Toxicology in the Fast Lane: Application of High-Throughput Bioassays to Detect Modulation of Key Enzymes and Receptors

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Background: Legislation at state, federal, and international levels is requiring rapid evaluation of the toxicity of numerous chemicals. Whole-animal toxicologic studies cannot yield the necessary throughput in a cost-effective fashion, leading to a critical need for a faster and more cost-effective toxicologic evaluation of xenobiotics.

Objectives: We tested whether mechanistically based screening assays can rapidly provide information on the potential for compounds to affect key enzymes and receptor targets, thus identifying those compounds requiring further in-depth analysis.

Methods: A library of 176 synthetic chemicals was prepared and examined in a high-throughput screening (HTS) manner using nine enzyme-based and five receptor-based bioassays.

Results: All the assays have high Z' values, indicating good discrimination among compounds in a reliable fashion, and thus are suitable for HTS assays. On average, three positive hits were obtained per assay. Although we identified compounds that were previously shown to inhibit a particular enzyme class or receptor, we surprisingly discovered that triclosan, a microbiocide present in personal care products, inhibits carboxylesterases and that dichlone, a fungicide, strongly inhibits the ryanodine receptors.

Conclusions: Considering the need to rapidly screen tens of thousands of anthropogenic compounds, our study shows the feasibility of using combined HTS assays as a novel approach toward obtaining toxicologic data on numerous biological end points. The HTS assay approach is very useful to quickly identify potentially hazardous compounds and to prioritize them for further in-depth studies.

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Although pharmaceuticals and pesticides are evaluated for toxicity at great cost, numerous anthropogenic compounds produced in sizable amounts and present in our everyday environment have not been tested for any toxicologic activity. The recent California Green Chemistry Report (California Department of Toxic Substances Control 2008) illustrates that far more chemicals are in common use than the ones tested for toxicity, and in most cases, there are few or no toxicity data for a large number of these chemicals. Novel international legislation, such as the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) program implemented in 2007 by the European Union (European Chemicals Agency 2007), requires that all chemicals used in the European Union at more than 1 metric ton/year/company be evaluated for their toxicity over the next decade. Ultimately, the European Union may develop an authorization system to control substances of very high concern and progressively replace them with suitable alternatives where economically and technically viable, unless there is an overall benefit for society of using the substance. The U.S. Environmental Protection Agency has several voluntary programs, including the High Production Volume Challenge Program (U.S. Environmental Protection Agency 1998), that allow compiling of chemical toxicity and hazard information for selected chemicals. It is very likely that additional national and international legislation will be enacted that will require generation of toxicity data for most of the chemicals produced in sizable quantity.

For almost 200 years, laboratory animal testing has been the major tool of toxicologists (Gad 2006). However, such tests have the disadvantages of being both time-consuming and very costly because they require use of large number of animals, and they are not always predictive of human risk. For the implementation of REACH, Scialli (2008) estimated that tens of million of animals will be used at a cost of several hundred thousand dollars per compound, making it very challenging to use experimental animals to complete analysis of the toxicologic effects of many chemicals in a reasonable time frame. Accordingly, there is a need for accurate toxicologic evaluation of xenobiotics to be faster and more cost-effective. Progress in molecular biology, biotechnology, and other fields have paved the way for toxicity testing to be quicker, less expensive, and more directly relevant to human exposures (Gibb 2008). Although it is certain that *in vitro* assays cannot yet replace animal testing (Tingle and Helsby 2006), they may provide essential information that can prioritize and dramatically reduce the use of animal testing assays (Silliman and Wang 2006). However, when considering the prospect of screening tens of thousands of chemicals against hundreds of *in vitro* assays, several important questions need to be answered. Can enzyme- or cell-based bioassays yield useful toxicologic information? Furthermore, can these assays be conducted in a high-throughput and reliable fashion, allowing the rapid screening of thousands of compounds for biological and toxicologic activities?

As part of the University of California–Davis Superfund Basic Research Program, whose aim is to identify biomarkers of exposure and effects of toxic substances, we have developed a library of techniques, including numerous enzyme- and cell-based screening assays (Ahn et al. 2008; Garrison et al. 1996; Han et al. 2004; Huang et al. 2007; Jones et al. 2005; Nagy et al. 2002; Rogers and Denison 2000; Shan and Hammock 2001). Although such assays are routinely used to find novel small chemical inhibitors in the pharmaceutical industry, we tested whether such mechanistically based screening assays can be used to rapidly provide information on the potential for compounds to produce specific biological toxic effects that would identify those requiring further in-depth study. More specifically, we tested whether these assays could be adapted for high-throughput screening (HTS). We

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selected a small (176 compounds) and structurally very diverse library from among commonly encountered environmental chemicals. We report the results of screening this library with nine enzyme-based and five receptor-based bioassays. These assays were selected because the proteins involved were shown to interact with xenobiotics, and because the *in vitro* effects of these xenobiotics could be related to the *in vivo* activity of these proteins and health effects.

Materials and Methods

A more detailed account of the materials and methods used is given in the Supplemental Materials, (doi:10.1289/ehp.0900834.S1 via http://dx.doi.org/).

Chemicals. Most chemicals used in the library were from commercial sources. Chemicals were at least 95% pure and used without further purification.

Environmental chemicals library. The library was prepared in 2-mL deep-well polypropylene 96-well assay plates. Every compound was dissolved at 10 mM in dimethyl sulfoxide (DMSO). Only compounds totally soluble at 10 mM in DMSO were included in the library. In each plate, the wells in the first column contained only DMSO to serve as controls. In the remainder of the plate, we dispensed one compound per well, with 88 compounds total per plate. We created two plates for a total of 176 compounds. A detailed description of the chemical contents in each plate is presented in the Supplemental Materials, Tables 1 and 2 (doi:10.1289/ehp.0900834.S1). The sealed plates were stored at –20°C until use. Upon use, the plates were diluted to the appropriate concentration using a robotic pipetting station.

Enzyme preparations. Recombinant human soluble epoxide hydrolase (sEH) was produced in a baculovirus expression system (Beetham et al. 1993) and purified by affinity chromatography (Wixtrom et al. 1988). Recombinant human carboxylesterases CES1, CES2, and CES3; fatty acid amide hydrolase (FAAH); and paraoxonase 2 (PON2) were expressed in baculovirus-insect cells as previously described (Huang et al. 2007; Nishi et al. 2006). The CESs were partially purified as previously described (Nishi et al. 2006), whereas microsomal preparations were used for FAAH and PON2 (Huang et al. 2007). Human liver cytosol and microsome extracts were obtained from BD Biosciences (San Jose, CA). Protein concentration was quantified using the Pierce BCA (bicinchoninic acid) assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) as the calibrating standard.

Enzyme assays. Although the conditions for each enzyme assay were different (for details, see Table 1), the enzymatic assays were all run in a similar format. Enzymes were used at a concentration that results in linear generation of product with increasing time and protein concentration, as well as yielding a signal that was 3–20 times greater than the background. BSA (0.1 mg/mL final concentration) was added to all buffers just before use to reduce nonspecific inhibition (McGovern et al. 2002). For glutathione *S*-transferase (GST) activities, the buffer was supplemented with 5 mM glutathione. For all the enzyme assays, we tested the compounds at final concentrations of 0.1 and 1 μM.

Kinetic assay conditions. The dissociation constant of triclosan for CES1 was determined following the method described by Dixon (1972) for competitive tight binding inhibitors, using cyano(6-methoxy-2-naphthyl) methyl acetate (CMNA) as the substrate (Shan and Hammock 2001). Inhibitor concentrations between 0 and 1,000 nM were incubated in triplicate for 5 min in sodium phosphate buffer (pH 7.4) at 30°C with 200 μL of the enzyme solution. Substrate at a final concentration of 5–100 μM was then added. Velocity of the reaction was measured as described above. For each substrate concentration, plots of velocity as a function of inhibitor concentration allow the determination of an apparent inhibition constant (K_{Iapp}). The plot of K_{Iapp} as a function of the substrate concentration allows the determination of K_I when the substrate concentration is zero. Results were expressed as the mean ± SD of three separate K_I measurements.

Cell-based bioassays. Table 2 presents an overview of the different cell-based bioassays used. For all test compounds, agonist activity in the aryl hydrocarbon receptor (AhR), androgen receptor (AR), and estrogen receptor (ER) assays was determined in the AhR, AR, and ER CALUX (chemically activated luciferase expression) bioassays, respectively. All three CALUX bioassays make use of different cell lines (H1L6.1c2, T47D-AR–positive, and BG1Luc4E2/ER-α–positive, respectively) that contain a stably transfected luciferase gene under the transcriptional control of DNA response elements for the activated AhR, AR, and ER, respectively (Garrison et al. 1996; Han et al. 2004; Rogers and Denison 2000). Activation of the receptor signaling pathway was determined by quantifying the luciferase activity in the absence or presence of a known agonist [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 17β-estradiol (E_2), or dihydrotestosterone (DHT)]. Results were expressed relative to luciferase activity maximally induced by a reference compound (1 nM TCDD for AhR, 10 nM DHT for AR, 1 nM E_2 for ER). For these assays, the primary screening of the library was done at 10 μM. Membranes enriched in ryanodine receptors (RyRs) were obtained either from

Table 1. Conditions for human enzyme-based bioassays.

Enzyme	Preparation used	Substrate	Concentration (μM)	Buffer	End point measured	Reference
sEH	Recombinant purified enzyme	CMNPC	5	Bis-Tris/HCl pH 7.0, 25 mM	Fluorescence kinetic	Jones et al. 2005
CES1	Recombinant partially purified enzyme	CMNA	50	Na_2PO_4 pH 7.4, 0.1 M	Fluorescence kinetic	Shan and Hammock 2001
CES2	Recombinant partially purified enzyme	CMNA	50	Na_2PO_4 pH 7.4, 0.1 M	Fluorescence kinetic	Shan and Hammock 2001
CES3	Recombinant partially purified enzyme	CMNA	50	Na_2PO_4 pH 7.4, 0.1 M	Fluorescence kinetic	Shan and Hammock 2001
FAAH	Recombinant microsomes	Octanoyl-MP	50	Na_2PO_4 pH 8.0, 0.1 M	Fluorescence kinetic	Huang et al. 2007
PON2	Recombinant microsomes	CMNA	50	Na_2PO_4 pH 7.4, 0.1 M	Fluorescence kinetic	Shan and Hammock 2001
GSTs	Pooled human liver cytosol	CDNB	1,000	K_2PO_4 pH 6.5, 0.1 M	Absorbance kinetic	Habig et al. 1974
CYP450 1A2 and 2C6	Pooled human liver microsomes	EROD	25	K_2PO_4 pH 7.4, 0.1 M	Fluorescence kinetic	Dutton et al. 1989
CYP450 2C9	Pooled human liver microsomes	Luciferin H	50	K_2PO_4 pH 7.4, 0.1 M	Luminescence	Cali et al. 2006

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CMNA, cyano(6-methoxy-2-naphthyl)methyl actetate; CMNPC, cyano(6-methoxy-naphthalen-2-yl)methyl *trans*-[3-phenyloxiran-2-yl) methyl] carbonate; EROD, ethoxyresorufin; Octanoyl-MP, *N*-(6-methoxypyridin-3-yl) octanamide.

