of agonist activity between 13% and 70% of CDCA
activity as well as antagonist activity to CDCA-induced FXR activa-
tion, a pharmacological behavior consistent with classification as a
partial agonist\textsuperscript{51}. Only a few FXR agonists showed efficacies similar to
CDCA, including the topical antiseptic 9-aminoacridine\textsuperscript{36} and the
teratogenic plant-derived steroid cyclopamine\textsuperscript{52}. About 1270 unique
compounds from the Tox21 10K compound collection had inhib-
itory effects on CDCA-induced FXR activity. More than 542 unique
of these active compounds appeared to be cytotoxic as detected
by viability assays in the same well, thus requiring cluster analysis
for chemical prioritization and orthogonal assays for further
confirmation.

The FXR-\textsuperscript{bla} assay is able to detect both weak and potent FXR
agonists. UDCA, a 7-beta isomer of CDCA previously reported as a
weak FXR agonist\textsuperscript{5} or a non-FXR ligand\textsuperscript{53}, exhibited partial agonist
activity at high micromolar concentrations in the FXR-\textsuperscript{bla} assay
(Figure 2a and Table S1). The potency of the other known FXR
agonist GW4064 in the FXR-\textsuperscript{bla} assay (Figure 2b and Table S1) is
comparable (<5-fold difference in EC\textsubscript{50} values) to the literature
value measured in a coactivator recruitment assay\textsuperscript{13}. The positive
control CDCA was the most efficacious FXR agonist compared to
the other cholic acids, DCA and LCA, which showed weak agonistic
effects (Figure 2a) and cytotoxicity at high concentrations (Table S1). The induction of FXR activity by these cholic acids is highly specific. It is likely due to the fact that cholic acids have a shape distinct from other endogenous steroids and the ligand binding domain of FXR adopts a unique orientation for binding of cholic acids⁶⁴. Both (E) and (Z) isomers of guggulsterone were confirmed as FXR antagonists in both the primary screening and confirmatory assays. (Z)-guggulsterone was used as the antagonist control in the FXR screening and its (E) isomer also showed antagonist response in the AR-bla assay. Although guggulsterones were first discovered as naturally occurring FXR antagonists, they also bind other steroid receptors including the glucocorticoid receptor, the mineralocorticoid receptor, and the progesterone receptor⁶⁵, making it complicated to interpret the effects of guggulsterones in FXR signaling and lipid metabolism⁶⁶.

The recently discovered FXR agonist ivermectin⁶⁷ and the other five avermectin macrolide antiparasitic agents (k3.3) identified in this study potently induced FXR-bla transactivation at submicromolar concentrations (Table S1). Ivermectin not only showed partial agonist activity but also exhibited antagonistic activity in the FXR-bla assay. This observation is consistent with an independent study in which ivermectin was identified in both biochemical and cell-based assays as a potent FXR antagonist⁶⁸. Ivermectin, an avermectin antiparasitic drug used in humans, reduces hyperglycemia and hyperlipidemia symptoms in the diabetic mice model at submicromolar concentrations via FXR-mediated signaling⁶⁸. Human patients with overdoses of ivermectin, abamectin, and emamectin have shown acute cardiotoxicity, acute neurotoxicity, or adverse effects on the gastrointestinal tract⁶⁹ although linkage to FXR-mediated effect is not known. Doramectin, an ivermectin analog for anti-parasitic treatment in dogs, was two-fold more potent and more efficacious than ivermectin in activating FXR in our screening. In addition, the two milbemycin macrolides—milbemectin and moxidectin which lack the disaccharide moiety present in avermectins exhibited weak and no activity in the FXR-bla assay (Table S1). These results indicate the importance of the disaccharide in forming hydrogen bonds with FXR as observed in the co-crystal structure of ivermectin-bound FXR-LBD⁷⁰.

