Title
Toxicology in the fast lane: application of high-throughput bioassays to detect modulation of key enzymes and receptors.

Permalink
https://escholarship.org/uc/item/338430qf

Journal
Environmental health perspectives, 117(12)

ISSN
0091-6765

Authors
Morisseau, Christophe
Merzlikin, Oleg
Lin, Amy
et al.

Publication Date
2009-12-01

DOI
10.1289/ehp.0900834

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

Christophe Morisseau,1 Oleg Merzlikin,1 Amy Lin,1 Guochun He,2 Wei Feng,3 Isela Padilla,3 Michael S. Denison,2 Isaac N. Pessah,3 and Bruce D. Hammock1

1Department of Entomology and Cancer Center, and 2Department of Environmental Toxicology, University of California at Davis, Davis, California, USA; 3Department of Veterinary Medicine Molecular Biosciences, School of Veterinary Medicine, University of California at Davis, Davis, California, USA

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 dichloro, 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.

KEY WORDS: bioassays, biomarkers, enzyme inhibition, high-throughput assays, triclocarban, triclosan. Environ Health Perspect 117:1867–1872 (2009). doi:10.1289/ehp.0900834 available via http://dx.doi.org/ [Online 31 July 2009]

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 has 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 Helzby 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...
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 paraxoax 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 microsomal 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 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 Ki 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 (E2), 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 E2 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/Cl pH 7.0, 25 mM | Fluorescence kinetic | Jones et al. 2005 |
| CES1   | Recombinant partially purified enzyme | CMNA     | 50                 | Na2PO4, pH 7.4, 0.1 M | Fluorescence kinetic | Shan and Hammock 2001 |
| CES2   | Recombinant partially purified enzyme | CMNA     | 50                 | Na2PO4, pH 7.4, 0.1 M | Fluorescence kinetic | Shan and Hammock 2001 |
| CES3   | Recombinant partially purified enzyme | CMNA     | 50                 | Na2PO4, pH 7.4, 0.1 M | Fluorescence kinetic | Shan and Hammock 2001 |
| FAAH   | Recombinant microsomes | Octanoyl-MP | 50                 | Na2PO4, pH 7.4, 0.1 M | Fluorescence kinetic | Shan and Hammock 2001 |
| PON2   | Recombinant microsomes | CMNA     | 50                 | Na2PO4, pH 7.4, 0.1 M | Fluorescence kinetic | Shan and Hammock 2001 |
| GSTs   | Pooled human liver cytosol | CDNB     | 1,000              | K2HPO4, pH 6.5, 0.1 M | Absorbance kinetic | Habig et al. 1974 |
| CYP450 1A2 and 2C6 | Pooled human liver microsomes | EROD     | 25                 | K2HPO4, pH 7.4, 0.1 M | Fluorescence kinetic | Dutton et al. 1989 |
| CYP450 2C9 | Pooled human liver microsomes | Luciferin | 5                  | K2HPO4, 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 acetate; CMNPC, cyano(6-methoxy-naphthalen-2-yl)methyl carbamate; EROD, ethoxyresoruﬁn; Octanoyl-MP, N-(6-methoxyxypirin-3-yl) methyl carbonate; EROD, ethoxyresoruﬁn; Octanoyl-MP, N-(6-methoxyxypirin-3-yl) methyl carbonate.

### Table 2. Conditions for cell-based bioassays.

| Human receptor | Acronym | Preparation used | Substrate | End point measured | Reference |
|----------------|---------|------------------|-----------|--------------------|-----------|
| 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 | [3H]Ry | Radioactivity | Pessall et al. 1987 |
| Rya

*Table 1 continued...*

| Human receptor | Acronym | Preparation used | Substrate | End point measured | Reference |
|----------------|---------|------------------|-----------|--------------------|-----------|
| Androgen receptor | AR | Recombinant cells | Luciferin | Luminescence | Rogers and Denison 2000 |
| Estrogen receptor | ER | Recombinant cells | Luciferin | Luminescence | Rogers and Denison 2000 |
| Rya

*Table 2 continued...*
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 (IC₅₀) was determined by measuring enzyme activities in the absence and presence of increasing concentrations of inhibitor (ranging from 0.5 to 10,000 nM). IC₅₀ 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 (EC₅₀) 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 [³H]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 [³H] 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 I (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 [carbofenthion, 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 lowering 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 | S/B* | S/N* | Z* | No. of positive results | Primary screen | Counterscreen |
|------------------|--------|--------|--------|--------------------------|----------------|----------------|
| Enzyme | | | | | | |
| sEH | 4.0 ± 0.1 | 38 ± 8 | 0.8 ± 0.1 | | 2 | 2 |
| CES1 | 11 ± 3 | 19 ± 2 | 0.9 ± 0.1 | | 4 | 2 |
| CES2 | 0.2 ± 0.9 | 108 ± 33 | 0.8 ± 0.1 | | 4 | 2 |
| CES3 | 8.1 ± 0.7 | 28 ± 7 | 0.7 ± 0.1 | | 7 | 4 |
| FAAH | 150 ± 10 | 35 ± 5 | 0.8 ± 0.1 | | 0 | — |
| PON1 | 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 |