Table 2. Conditions for cell-based bioassays.

Human receptor	Acronym	Preparation used	Substrate	End point measured	Reference
Aryl hydrocarbon receptor	AhR	Recombinant cells	Luciferin	Luminescence	Han et al. 2004
Androgen receptor	AR	Recombinant cells	Luciferin	Luminescence	Rogers and Denison 2000
Estrogen receptor	ER	Recombinant cells	Luciferin	Luminescence	Rogers and Denison 2000
Ryanodine receptor 1	RyR1	Skeletal muscle membranes	[^{3}H]Ry	Radioactivity	Pessah et al. 1987
Ryanodine receptor 2	RyR2	Ventricular muscle membranes	[^{3}H]Ry	Radioactivity	Pessah et al. 1990

adult rabbit skeletal muscle, a pure type 1 ryanodine receptor (RyR1) source (Saito et al. 1984), or from cardiac ventricular tissue, a pure type 2 ryanodine receptor (RyR2) source (Pessah et al. 1990). Activation or inhibition of the receptors was measured by quantifying the ability of the tested compound at 5 μM to enhance or inhibit the basal binding of [^{3}H]Ry (2 nM) in the presence of 20 μM $CaCl_2$. After a 3-hr incubation at 37°C, the reactions were quenched by filtration through GF/B-grade glass fiber filters and washed twice with ice-cold harvest buffer containing 20 μM $CaCl_2$. [^{3}H]Ry binding was quantified by measuring the radioactivity collected on the filter.

Selection of positive hits and counterscreening. For the enzyme assays, a compound was selected as a positive hit if it resulted in > 50% inhibition at the lower concentration (100 nM) and if it resulted in more than 60% inhibition at the higher concentration (1 μM). For the cell-based assays, we selected compounds that significantly (*t*-test and *F*-test, $p < 0.01$) induced the receptor activation of gene expression. For counterscreening, fresh solutions of all positive compounds were prepared in DMSO. For the enzyme assays, the concentration of each compound that inhibited 50% of the enzyme activity (IC_{50}) was determined by measuring enzyme activities in the absence and presence of increasing concentrations of inhibitor (ranging from 0.5 to 10,000 nM). IC_{50} values were calculated by nonlinear regression of at least five data points using SigmaPlot, version 9.01 (Systat Software Inc., Chicago, IL). Results are provided as the mean ± SD of at least three separate measurements. Similarly, half-maximal effective concentration (EC_{50}) values for agonists of the AhR and ER bioassays were determined, and the results are presented as the mean of triplicate analysis. For the assay of [^{3}H]Ry binding to RyR1 or RyR2, the influence of 5 μM of each compound was screened for its ability to either enhance or inhibit specific radioligand binding more than twice the baseline (defined as the level of [^{3}H] Ry-specific binding in the presence of DMSO alone). Therefore, a positive hit on RyR1 or RyR2 was defined as ≥ 200% of control binding for activators, or ≤ 50% of control for inhibitors.

Results and Discussion

Assays characteristics and positive hits selection. Using results from the blank and full activity controls, we evaluated the suitability of each assay for use as HTS assays. We therefore calculated the signal-to-background ratio (S/B), the signal-to-noise ratio (S/N), and the *Z′* factor as defined by Zhang et al. (1999). As shown in Table 3, we found that S/B ratios varied from 2.5 to > 150, with the lowest value for the absorbance-based assay (GSTs) and the highest for the radioactive-based assays (RyRs). Similarly, the S/N ratios varied greatly, with a lower value for the absorbance assay and the higher values for the radioactive-based assays. In general, the enzyme-based assays yielded higher *Z′* factors than did the cell-based bioassays. For the enzyme assays, *Z′* values were > 0.7, indicating very good and reliable assays that are easily suitable for HTS assays. Although the cell-based assays yielded lower *Z′* factors, the values were still > 0.5, suggesting that the discrimination is adequate and that these assays could be used in HTS assays. Nevertheless, for the RyR assays, a larger separation band and higher *Z′* factor could be obtained by reducing the SD of the signal, which was around 20%.

The aims of the primary screening were to identify all possible positive hits and to ensure there were no false negatives. Thus, for the primary screening of the library, we tested the xenobiotics at relative high concentrations (0.1 and 1 μM for the enzymes, and 5 and 10 μM for the receptors), which should be far higher than blood concentrations resulting from exposure. Thus, it is unlikely that compounds found negative in the primary screening will be false negative and affect the tested proteins *in vivo*. Generally, testing higher concentrations result in solubility problems for an increasing proportion of compounds. Based on our definition of positive hits (described above), for the 14 assays we obtained a total of 69 positive results (Table 3), which represent on average five positive hits per assay, or 3% of the library. For FAAH, GST, and AR bioassays, we obtained no hits from the screening. There were twice as many positive hits from chemicals in plate II (42) than from those in plate I (27) [see Supplemental Material, Figure 1 (doi:10.1289/ehp.0900834. S1)]. The latter plate contained numerous triazine herbicides that did not result in any significant inhibition in any assay. Although three compounds [carbophenothion, triclosan, and triphenyl phosphate (TPP)] gave positive results with three enzymes or more, all the target enzymes were esterases.

Even if the assays are of high quality, as defined by their S/B, S/N, and *Z′* factors (described above), false positives are bound to happen as they are dependent on the compounds tested and not on the assays. False positives are mostly due to nonspecific binding, alteration of the reporting signal (quenching of the fluorescence signal, cytotoxicity to the cells, etc.), and chemical modifications during storage of the chemicals. The purpose of the counterscreening is to eliminate false positives. To reduce nonspecific inhibition, BSA (0.1 mg/mL final concentration) was added to all buffers just before use (McGovern et al. 2002). To eliminate alteration of the reporting signal, we tested the ability of each positive hit to quench the fluorescent or luminescent signal as well as its possible cytotoxic effect. Unfortunately, it is not possible to run such controls in the primary screen format. Finally, to reduce false positives resulting from some chemical modification upon storage, we prepared a fresh solution of each positive hit just before counterscreening. Out of the 69 positive hits initially found in the library screening, individual counterscreening analysis confirmed that 39 of them are effectively positive hits (see definition above), indicating an approximately 40% false-positive rate for the primary screening. This relatively high number of false positives reflects the high concentrations used for the primary screening. A lower screening concentration will have a lower number of false positives but will significantly increase the chance of false negatives, which is not desirable. Overall, using this two-step screening method, we found that 98% of the compounds tested have no effects on the tested assays.

Individual enzymes and receptors results. For all the positive hits selected from the library screening, we determined their individual

Table 3. Characteristics and positive primary screen results for enzyme- and cell-based bioassays.

Assay	Assay characteristics			No. of positive results	
	S/B[a]	S/N[a]	*Z′*[a]	Primary screen	Counterscreen
Enzyme					
sEH	4.0 ± 0.1	38 ± 8	0.8 ± 0.1	2	2
CES1	11 ± 3	19 ± 2	0.8 ± 0.1	4	2
CES2	9.2 ± 0.9	106 ± 33	0.8 ± 0.1	4	3
CES3	6.1 ± 0.7	28 ± 7	0.7 ± 0.1	7	4
FAAH	150 ± 10	35 ± 5	0.8 ± 0.1	0	—
PON2	18 ± 2	134 ± 31	0.8 ± 0.04	4	3
GSTs	2.4 ± 0.5	28 ± 9	0.7 ± 0.05	0	—
CYP450 1A2 and 2C6	13 ± 5	48 ± 10	0.7 ± 0.1	1	0
CYP450 2C9	19 ± 4	79 ± 18	0.7 ± 0.05	12	7
Receptor					
AhR	32 ± 1	410 ± 30	0.6 ± 0.2	3	2
AR	18 ± 2	180 ± 70	0.7 ± 0.1	0	—
ER	5 ± 1	80 ± 40	0.6 ± 0.1	8	5
RyR1	170 ± 30	500 ± 90	0.5 ± 0.1	12	8
RyR2	100 ± 10	310 ± 40	0.6 ± 0.1	12	4

[a]Results are mean ± SD of at least four independent measurements.

inhibition or induction potency (IC_{50} or EC_{50}) toward an enzyme or a receptor (Table 4), except for the RyR assays, which are the subject of a forthcoming study. As expected, we found that sEH was strongly inhibited by two urea-containing compounds, which are a well-established class of sEH inhibitors (Morisseau et al. 1999): siduron and triclocarban [trichlorocarbanilide (TCC)]. Although siduron uses are limited, TCC is present in numerous personal care products (Ahn et al. 2008), suggesting a large exposure risk. Animal models have shown that inhibition of the sEH affects human health by altering homeostasis, blood pressure, inflammation, and pain (Morisseau and Hammock 2008).

Inhibition of the CESs by organophosphate xenobiotics (Table 4), such as carbophenothion, parathion, phosdrin, and TPP, was expected, because such compounds are common mechanistic suicide inhibitors of serine hydrolases after activation to the oxon form (Casida and Quistad 2005). Because the CESs are only slowly reactivated, there is thus a cumulative risk. Although many organophosphate insecticides have been or are being phased out around the world, TPP continues to be used both as a plasticizer and a fire retardant in electronic components. Burning or leaching of TPP from electronic waste could result in its presence in water (Owens et al. 2007). Given the role of CES in the metabolism of ester- and amide-containing xenobiotics (Satoh and Hosokawa 2006), CES inhibition could lead to increased toxicity of xenobiotics. In general, CES inhibitors contain a carbonyl that reacts with the active-site serine to form a tetrahedral intermediate (Harada et al. 2009). Thus, the inhibition of CES1 and CES2 by triclosan, present in numerous personal care products (Ahn et al. 2008), was unexpected. To understand the mechanism of action of triclosan, we determined its kinetic constant [see Supplemental Material, Figure 2 (doi:10.1289/ehp.0900834.S1)]. We found that triclosan inhibits CES1 by a competitive mechanism and a K_I of 105 ± 5 nM. Although not the most potent of known CES1 inhibitors, triclosan represents a lead compound for a new class of esterase inhibitors.