We identified 63% (12 of 19) of the tested dihydropyridine-class calcium channel blockers as FXR agonists or FXR antagonists with low to high micromolar potencies (Table S1 and Figure S2). Two recently reported FXR antagonists, nimodipine and felodipine⁷¹, were identified as an antagonist and an agonist in the FXR-bla assay, respectively. The dihydropyridine drugs in the Tox21 10K compound collection were grouped into two clusters represented by nicardipine (k10.12) and nifedipine (k11.12). The two clusters exhibited distinct FXR activity and selectivity towards the other five nuclear receptors (Figure S2). The major structural difference of the two clusters is the extra phenyl group in the nicardipine-like compounds which may introduce additional steric hindrance and hydrophobic interactions in binding of FXR and other nuclear receptors. When compared with other functionally related receptors such as the pregnancy X receptor (PXR), the majority of the FXR-active dihydropyridines are more potent in activating PXR⁷². Accidents of toddlers over-dosed with nifedipine, a dihydropyridine drug used to treat hypertension and to control angina, resulted in hypotension, lethargy, and/or vomiting⁷³. Adults overdosed with nifedipine showed symptoms such as hypotension, sinus tachycardia, and/or sinoatrial and atrioventricular nodal depression⁷⁴. Although therapeutic doses of dihydropyridine drugs normally yield submicromolar serum concentrations⁷⁵ and no direct link between FXR and overdose of dihydropyridines has been reported, potential impacts of dihydropyridines on FXR-related physiological function need further evaluation as cases of overdose of these drugs increase.

Sixty-seven percent (12 of 17) of the tested pyrethroid insecticides were identified as potential FXR-specific antagonists (Table S1 and Figure 5). These pyrethroids inhibited CDCA-induced FXR transactivation at high micromolar concentrations. Pyrethroid insecticides kill insects by over-stimulating the voltage-gated sodium channels⁷⁶. Humans are exposed by pyrethroids through ingestion or occupational exposure, and can result in paresthesia⁷⁷, gastrointestinal irritation⁷⁸, or abnormal glucose regulation⁷⁹. However, human exposure of pyrethroids would likely have to reach very high levels to trigger FXR effects, doses at which other modes of activity/toxicity would likely already have been manifested. One interesting finding for pyrethroids is a relationship between structure and FXR activity. The alphacyano pyrethroids (k28.24) were more enriched in FXR antagonists and more FXR-specific than the noncyano pyrethroids (k28.23) (Table S5). The main structural difference between the two clusters is the substitution of vinyl hydrogen to a cyano group in the k28.24 pyrethroids. While the majority of k28.24 pyrethroids exhibited FXR-specific antagonistic effect, cyhalothrin, fenvalerate and flucythrinate, also showed antagonist activity in other nuclear receptor assays including the AR-bla and the VDR-bla assays (Figure 5). These three compounds have an aryl group instead of the acid moiety present in most pyrethroids. These data suggest that FXR could be a novel mammalian target of pyrethroids, and that the structural differences of the FXR-active pyrethroids play a crucial role in compound activity and selectivity.