*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 phosphotriothioate, this is likely due to traces of oxim 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 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$^3$-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 signaling transcription was activated by $o,p'$-DDE (dichlorodiphenyltrichloroethane) and its metabolites $o,p'$-DDE and $p,o'$-DDD (dichlorodiphenyl-dichloroethylene) 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 ($o,p'$-DDE 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$^3$-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 that of 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

| Assay | Compound | IC$_{50}$ or EC$_{50}$ (nM) | Use |
|-------|----------|--------------------------|-----|
| sEH   | Siduron  | 32 ± 1                   | Herbicide |
|       | TCC      | 13 ± 1                   | Microbiocide |
| CES1  | Triclosan| 210 ± 20                 | Microbiocide |
| CES2  | Carbophenothion | 43 ± 3 | Flame retardant |
|       | TCC      | 580 ± 30                 | Microbiocide |
| CES3  | Carbophenothion | 110 ± 15 | Insecticide |
|       | Parathion| 4.9 ± 0.4                | Insecticide |
|       | Phosdrin | 11 ± 1                   | Insecticide |
|       | Primiphos-ethyl | 180 ± 20 | Insecticide |
| PON2  | Carbophenothion | 110 ± 6 | Insecticide |
|       | Tributyl phosphorothioate | 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 |
|       | Fenaridine | 1,500 ± 100 | Antianadren |
|       | 2-Methylheptyl-4,6-dinitrophenyl crotonate | 120 ± 1 | Pesticide |
|       | Pentachlorophenol | 850 ± 10 | Herbicide |
|       | Pyrethrum | 2,300 ±100 | Insecticide |
|       | Triclosan | 650 ± 40 | Microbiocide |
| AhR   | CPB      | 11,400                   | 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 |

*Values are IC$_{50}$ for the enzyme-based assays (sEH to CYP450 2C9) and EC$_{50}$ 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 [3H]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 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 [3H]Ry binding to RyR1 was increased almost 3-fold by chlorpyrifos and α,p′-DDE. Countercurrent 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 enzyme 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 RyR by chloranil and dichloro-1,4-quinone. 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 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.

**References**

Ahn KC, Zhao B, Chen J, Cherednichenko G, Sammarti E, Denison MS, et al. 2008. In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. Environ Health Perspect 116:1203–1210.

Beetham JK, Tian T, Hammock BD. 1993. cDNA cloning and expression of a soluble epoxide hydrolase from human liver. Arch Biochem Biophys 305:197–201.

Bellinger AM, Mongillo M, Marks AR. 2008. Stressed out: the skeletal muscle ryanodine receptor as a target of stress. J Clin Invest 118:445–453.

Berridge MJ. 2006. Calcium microdomains: organization and function. Cell Calcium 40:405–412.

Bondefeld-Jørgensen EC, Long M, Hofmeier MV, Vinggaard AM. 2007. Endocrine-disrupting potential of bisphenol A, bisphenol A dimethylacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. Environ Health Perspect 115(suppl 1):89–97.

Cali JJ, Ma D, Sobol M, Simpson DJ, Frackman S, Good TD, et al. 2006. Luminogenic cytochrome P450 assays. Expert Opin Drug Metab Toxicol 2:439–445.

California Department of Toxic Substances Control. 2008. California Green Chemistry Initiative Final Report. Available: http://www.dtsc.ca.gov/PollutionPrevention/GreenChemistryInitiative/upload/GREEN_Chem.pdf [accessed 8 June 2009].

Casida JE, Quistad GB. 2005. Serine hydrolase targets of organophosphorus toxicants. Chem Biol Interact 157–158:277–283.

Chen CW, Kurd C, Vorojekina DP, Arnold SF, Notides AC. 1997. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem Pharmacol 53:1161–1172.

Denison MS, Heath-Pagliuso S. 1998. The Ah receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. Bull Environ Contam Toxicol 61:557–568.

Denison MS, Nagy SR. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol. 43:309–334.

Dixon M. 1972. Graphical determination of Km and Vmax. Biochem J 129:197–202.

Dutton DR, Parkinson A. 1989. Reduction of 7-alkoxyresorufins by NAPDH:cytochrome P450 reductase and its differential effects on their O-dealkylation by rat liver microsomal cytochrome P450. Arch Biochem Biophys 269:17–29.

European Chemicals Agency. 2007. REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals. Available: http://echa.europa.eu/reach_en.asp [accessed 8 June 2009].

Feng W, Liu G, Xia R, Abramson JJ, Bassan IH. 1999. Site-selective modification of hyperreactive cysteines of ryanodine receptor complex by quinones. Mol Pharmacol 55:821–831.
Gad SC. 2006. Introduction. In: Animal Models in Toxicology, 2nd ed (Gad SC, ed). New York:Informa HealthCare, 1–18.