PON2 was first identified as an enzyme that protects humans from environmental poisoning by organophosphate derivatives (James 2006); thus, one could expect apparent inhibition of this enzyme by organophosphates as we observed (Table 4). For carbophenothion and tributyl phosphotrithioite, this is likely due to traces of oxon impurities. Interestingly, we found that, in addition to CES1 and CES2, TPP can also significantly reduce PON2 activity. Inhibition of PON2 could lead to increased atherosclerosis and cardiovascular risk (James 2006). Taken together, exposure to TPP could affect human health through various modes of action.

For the two cytochrome P450 (CYP450) activities tested, significant inhibition was observed only for CYP450 2C9 (Table 4). 2-Methylheptyl-4,6-dinitrophenyl crotonate, the active ingredient in the fungicide dinocap, was the only very potent inhibitor of this CYP450 found. Interestingly CYP450 2C9 is involved in the production of antiinflammatory and antihypertensive epoxyeicosatrienoic acids from arachidonic acid; thus, inhibition of this CYP450 could lead to increased cardiovascular risk (Morisseau and Hammock 2008).

Screening results for the three nuclear receptor signaling pathways (AhR, ER, and AR) identified seven compounds with significant agonist activity: two for AhR, five for ER, and none for AR. Interestingly, even given the promiscuity of AhR ligand binding (Denison and Heath-Pagliuso 1998; Denison and Nagy 2003), only two fungicide chemicals, 2-(4-chlorophenyl)-benzothiazole (CPB) and dichlone, induced AhR-dependent gene expression, and they were relatively weak inducers. CPB and dichlone EC_{50} values for induction (Table 4) were approximately 5×10^5-fold less potent than the prototypical AhR agonist TCDD. Although dichlone is a newly identified AhR agonist, CPB was previously reported to induce AhR-dependent expression of cytochrome CYP450 1A1 in human and mouse cell lines (Kärenlampi et al. 1989). As expected, we found that ER signal transcription was activated by *o,p′*-DDT (dichlorodiphenyltrichloroethane) and its metabolites *o,p′*-DDE (dichlorodiphenyldichloroethylene) and *o,p′*-DDD (dichlorodiphenyldichloroethane) (Chen et al. 1997; Rogers and Denison 2000), and our screening identified *o,p′*-DDE and *o,p′*-DDD as activators also (*o,p′*-DDT was not present in the screened library). In our system, the EC_{50} for induction by *o,p′*-DDE and *o,p′*-DDD was approximately 10^5-fold less potent than that of E_2 (Rogers and Denison 2000). Similarly, bisphenol A (BPA) and lindane have also been previously identified as ER agonists (Bonefeld-Jørgensen et al. 2007; Maranghi et al. 2007; Steinmetz et al. 1996; Vandenberg et al. 2009), although lindane has been suggested to activate ER-dependent gene expression through a nonclassical mechanism (Steinmetz et al. 1996). BPA was the most potent ER agonist identified, only 3,000-fold less potent than E_2, whereas lindane was the weakest. Taken together, the relatively low potency of these agonists coupled with existing controversies regarding exposure and health risks associated with BPA and other endocrine-disrupting chemicals (Vandenberg et al. 2009) suggests that the adverse effects of these chemicals remain to be determined.

Our primary screen revealed that numerous compounds affected the RyRs, such as triclosan, which we previously showed to increase [^{3}H]Ry binding to RyR1 (Ahn et al. 2008). For counterscreening, we concentrated on the 12 chemicals that produced the most significant RyR effect (Figure 1). Overall, the profiles

Table 4. Positive counterscreen results for the enzyme assays and nuclear receptor–based bioassays.

Assay	Compound	IC_{50} or EC_{50} (nM)[a]	Use
sEH	Siduron	33 ± 3	Herbicide
	TCC	13 ± 1	Microbiocide
CES1	Triclosan	210 ± 20	Microbiocide
	TPP	43 ± 3	Flame retardant
CES2	Carbophenothion	34 ± 1	Insecticide
	Triclosan	580 ± 30	Microbiocide
	TPP	50 ± 2	Flame retardant
CES3	Carbophenothion	110 ± 15	Insecticide
	Parathion	4.9 ± 0.4	Insecticide
	Phosdrin	1.1 ± 0.1	Insecticide
	Primiphos-ethyl	180 ± 20	Insecticide
PON2	Carbophenothion	110 ± 6	Insecticide
	Tributyl phosphorotrithioite	120 ± 10	Herbicide
	TPP	85 ± 8	Flame retardant
CYP450 2C9	2-Butan-2-yl-4,6-dinitro-phenol	1,900 ± 100	Pesticide
	Chlorpyrifos	3,200 ± 200	Insecticide
	Finasteride	1,500 ± 100	Antiandrogen
	2-Methylheptyl-4,6-dinitrophenyl crotonate	120 ± 1	Fungicide
	Pentachlorophenol	850 ± 10	Herbicide
	Pyrethrum	2,300 ±100	Insecticide
	Triclosan	650 ± 40	Microbiocide
AhR	CPB	11,400	Fungicide
	Dichlone	> 10,000	Fungicide
ER	BPA	330	Plastic monomer
	o,p-DDD (dichlorodiphenyldichloroethane)	1,200	Insecticide
	o,p-DDE (dichlorodiphenyldichloroethylene)	1,200	Insecticide
	Endrin	13,000	Pesticide
	Lindane	> 50,000	Insecticide

[a]Values are IC_{50}s for the enzyme-based assays (sEH to CYP450 2C9) and EC_{50}s for the receptor-based assays (AhR and ER). Results are mean ± SD of at least three independent measurements.

for both receptors are similar, with the profile of RyR2 being more attenuated than that for RyR1. For the latter protein, we found eight compounds (at 5 μM) that significantly affected the binding of [^{3}H]Ry: five of them inhibited the binding, and three increased it. For RyR2, we found four compounds that significantly inhibited this receptor. For both receptors, the largest effect was observed for chloranil (IC_{50} < 1.0 μM) and dichlone (IC_{50} < 1.0 μM), which both contain in their structure a 2,3-dichloro-1,4-quinone. These results are consistent with our previously published work showing that naphthoquinones and benzoquinones are capable of selectively modifying RyR1 channels in a time- and concentration-dependent manner (Feng et al. 1999). Interestingly, we found that [^{3}H]Ry binding to RyR1 was increased almost 3-fold by chlorpyriphos and *o,p′*-DDE. Counterscreening results suggested that baythroid, α-cypermethrin, deltamethrin, and *N*-cyclohexyl-2-benzothiazyl sulfonamide have no significant effect on either RyR at 5 μM. Obtaining a compound that interacts specifically with only one of the RyRs or has opposing effects on both proteins will be scientifically very important. The deltamethrin scaffold could be a lead toward such compounds, because deltamethrin seemed to have opposing effects on both RyRs. RyR1 and RyR2 are major components of skeletal and cardiac muscle excitation contraction coupling, and several heritable mutations in these proteins have been associated with myogenic disorders (Bellinger et al. 2008). In addition, RyR1 and RyR2 are the major isoforms expressed in neurons and are responsible for producing temporally and spatially defined Ca^{2+} signals important for neuronal growth and plasticity (Berridge 2006). Deregulation of RyR function and expression contributes to alterations in activity-dependent dendritic growth and plasticity (Kenet et al. 2007; Roegge et al. 2006; Yang et al. 2009) and the balance of excitatory and inhibitory neurotransmission in the hippocampus CA1 region (Kim et al. 2009). Thus, exposure to the RyR channel activators and inhibitors identified here could trigger adverse contractile responses in muscle cells and affect proper brain development, especially in susceptible individuals.

Conclusion

The HTS method described herein allowed the elimination of 98% of the compounds as negative hits. Furthermore, we were able to correctly identify compounds that were previously shown to inhibit or induce a particular enzymes or receptor; however, we also discovered new effects of some xenobiotics. For example, the inhibition of CES1 and CES2 by triclosan was totally unexpected, as was the inhibition of the RyRs by chloranil and dichlone. These *in vitro* results raise significant biological/toxicologic questions and further *in vivo* studies are necessary before drawing any conclusions on the health risks associated with any of these compounds by these specific mechanisms. Overall, our study shows the feasibility of using combined HTS assays as an approach toward obtaining toxicologic data on the many thousands of anthropogenic compounds for which there is little if any information. Furthermore, the HTS assays were very useful for quickly identifying compounds of potential risk for further studies, thus concentrating resources on the potentially most significant chemicals.

The National Library of Medicine has developed the infrastructure to screen compounds on possible pharmacologic leads and to report the data in an easily accessible publically available format; this is part of the National Institutes of Health Molecular Libraries Roadmap initiative. The results for the screening of sEH in this system are available online (National Center for Biotechnology Information 2009); the AhR CALUX bioassay is currently used in the same program. One useful rapid approach would be for investigators or the National Institute of Environmental Health Sciences to propose toxicologically relevant assays and also provide environmentally or industrially important compounds to the system.

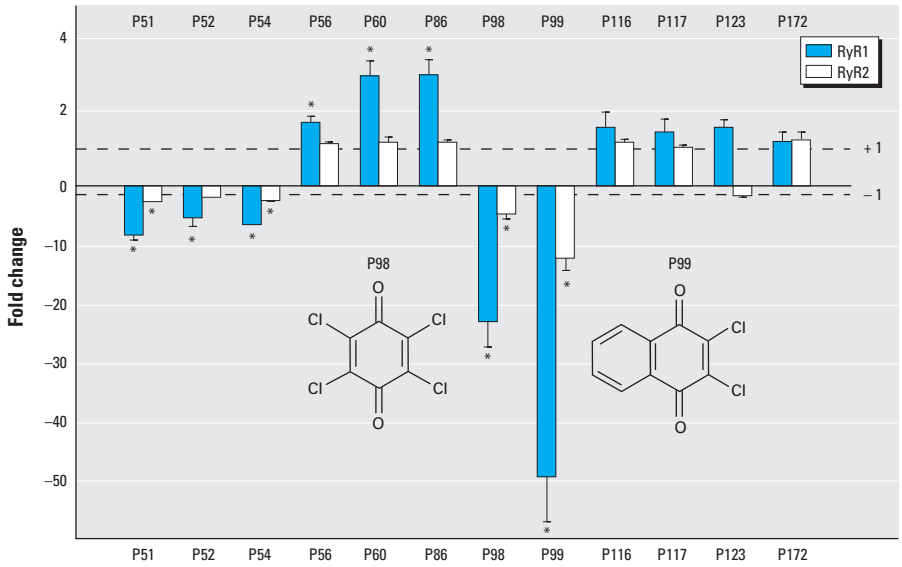

Figure 1. Effect of 5 μM of ferbam (P51), maneb (P52), tetramethyl-thiuram disulfide (P54), pirimiphos-methyl (P56), chlorpyrifos (P60), *o,p′*-DDE (P86), chloranil (P98), dichlone (P99), baythroid (P116), α-cypermethrin (P117), deltamethrin (P123), and *N*-cyclohexyl-2-benzothiazyl sulfonamide (P172) on the binding of [^{3}H]Ry to the RyRs compared with DMSO control. A positive value indicates that the binding of [^{3}H]Ry was increased; a negative value indicates that the binding of [^{3}H]Ry was inhibited. The dashed lines at 1 and –1 are reference lines for no change in the binding of [^{3}H]Ry to the receptors.
*$p < 0.01$.