Our data provides interesting evidence that chemicals known to inhibit tubulin are active as FXR antagonists. All tested tubulin inhibitors including benzimidazoles (e.g., nocardazole), colchicine, docetaxel, griseofulvin, podofilox, and vinca alkaloids (vinblastine, vincristine, and vinorelbine) exhibited FXR antagonist activity at submicromolar to low micromolar concentrations in the FXR-bla assay screening (Figure 5). Despite the fact that most of these FXR-active tubulin inhibitors are mitotic inhibitors, concentration-dependent inhibitory effects of these compounds in the FXR-bla assay were only observed in the antagonist screening. The FXR-active benzimidazoles, vinca alkaloids, colchicine, and podofilox are 5 to 100-fold more potent in inhibiting CDCA-induced FXR activity than activating PXR⁸⁰. Benzimidazoles have been identified as FXR agonists with lipid lowering effects in vivo⁸¹. However, the FXR-active benzimidazoles identified from the screen were unable to induce FXR-bla transactivation and to alter FXR coactivator recruitment (Table 1). Compared to the reported benzimidazole-based FXR agonists, the nocardazole-like benzimidazoles lack an alkylated N3 functionality important for FXR binding. Therefore these benzimidazoles are likely to modulate FXR signaling via alternative mechanisms. Tubulin inhibitors and microtubules have been implicated in regulating a number of nuclear receptors including AR⁸², the glucocorticoid receptor (GR)⁸³, PXR⁸⁴, and the retinoic acid receptor (RAR)⁸⁵ as well as the aryl hydrocarbon receptor (AhR)⁸⁶. Those studies suggest that microtubule inhibitors affect nuclear receptor trafficking by not allowing coregulators to move from the cytoplasm to the nucleus. Additionally, ERα was reported to activate histone deacetylase 6 (HDAC6) to deacetylate tubulins in human breast cancer MCF-7 cells⁸⁷. Future work should interrogate the role of microtubules in FXR signaling and evaluate effects of tubulin inhibitors on FXR target genes to determine if these compounds are acting directly or indirectly on FXR activity.

In summary, the present study identified several key scaffolds of FXR-active drugs and environmental chemicals. Because FXR regulates diverse metabolic pathways, it has emerged as an important drug target and a potential toxicity mediator. Complete structure-activity relationships of anthracyclines, avermectins, dihydropyridines, and pyrethroids require further testing of a larger number of tailor-designed analogs to identify the pharmacophore important for FXR binding. Systematic evaluation of compound selectivity of FXR-active environmental chemicals against other functionally related receptors like PXR, liver X receptor (LXR), and constitutive androstane receptor (CAR)⁸⁸, and G protein-coupled bile acid receptor
The Tox21 10K compound library (http://www.epa.gov/nct/daston/sdf_tox21.html) is provided by the National Toxicology Program (NTP), the Environmental Protection Agency (EPA), and the NIH Chemical Genomics Center/National Center for Advancing Translational Sciences. Each compound was prepared as previously described and serially diluted in 1536-well microplates to yield 15 concentrations generally ranging from 1 nM to 92 μM (final concentrations in the assay wells).

Cell culture. The cell line and the cell culture reagents were purchased from the Life Technologies (Carlsbad, CA, USA). The GeneBellAER® FXR-UAS-bla HEK293T cells stably expressing an FXR-driven β-lactamase reporter gene were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Cat. No. 10569-010) supplemented with 5.5 mM (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.3), 0.1 mM non-essential amino acids (NEAA), 100 μg/mL hygromycin, 100 μg/mL zeocin, 100 U/mL penicillin, 1 mg/mL streptomycin, and 10% dialyzed fetal bovine serum (FBS). Cells were grown at 37°C in 5% CO2 in a humidified incubator and passed at 70–80% confluency. To prepare FXR-UAS-bla cells for assays, cells were washed with Dulbecco’s phosphate-buffered saline (DPBS), detached with 0.05% Trypsin/EDTA, and re-suspended in phenol red-free DMEM containing 1 mM sodium pyruvate, 0.1 mM NEAA, 100 U/mL penicillin, 1 mg/mL streptomycin, and 2% charcoal-stripped FBS.