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Toxicology in the Fast Lane: Application of High-Throughput Bioassays to Detect Modulation of Key Enzymes and Receptors (Supplemental materials)

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Materials and methods

Chemicals. Most chemicals used in the library were obtained from Chem Service Inc. (West Chester, PA) and Sigma Chemical Co (St Louis, MO). Chemicals were at least 95% pure, and used without further purification. Cyano(6-methoxy-naphthalen-2-yl)methyl *trans*-[(3-phenyloxiran-2-yl)methyl] carbonate (CMNPC), cyano(6-methoxy-2-naphthyl)methyl acetate (CMNA), N-(6-methoxypyridin-3-yl) octanamide (Octanoyl-MP) were prepared previously in the laboratory (Huang et al. 2007; Jones et al. 2005; Shan et al. 2001). 1-Chloro-2,4-dinitrobenzene (CDNB), glutathione and ethoxyresorufin (EROD) were obtained form Sigma-Aldrich. Luciferin H was bought from Promega (Madison, WI). We obtained 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from S. Safe (Texas A&M University, College Station, TX). We purchased dimethyl sulfoxide (DMSO), 17β-estradiol (E_2), and phenol red-free Dulbecco's modified Eagle medium (DMEM) from Sigma Chemical Co. (St. Louis, MO); cell culture reagents and media from Gibco/BRL (Grand Island, NY); and dihydrotestosterone (DHT) from Dr. B. Wilson (UC Davis). We purchased [^{3}H]ryanodine ([^{3}H]Ry, 60–90 Ci/mmol; > 99% pure) from Perkin-Elmer New England Nuclear (Wilmington, DE) and unlabeled Ry (> 99% by ultraviolet-HPLC) from Calbiochem (San Diego, CA). All chemicals and solvents were used without further purification.

Environmental chemicals library. The library was prepared in 2 mL deep well polypropylene 96-well assay blocks (Fisher Scientific, Santa Clara, CA; # 07200700). For every compound, a 1 mL solution at 10 mM in DMSO was prepared in a 2 mL glass vial and the solution was transferred into the assay block using a clean glass syringe. Only compounds totally soluble at 10 mM in DMSO were kept inside the library. In each plate we dispensed 1 mL of DMSO in the first column wells to serve as controls. In the remainder of the plate, we dispensed one compound per well, with 88 compounds total per plate. We created two plates with different

chemicals for a total of 176 compounds. A detail description of the chemical contents in each plate is presented in the supplemental materials. The plates were tightly sealed with EVA copolymer sealing mats (Fisher Scientific #07201112). The plates were then sealed in a 2-mil thick plastic bag, to avoid condensation, and stored at -20°C until use. Upon usage, the plates were let to warm-up at room temperature overnight before to be removed from the plastic bag. Using a robotic pipetting station (Quadra 96 – 96 well automated pipettor; Tomtec, Hamden, CT), each well was first mixed and the compound solutions were diluted 10-fold in DMSO (down to 1 mM) and then in the appropriate buffer and transferred into 96 well plates.

Enzyme-based assays.

Hydrolases and GSTs. The sEH activity was measured following the method of Jones et al. 2005; the CESs and PON2 activities were measured following the method of Shan and Hammock, 2001; the FAAH activity was measured following the method of Huang et al. 2007; and the liver cytosolic activity was measured following the method of Habig et al. 1974. For the assay 96-well plates containing 20μL of 10x concentrated test-compound solutions, 150 μL of the appropriate buffer were added in wells A1 to D1 (these four wells served as background control, while wells E1 to H1 served as full activity control), and 150 μL of the enzyme diluted in the same buffer were added to the rest of the plate using our Miniprep robotic system (Tecan, Durham, NC),. The plate was then mixed and incubated at 30 °C for 5 minutes. Across the plate, 30 μL of the working substrate solution (267 μL of 100x substrate solution in DMSO or ethanol diluted with 3,763 μL of buffer) were added quickly to yield the concentration of substrate given in Table 1. The activity was immediately measured at 30 °C kinetically for 10 min in a Spectramax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) in either fluorescent or absorbance mode using the published optimal wavelength for each substrate.

P450 1A2 & 2C6. Microsomal 7-ethoxyresorufin dealkylation activity (EROD) was

measured following a modified method described by Dutton and Parkinson (1989). To the back plates containing 20 µL of the 10x inhibitor dilution, 160 µL of the human liver microsomal preparation diluted in buffer were added across the plate, except in wells A1 to D1 that received 160 µL of buffer only (these wells served as background control). Using a repeating syringe, 2 µL of 100x EROD solution in DMSO were added to each well. The plate was then mixed and incubated at 30°C for 5 minutes. The enzymatic reaction was started by the addition across the plate of 20 µL of NADPH generating system (Watanabe and Hammock 2001). The resorufin formed was detected fluorometrically ($\lambda_{ex.}$ 535 mn; $\lambda_{em.}$ 585 nm) for 30 min at 30°C in a Spectramax M2 fluorometer.

P450 2C9. The Luciferin-H activity was performed following the method described by (Cali et al. 2006). To the white plate containing 20 µL of the 10x inhibitor dilution, 160 µL of the human liver microsomal preparation diluted in buffer were added across the plate, except in wells A1 to D1 that received 160 µL of buffer only (these wells served as background control). Using a repeating syringe, 2 µL of 100x luciferin-H solution in DMSO were added to each well. The plate was then mixed and incubated at 30°C for 5 minutes. The enzymatic reaction was started by the addition across the plate of 20 µL of NADPH generating system (Watanabe and Hammock 2001). The plates were mixed and incubated at 30°C for 30 minutes. The reaction was stopped and the produced luciferin was revealed by adding 100 µL of luciferase solution provided in the kit from Promega. After 15 minutes at 30°C, the luminescence was measured on a Spectrafluor plus lumimeter (Tecan).

Cell-based bioassay.

Aryl hydrocarbon Receptor (AhR) bioassay. Recombinant mouse hepatoma (H1L6.1c2) cells were grown and maintained as previously described (Garrison et al. 1996; Han et al. 2004). These cells contain the stably integrated, dioxin-responsive–element (DRE)-driven firefly

luciferase reporter gene plasmid pGudLuc6.1. Transcriptional activation of the plasmid occurs in a ligand-, dose-, time- and AhR-dependent manner. Cells were plated into white, clear-bottomed 96-well tissue culture dishes at 75,000 cells/well and allowed to attach for 24 hr. Cells were incubated with carrier solvent DMSO (1% final solvent concentration), TCDD (1 nM), or the indicated compound (10 μM) for 24 hr at 37°C. For luciferase measurement, sample wells were washed twice with phosphate-buffered saline, followed by addition of cell lysis buffer (Promega, Madison, WI); the plates were then shaken for 20 min at room temperature to allow cell lysis. We measured luciferase activity in each well using a Orion microplate luminometer (Berthold, Oak Ridge, TN) with automatic injection of Promega stabilized luciferase reagent. Luciferase activity in each well was expressed relative to that maximally induced by TCDD.

Androgen Receptor (AR) bioassays. For the cell-based human AR-responsive bioassay, recombinant human cells [T47D-androgen-responsive element (ARE)] were grown and maintained as described above for H1L6.1c2 cells. The T47D-ARE cells contain a stably integrated AR-responsive firefly luciferase reporter gene plasmid, pGudLuc7ARE (Rogers and Denison 2000). Cells were plated into white, clear-bottomed 96-well tissue culture dishes at 75,000 cells/well and allowed to attach for 24 hr. Cells were incubated with carrier solvent (DMSO; 1% final solvent concentration), dihydrotestosterone (DHT, 10 nM), or the indicated compound (10 μM) for 24 hr at 37°C. Luciferase activity was measured as described above and activity in each well expressed relative to that maximally induced by DHT.

Estrogen Receptor (ER) bioassay. Recombinant human ovarian cancer cells (BG1Luc4E$_2$, ER-α–positive) were grown and maintained as previously described (Rogers and Denison 2000). These cells contain a stably integrated, ER-responsive firefly luciferase reporter plasmid, pGudLuc7ERE. Cells were maintained in estrogen-stripped media for 5 days before they were plated into white, clear-bottomed 96-well tissue culture dishes at 75,000 cells/well and

allowed to attach for 24 hr. Cells were then incubated with carrier solvent (DMSO: 1% final solvent concentration), 17β-estradiol (E_2, 1 nM), or the indicated compound (10 μM) for 24 hr at 37°C. Luciferase activity was measured as described above and activity expressed relative to that maximally induced by E_2.

Ryanodine receptor 1 and 2 (RyR1 and RyR2) bioassay. Sarcoplasmic reticulum (SR) membrane vesicles enriched in ryanodine receptor (RyR1) were prepared from back and hind limb skeletal muscles of New Zealand White rabbits according to the method of Saito et al. (1984). Heavy SR enriched in RyR2 from rat cardiac ventricles was prepared by sucrose-density gradient centrifugation, as described previously by Pessah et al. (1990). The preparations were stored in 10% sucrose, and 5 mM imidazole (pH 7.4) at -80°C until needed. Equilibrium of specific high-affinity [^{3}H]ryanodine ([^{3}H]Ry) binding were determined according to the method of Pessah et al. (1987). [^{3}H]Ry binds with high affinity and specificity to the open state of RyR1 and RyR2 and therefore provides a convenient measure of ligands that influence channel conformation (Pessah et al. 1985 and 1987; Zimanyi et al. 1991). SR vesicles enriched with RyR1 (50 μg protein/ml) or RyR2 (100 μg protein/mL) were incubated with a compound (5 μM, and its solvent dimethyl sulfoxide (DMSO) served for control) in assay buffer containing HEPES (20 mM, pH 7.4), KCl (250 mM), NaCl (15 mM), [^{3}H]Ry (2 nM) and $CaCl_2$ (20μM, adjusted with EGTA; Brooks and Storey, 1992). Nonspecific binding was determined by incubating SR with 1000-fold excess unlabeled ryanodine in the absence or presence of the compound. The binding reactions were kept in 37°C for 3hr and then quenched by filtration through GF/B glass fiber filters and washed twice with ice-cold harvest buffer (20 mM Tris-HCl, 250 mM KCl, 15 mM NaCl, and 20 μM $CaCl_2$, pH 7.4). Total n = 8 samples/compound or DMSO from two independent measurements under the identical conditions were taken for data analysis.

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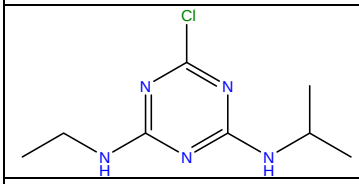
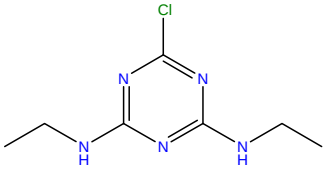
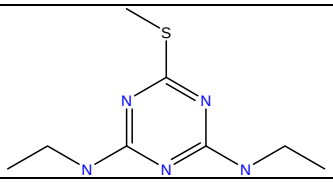
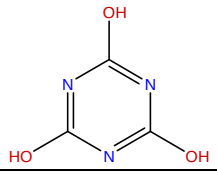
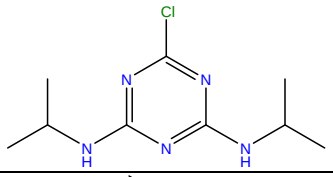
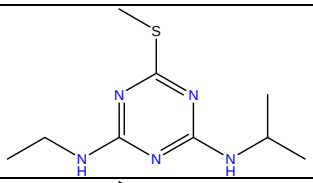
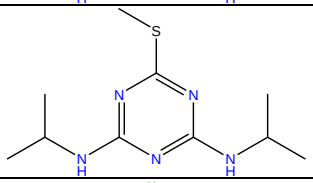
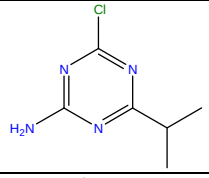
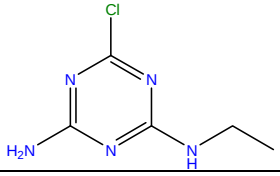
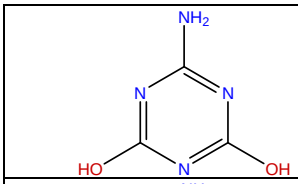
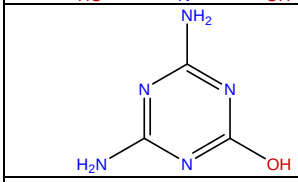
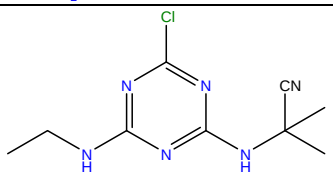
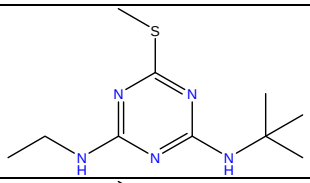
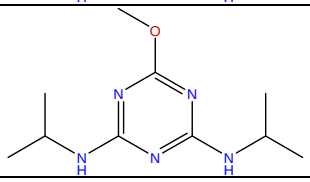
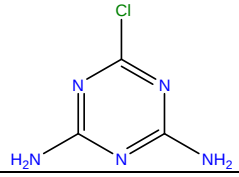
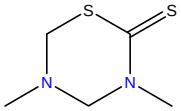
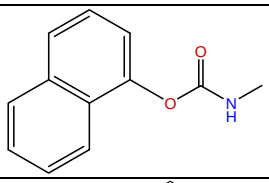
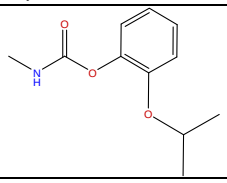
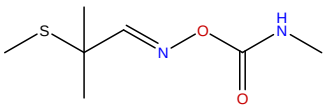
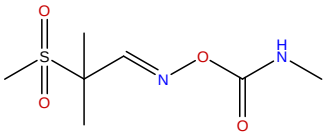
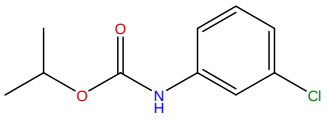
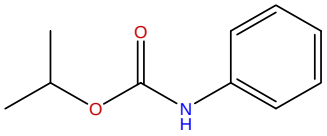
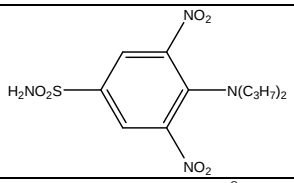
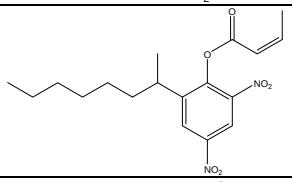
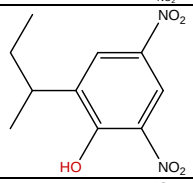
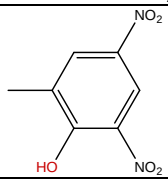
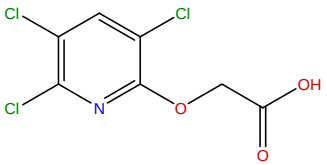
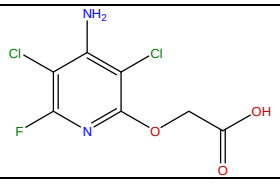
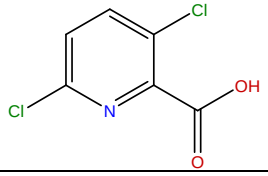
Supplemental Material, Table 1. Overall composition of library of chemicals tested.

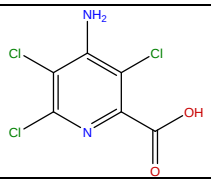
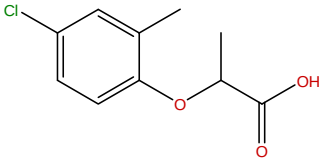
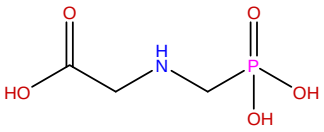
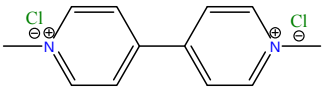
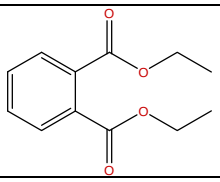
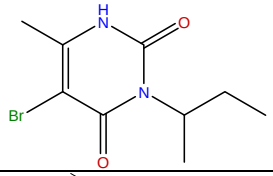
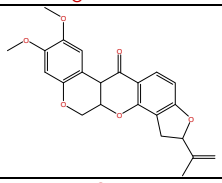
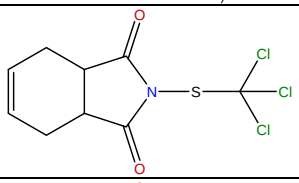
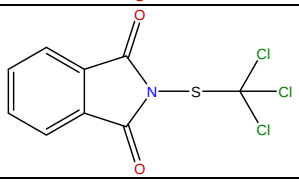
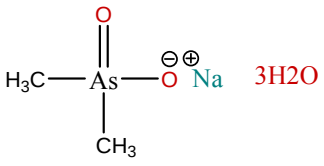
Pesticides (155)		Industrial products (32)	
Fungicide	20	Detergent	4
Herbicide	63	Exhaust pollutant	4
Insecticide	63	Flame retardant	5
Metabolite	4	Food additive	2
Microbiocide	3	Pharmaceutical drug	5
Nematocide	1	Plant growth regulator	9
Piscicide	1	Plastic product	3

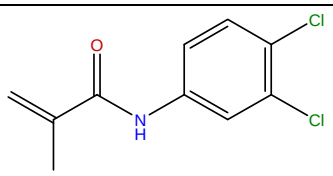
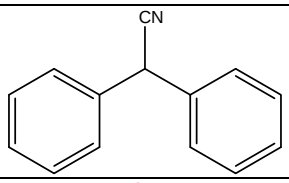
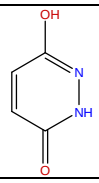
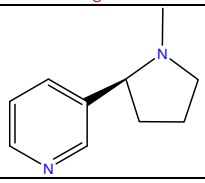
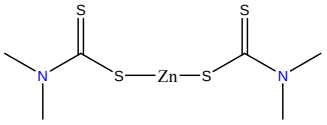
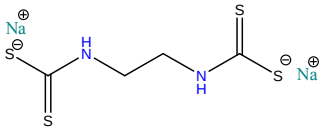
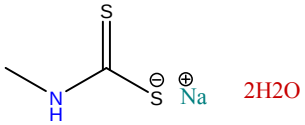
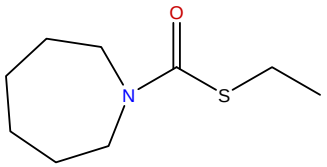
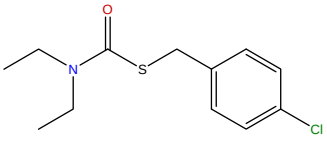
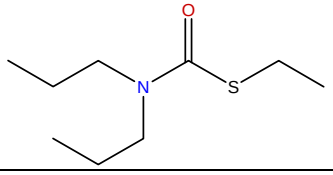
Supplemental Material, Table 2. Detail composition of the library of compounds used.