qHTS of beta-lactamase reporter gene and cell viability assays. The online screening procedures to profile the Tox21 compound collection against androgen receptors (AR), estrogen receptor alpha (ERα), farnesoid X receptor (FXR), peroxisome proliferator-activated receptor delta and gamma (PPARδ/γ), and vitamin D receptor (VDR) were adapted from the previously reported procedures and the results were deposited to the PubChem BioAssay database (Table S4). Titration protocols of the FXR-bla and viability screening on the Tox21 compound collection are described as follows (Table S6 and Table S7). Five μL of suspended FXR-bla cells at a cell density of 1000 cells/μL was plated in 1536-well tissue culture-treated black clear bottom plates (Greiner Bio One North America, Monroe, NC, USA) using a 3-μL multipipette reagent dispenser (Thermo Fisher Scientific, Waltham, MA, USA). The standard procedure to screen FXR agonists starts with cell incubation at 37°C/5% CO2 for 5 hours, followed by addition of 23 μL of compound solution on a compound transfer workstation (Kalypsys, San Diego, CA, USA). Chenodeoxycholic acid (CDCA) and DMSO (Sigma-Aldrich Corp., St. Louis, MO, USA) were used as positive controls and negative agonist controls, respectively. To screen compounds that antagonize CDCA-induced transactivation of FXR, an extra 1 μL of DMSO was added on the top of the cell/compound mixtures to achieve a final agonist concentration of 50 nM. After 16 hours of incubation at 37°C/5% CO2, 1 μL of CCF2-AM substrate reagents was added to each well using a Bioraptr Flying Reagent Dispenser (Beckman Coulter, Fullerton, CA, USA) followed by a 2 hour incubation at room temperature in the dark. Samples were excited at 405 nm and the resulting fluorescence emission intensity values at 460 nm and 530 nm were measured using a 96-well plate reader (Perkin Elmer, Shelton, CT, USA). The same assay protocols of FXR-reagent (Promega, Madison, WI, USA) to each well and incubating the plates at room temperature in the dark. Samples were excited at 405 nm and the resulting fluorescence emission intensity values at 495 nm and 520 nm with 90 μs delay time and 300 μs integration time were acquired on the EnVision plate reader (Perkin Elmer, Shelton, CT, USA). The antagonist assay was conducted in the presence of 50 μM CDCA to induce coactivator recruitment. The TR-FRET values were normalized using DMSO as 0% FXR activity and 50 μM CDCA as 100% FXR activity for both agonist and antagonist mode screening.

<ref>FXR coreactor recruitment assay</ref>. The glutatione S-transferase (GST)-tagged FXR-LBD protein, ferbur (Tb)-labelled goat anti-GST antibody, and fluorescein-labeled SRC2-2 coactivator peptide were purchased from the Life Technologies (Carlsbad, CA, USA). The ability of an FXR agonist to recruit a coactivator is reported by FRET between the donor Tb-labeled antibody (λex = 340 nm and λem = 495 nm) and the acceptor fluorescein-labeled peptide (λex = 520 nm), where the agonist induces conformational change of FXR-LBD and leads to coactivator recruitment. Six μL of 5 nM GST-tagged FXR-LBD, 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 5 nM Tb-labeled anti-GST antibody, and 500 nM fluorescein-labeled SRC2-2 were plated in a 1536-well black solid bottom plate (Greiner Bio One North America, Monroe, NC, USA) using a Mantis single-channel liquid dispenser (StorkeTech, Inc., Waltham, MA, USA). Eight concentrations in DMSO was added to the corresponding well using a compound transfer workstation (Wako Automation, San Diego, CA, USA). The reaction was incubated in the dark at room temperature for 30 minutes. Samples were excited at 340 nm, and the resulting emission intensity values at 495 nm and 520 nm with 10 μs delay time and 300 μs integration time were acquired on the EnVision plate reader (Perkin Elmer, Shelton, CT, USA). The antagonist assay was conducted in the presence of 50 μM CDCA to induce coactivator recruitment. The TR-FRET ratio values were normalized using DMSO as 0% FXR activity and 50 μM CDCA as 100% FXR activity for both agonist and antagonist mode screening.

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**Author contributions**

C.W.H., K.H. and M.X. designed the study. C.W.H., J.Z., M.X. and R.H. performed the experiments and analyzed the data. C.W.H., R.H. and M.X. wrote the manuscript. J.H.H., X.C., J.H. and K.H. edited the manuscript.

**Additional information**

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