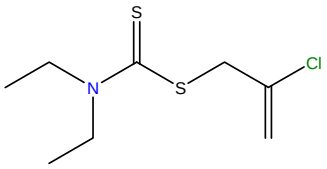
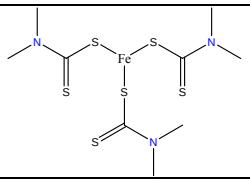
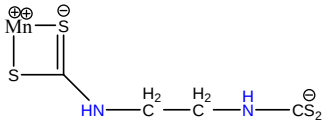
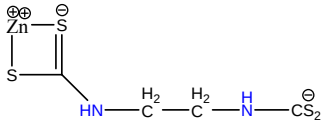
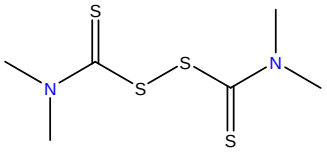
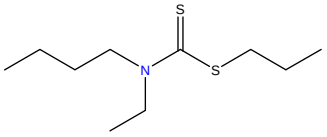
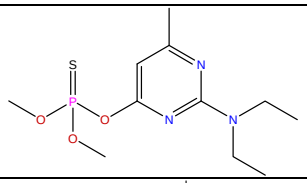
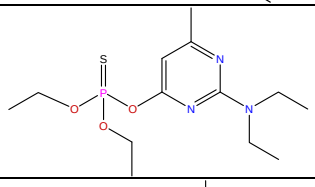
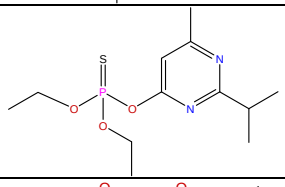
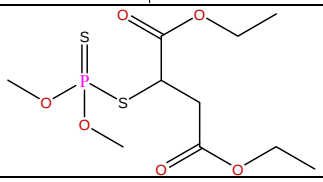
Structure	#	Name	Plate	Row	Column	Usage
	P1	Atrazine	I	A	2	Herbicide
	P2	Simazine	I	B	2	Herbicide
	P3	Simetryn	I	C	2	Herbicide
	P4	Cyanuric acid	I	D	2	Herbicide
	P5	Propazine	I	E	2	Herbicide
	P6	Ametryn	I	F	2	Herbicide
	P7	Prometryn	I	G	2	Herbicide
	P8	2-Chloro-4-isopropyl-6-amino-s-triazine	I	H	2	Herbicide
	P9	2-Chloro-4-ethylamino-6-amino-s-triazine	I	A	3	Herbicide

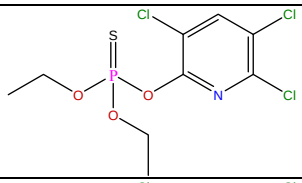
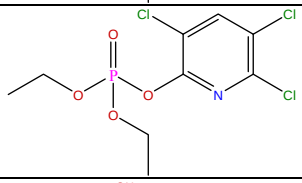
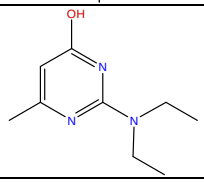
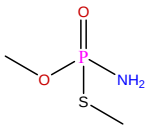
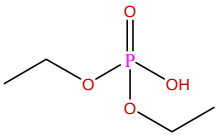
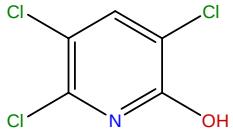
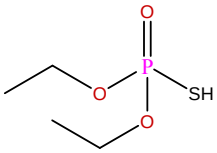
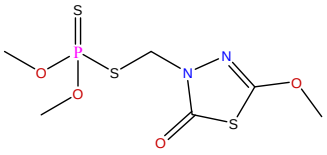
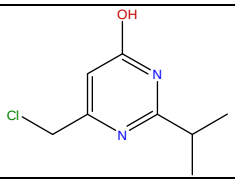
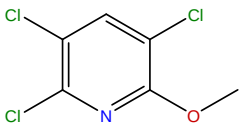
	P10	Ammelide	I	B	3	Herbicide
	P11	Ammeline	I	C	3	Herbicide
	P12	Cyanazine	I	D	3	Herbicide
	P13	Terbutryn	I	E	3	Herbicide
	P14	Prometon	I	F	3	Herbicide
	P15	2-Chloro-4,6-diamino-s-triazine	I	G	3	Herbicide
	P16	Dazomet	I	H	3	Fungicide
	P17	Carbaryl	I	A	4	Insecticide
	P18	Propoxur	I	B	4	Insecticide
	P19	Aldicarb	I	C	4	Insecticide

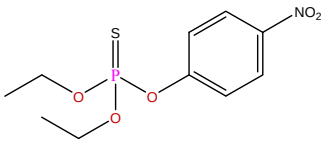
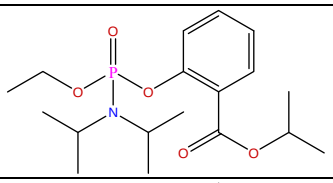
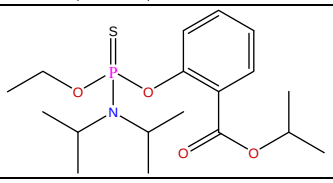
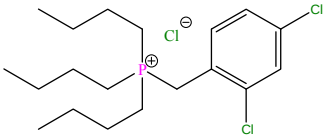
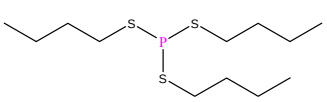
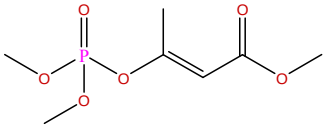
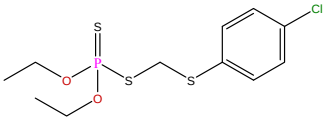
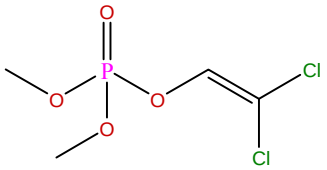
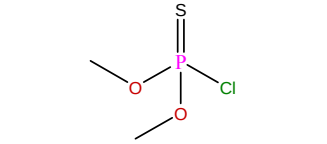
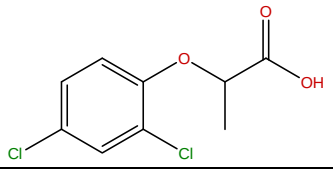
	P20	Aldoxycarb	I	D	4	Insecticide Nematocide
	P21	Isopropyl-N-[m-chlorophenyl] carbamate	I	E	4	Herbicide
	P22	Isopropyl-N-phenylcarbamate	I	F	4	Herbicide
	P23	Oryzalin	I	G	4	Herbicide
	P24	2-methylheptyl-4,6-dinitrophenyl Crotonate	I	H	4	Fungicide Acramicide
	P25	DNBP	I	A	5	Herbicide
	P26	4.6-Dinitro-o-cresol	I	B	5	Fungicide Insecticide Herbicide
	P27	Triclopyr	I	C	5	Herbicide
	P28	Fluroxypyr	I	D	5	Herbicide
	P29	Clopyralid	I	E	5	Herbicide

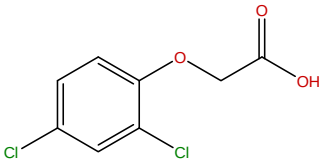
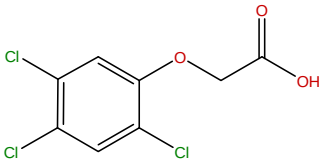
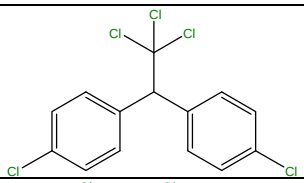
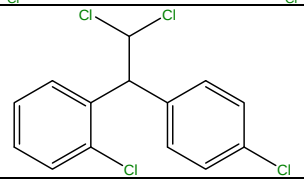
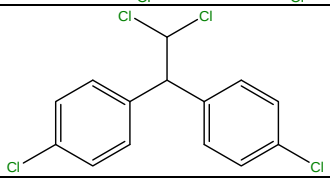
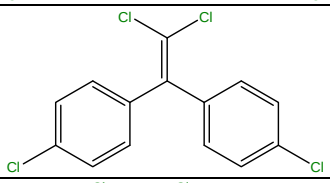
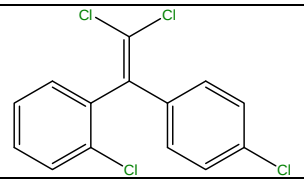
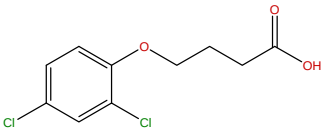
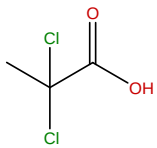
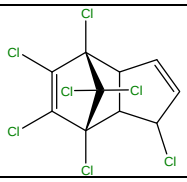
	P30	Picloram	I	F	5	Herbicide
	P31	Mecoprop	I	G	5	Herbicide
	P32	Glyphosate	I	H	5	Herbicide
	P33	Paraquat dichloride	I	A	6	Herbicide
	P34	Diethyl phthalate	I	B	6	Plasticizer
	P35	Bromacil	I	C	6	Herbicide
	P36	Rotenone	I	D	6	Insecticide Piscicide
	P37	Captan	I	E	6	Fungicide
	P38	Folpet	I	F	6	Fungicide
	P39	Cacodylic acid, Na salt	I	G	6	Herbicide

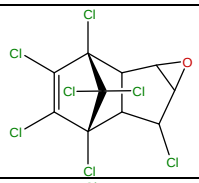
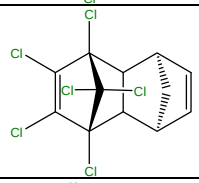
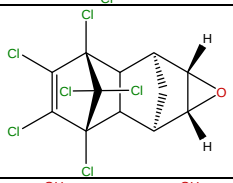
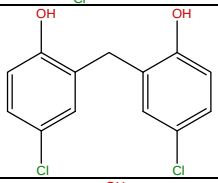
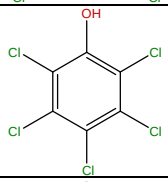
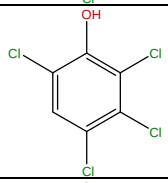
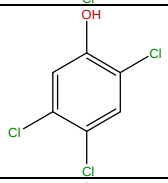
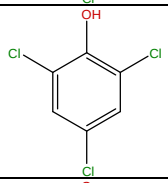
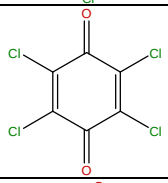
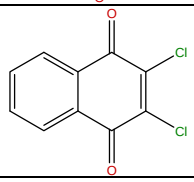
	P40	Chloranocryl	I	H	6	Herbicide
	P41	Diphenyl acetonitrile	I	A	7	Pesticide
	P42	Maleic acid hydrazide	I	B	7	Herbicide
	P43	Nicotine	I	C	7	Insecticide
	P44	Ziram	I	D	7	Fungicide
	P45	Nabam	I	E	7	Fungicide
	P46	Metam sodium	I	F	7	Fungicide Herbicide
	P47	Molinate	I	G	7	Herbicide
	P48	Thiobencarb	I	H	7	Herbicide
	P49	Eptam	I	A	8	Fungicide

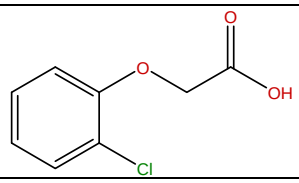
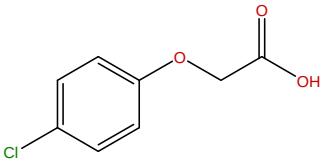
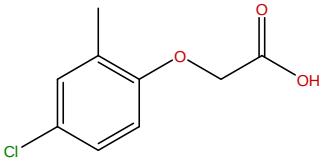
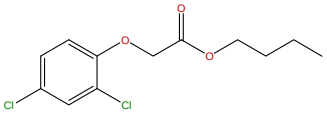
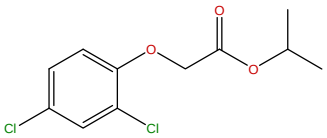
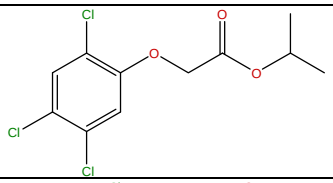
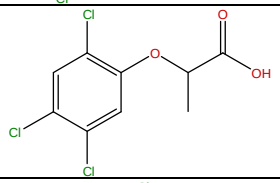
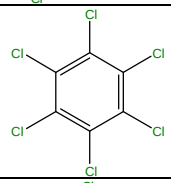
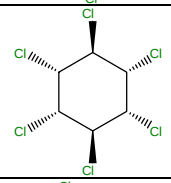
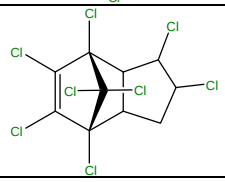
	P50	CDEC	I	B	8	Fungicide
	P51	Ferbam	I	C	8	Fungicide
	P52	Maneb	I	D	8	Fungicide
	P53	Zineb	I	E	8	Fungicide
	P54	Tetramethyl-thiuram disulfide	I	F	8	Fungicide
	P55	S-propyl butylethyl-thiocarbamate	I	G	8	Herbicide
	P56	Pirimiphos – methyl	I	H	8	Insecticide
	P57	Pirimiphos – ethyl	I	A	9	Insecticide
	P58	Diazinon	I	B	9	Insecticide
	P59	Malathion	I	C	9	Insecticide

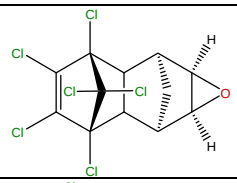
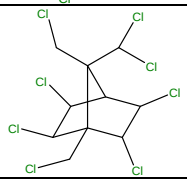
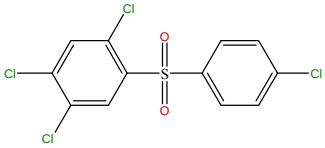
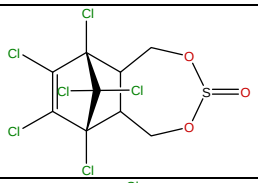
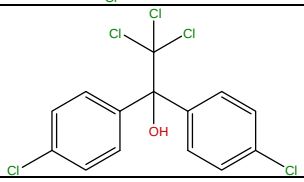
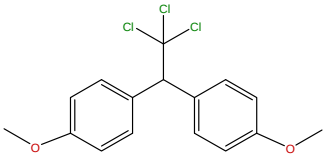
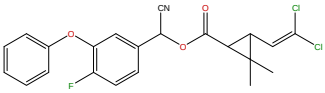
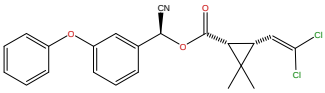
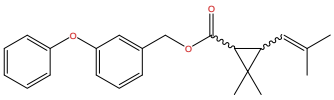
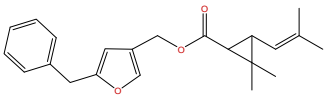
	P60	Chlorpyrifos	I	D	9	Insecticide
	P61	Chlorpyrifos oxon	I	E	9	Insecticide
	P62	2-diethylamino-6-methylpyrimidin-4-ol	I	F	9	Insecticide metabolite
	P63	Methamidophos	I	G	9	Insecticide
	P64	Diethyl phosphate	I	H	9	Insecticide
	P65	3,5,6-Trichloro-2-pyridinol	I	A	10	Insecticide metabolite
	P66	O,O-Diethylthio-phosphate	I	B	10	Insecticide
	P67	Methidathion	I	C	10	Insecticide
	P68	6-Chloromethyl-4-hydroxy-2-isopropyl pyrimidine	I	D	10	Insecticide metabolite
	P69	2-Methoxy-3,5,6-trichloropyridine	I	E	10	Insecticide metabolite

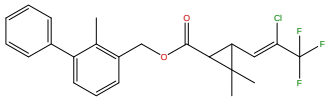
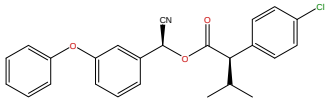
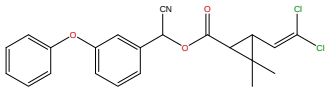
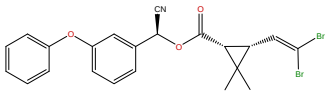
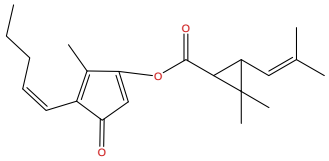
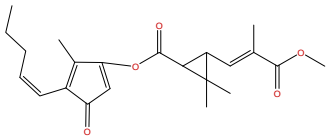
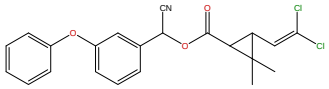
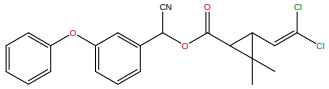
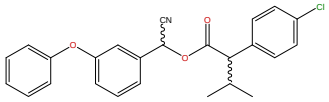
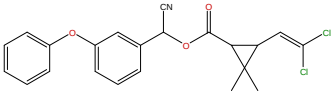
	P70	Parathion	I	F	10	Insecticide
	P71	des-N-Isopropyl isophenphos oxygen analog	I	G	10	Insecticide
	P72	des-N-Isopropyl isophenphos	I	H	10	Insecticide
	P73	Tributyl (2,4-dichlorobenzyl)-phosphonium chloride	I	A	11	Herbicide
	P74	Tributyl phosphoro-trithioite	I	B	11	Herbicide
	P75	Phosdrin	I	C	11	Insecticide
	P76	Carbophenothion	I	D	11	Insecticide
	P77	DDVP	I	E	11	Insecticide
	P78	O,O-dimethyl phosphochloridothioate	I	F	11	Herbicide
	P79	Dichlorprop	I	G	11	Herbicide

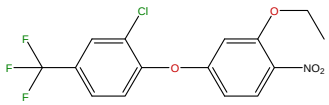
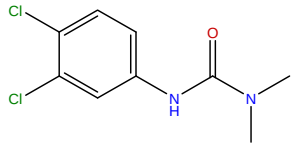
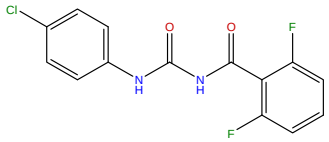
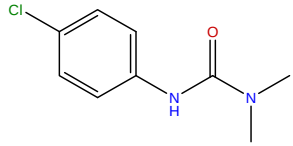
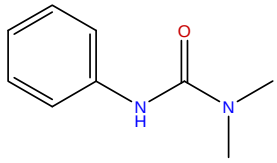
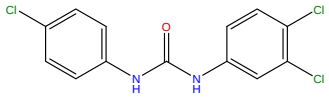
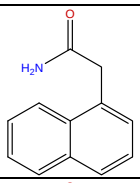
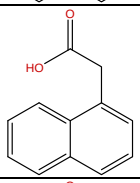
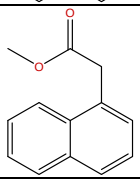
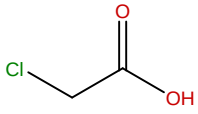
	P80	2,4-D	I	H	11	Herbicide
	P81	2,4,5-T	I	A	12	Herbicide
	P82	p,p-DDT	I	B	12	Insecticide
	P83	o,p'-DDD	I	C	12	Insecticide
	P84	p,p-DDD	I	D	12	Insecticide
	P85	p,p-DDE	I	E	12	Insecticide
	P86	o,p'-DDE	I	F	12	Insecticide
	P87	2,4-DB	I	G	12	Herbicide
	P88	Dalapon	I	H	12	Herbicide
	P89	Heptachlor	II	A	2	Insecticide

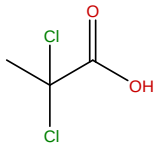
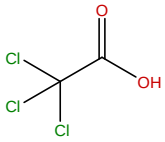
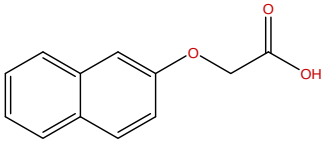
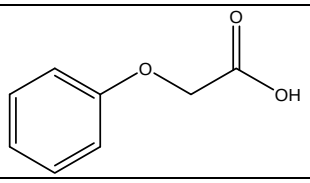
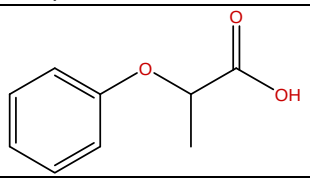
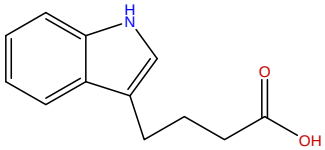
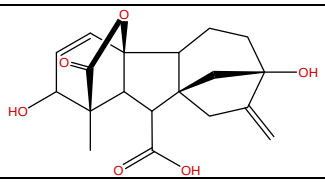
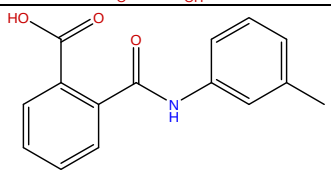
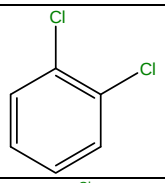
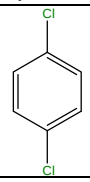
	P90	Heptachlor epoxide	II	B	2	Insecticide
	P91	Aldrin	II	C	2	Insecticide
	P92	Dieldrin	II	D	2	Insecticide
	P93	2,2'-Methylenebis(4-chlorophenol)	II	E	2	Microbiocide
	P94	Pentachlorophenol	II	F	2	Fungicide Herbicide Insecticide
	P95	2,3,4,6-Tetrachlorophenol	II	G	2	Herbicide
	P96	2,4,5-Trichlorophenol	II	H	2	Herbicide
	P97	2,4,6-Trichlorophenol	II	A	3	Fungicide Herbicide Insecticide
	P98	Chloranil	II	B	3	Fungicide
	P99	Dichlone	II	C	3	Fungicide

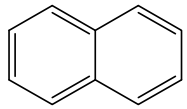
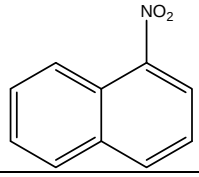
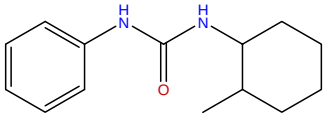
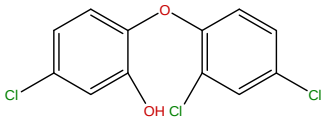
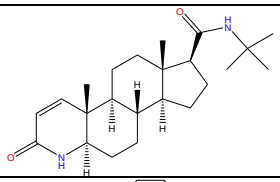
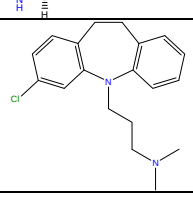
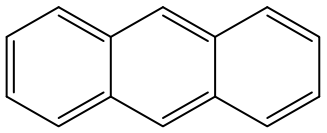
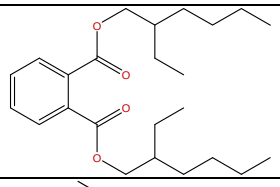
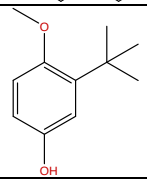
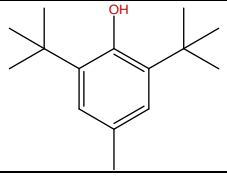
	P100	o-Chlorophenoxy acetic acid	II	D	3	Herbicide
	P101	p-Chlorophenoxy acetic acid	II	E	3	Herbicide
	P102	MCPA	II	F	3	Herbicide
	P103	2,4-Dichlorophenoxy acetic acid, butyl ester	II	G	3	Herbicide
	P104	2,4-Dichlorophenoxy acetic acid, isopropyl ester	II	H	3	Herbicide
	P105	2,4,5-Trichlorophenoxy acetic acid, isopropyl ester	II	A	4	Herbicide
	P106	Silvex	II	B	4	Herbicide
	P107	Benzene hexachloride	II	D	4	Insecticide
	P108	Lindane	II	E	4	Insecticide
	P109	Chlorodane	II	F	4	Insecticide

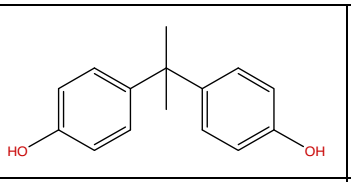
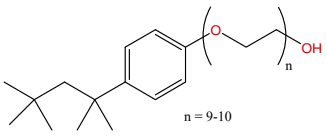
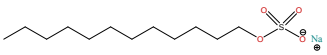
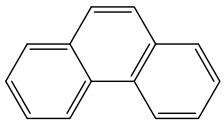
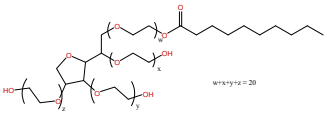
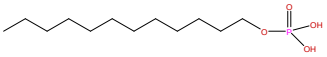
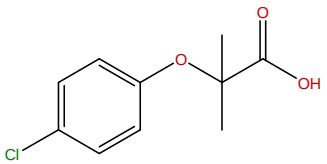
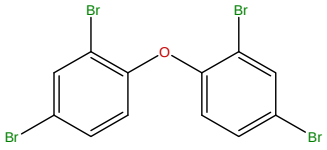
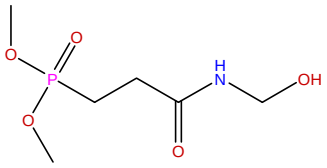
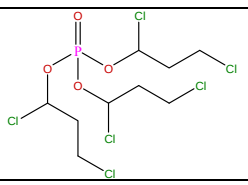
	P110	Endrin	II	G	4	Insecticide
	P111	Toxaphene	II	H	4	Insecticide
	P112	Tedion	II	C	4	Insecticide
	P113	Thiodan	II	A	5	Insecticide
	P114	4,4'-Dichloro-a-(trichloromethyl)-benzhydrol	II	B	5	Insecticide
	P115	Methoxychlor	II	C	5	Insecticide
	P116	Baythroid	II	D	5	Insecticide
	P117	a-Cypermethrin	II	E	5	Insecticide
	P118	d-(*cis*/*trans*) Phenothrin	II	F	5	Insecticide
	P119	Resmethrin	II	G	5	Insecticide

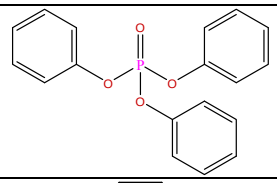
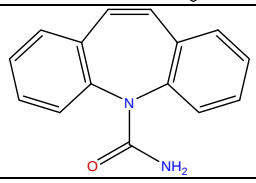
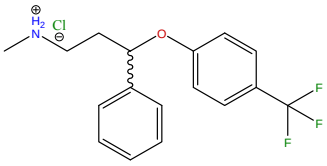
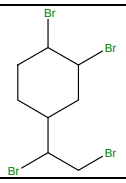
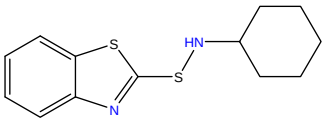
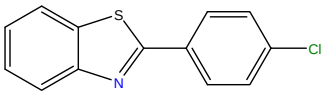
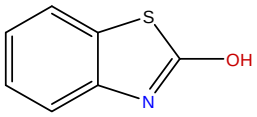
	P120	Bifenthrin	II	H	5	Insecticide
	P121	Asana	II	A	6	Insecticide
	P122	*zeta*-Cypermethrin	II	B	6	Insecticide
	P123	Deltamethrin	II	C	6	Insecticide
	P124 A	Pyrethrum	II	D	6	Insecticide
	P124 B	Pyrethrum	II	E	6	Insecticide
	P125	Cypermethrin (mix of isomers)	II	F	6	Insecticide
	P126	*trans*-Cypermethrin	II	G	6	Insecticide
	P127	Sanmarton	II	H	6	Insecticide
	P128	*cis*-Cypermethrin	II	A	7	Insecticide

	P129	Oxyfluorfen	II	B	7	Herbicide
	P130	Diuron	II	C	7	Herbicide
	P131	Diflubenzuron	II	D	7	Insecticide
	P132	Monuron	II	E	7	Herbicide
	P133	Fenuron	II	F	7	Herbicide
	P134	3,4,4'-Trichloro-carbanilide (Trichlocarban)	II	G	7	Microbiocide
	P135	1-Naphthalene acetamide	II	H	7	Plant growth Regulator
	P136	1-Naphthalene acetic acid	II	A	8	Plant growth Regulator
	P137	1-Naphthaleneacetic acid, methyl ester	II	B	8	Plant growth Regulator
	P138	Chloracetic acid	II	C	8	Herbicide

	P139	2,2-Dichloropropionic acid	II	D	8	Herbicide
	P140	Trichloroacetic acid	II	E	8	Herbicide
	P141	2-Naphthoxyacetic acid	II	F	8	Plant Growth Regulator
	P142	Phenoxyacetic acid	II	G	8	Plant Growth Regulator
	P143	2-Phenoxy propionic acid	II	H	8	Plant Growth Regulator
	P144	3-Indolebutyric acid	II	A	9	Plant Growth Regulator
	P145	Gibberellic acid	II	B	9	Plant Growth Regulator
	P146	N-*m*-Tolyl-phthalamic acid	II	C	9	Plant Growth Regulator
	P147	*o*-Dichloro-benzene	II	D	9	Insecticide
	P148	*p*-Dichloro-benzene	II	E	9	Insecticide

	P149	Naphthalene	II	F	9	Insecticide Exhaust pollutant
	P150	1-Nitro-naphthalene	II	G	9	Exhaust pollutant
	P151	Siduron	II	H	9	Herbicide
	P152	Irgasan (Triclosan)	II	A	10	Microbiocide
	P153	Finasteride	II	B	10	Anti-androgen
	P154	Clomipramine	II	C	10	Anti-depressant
	P155	Anthracene	II	D	10	Insecticide Exhaust pollutant
	P156	DEHP	II	E	10	Plasticizer
	P157	BHA	II	F	10	Food additive
	P158	BHT	II	G	10	Food additive

	P159	Bisphenol A	II	H	10	Plastic monomer
	P160	Triton X-100	II	A	11	Detergent
	P161	SDS	II	B	11	Detergent
	P162	Phenanthrene	II	C	11	Exhaust pollutant
	P162	Tween - 20	II	D	11	Detergent
	P163	n-Dodecyl phosphoric acid	II	E	11	Detergent
	P164	Clofibric acid	II	F	11	Lipid regulator
	P165	PBDE-47	II	G	11	Flame retardant
	P166	Pyrovatex CP	II	H	11	Flame retardant
	P167	Amgard CJ	II	A	12	Flame retardant

	P168	Triphenyl phosphate	II	B	12	Flame retardant Plasticizer
	P169	Carbamazepine	II	C	12	Anti-convulsant
	P170	Fluoxetine HCl	II	D	12	Anti-depressant
	P171	1,2-Dibromo-4-(1,2-dibromo-ethyl)cyclohexane	II	E	12	Flame retardant
	P172	N-Cyclohexyl-2-benzothiazyl sulfenamide	II	F	12	Fungicide
	P173	2-(4-Chloro-phenyl)-benzothiazole	II	G	12	Fungicide
	P174	2-Hydroxy-benzothiazole	II	H	12	Fungicide

Supplemental Material, Figure 1: Positive hits map from primary screening of 9 enzymes activities and 5 receptors bioassay.

Plate I	2	3	4	5	6	7	8	9	10	11	12
A			1	1				1			
B				1						1	
C					1		1			1	1
D				1	1		2	2		3	
E							1	1			
F							2		1		2
G											
H							2				

Plate II	2	3	4	5	6	7	8	9	10	11	12
A					1				4		
B		1							2		4
C		2									
D			1	1	2						
E	1			1	1				1		
F	1		1								1
G						1					1
H								1	1		

Supplemental Material, Figure 2: Determination of the K_I of triclosan with the Human CES1 Using CMNA as Substrate. For each substrate concentration (5 to 100 μM), the velocity is plotted as a function of triclosan concentration (0 to 1000 nM), allowing the determination of an apparent inhibition constant (K_{Iapp}). K_{Iapp}s are plotted as a function of the substrate concentration (insert). For [S] = 0, a K_I value of 103 nM was found.