Garrison PM, Tulis K, Aarts JM, Brouwer A, Giesy JP, Denison MS. 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. Fundam Appl Toxicol 30:194–203.

Gibbs S. 2008. Toxicity testing in the 21st century: a vision and a strategy. Reprod Toxicol 25:138–138.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130–7139.

Han D, Naspy SR, Denison MS. 2004. Comparison of recombinant cell bioassays for the detection of Ah receptor agonists. Biofactors 20:11–22.

Harada T, Nakagawa Y, Wadkins RM, Potter PM, Wheelock CE. 2009. Comparison of benzil and trifluoromethyl ketone (TFK)-mediated carboxylesterase inhibition using classical and 3D-quantitative structure-activity relationship analysis. Bioorg Med Chem Med 17:149–164.

Huang H, Nishi K, Tsai H-J, Hammock BD. 2007. Development of highly sensitive fluorescent assays for fatty acid amide hydrolase. Anal Biochem 363:12–21.

James RW. 2006. A long and winding road: defining the biological role and clinical importance of paracoxons. Clin Chem Lab Med 44:1052–1059.

Jones PD, Wolf NM, Morisseau C, Whetstone P, Hock B, Barlow JM, Nebel DA. 2009. Characterization of pyrethroid hydrolysis by the human liver carboxylesterases hCE-1 and hCE-2. Arch Biochem Biophys 485:115–123.

Kåre-Olafsdóttir SG, Tuomi K, Korkalainen M, Raunio H. 1989. Animal Models in Toxicology. 2nd ed (Gad SC, ed). New York:Informa HealthCare, 1–18.

Kahlenberg MD, Pessah IN, Durie EL, Schiedt MJ, Zimani I. 1990. Anthraquinone-based endocrine disruptors: development of stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. In Vitro Mol Toxicol 3:67–82.

Kärenlampi SO, Tuomi K, Korkalainen M, Raunio H. 1989. Abnormal auditory cortex development in PCB exposed rats. Proc Natl Acad Sci USA 104:7646–7651.

Klintsova AY, et al. 2006. Purkinje cell and cerebellar morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. J Cell Biol 99:875–885.

Kreyling WG, Meinhardt R, Jelinek J, Haller S, Bobbitt J, Hecker M, Collas P, Dauth J. 1999. Potential urea and carbamate inhibitors of soluble epoxide hydrolases. Proc Natl Acad Sci USA 96:8849–8854.

Kreppel L, Santer L, Gerber M, Huber C, Moosmayer S, Hinrichs R, Engel J, Singer M. 2006. Characterization of pyrethroid hydrolysis by the human liver carboxylesterases hCE-1 and hCE-2. Arch Biochem Biophys 455:115–123.

Labban J, Bensaid M, Meklat S, Daradoumis T, El Shabrawi J, Charara F, Girard M, Sbhi M, Gueddache S. 2009. Interactions between estrogenic and anti-estrogenic compounds in human breast cancer cells. Cancer Res 66:1494–1501.

Laughren MP, Desikan V, Tani S, Koyama T, Maruyama S, Morita H. 2000. Evaluation of a green fluorescent protein-based cell bioassay for the rapid and inexpensive detection and characterization of AhR agonists. Toxicol Sci 65:200–210.

Mackay CR, Satterfield NG, Spaink HP. 2002. Development of a green fluorescent protein-based cell bioassay for the rapid and inexpensive detection and characterization of AhR agonists. Toxicol Sci 65:200–210.

National Center for Biotechnology Information. 2009. PubChem. Available: http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=707 [accessed 3 November 2009].

Nishi K, Huang H, Kamita SG, Kim I-H, Morisseau C, Hammock BD. 2006. Characterization of pyrethroid hydrolysis by the human liver carboxylesterases hCE-1 and hCE-2. Arch Biochem Biophys 455:115–123.

Nishino K, Wixtrom RN, Silva MH, Hammock BD. 1988. Affinity purification of cytosolic epoxide hydrolase and application to inhibition studies. Anal Biochem 170:66–75.

Ogg JN. 1997. The criteria for the acceptance of Ah-receptor agonists. Fundam Appl Toxicol 36:135–148.

Pestana PM, Branco BC, Pessah IN, Durie EL, Schiedt MJ, Zimani I, Källberg G, Ekberg O, Friesen EH, Schayevitz JR. 2005. Inhibition of cytochrome P450IA1 in a human and a mouse cell line. Toxicol Sci 84:594–609.

Pessah IN, Durie EL, Schiedt MJ, Zimani I. 1990. Anthraquinone-based endocrine disruptors: development of stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. In Vitro Mol Toxicol 3:67–82.

Saito A, Seiler S, Chu A, Fleischer S. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. J Cell Biol 99:875–885.

Sanborn JR, et al. 1999. Potent urea and carbamate inhibitors of soluble epoxide hydrolases. Proc Natl Acad Sci USA 96:8849–8854.

Shan G, Hammock BD. 2001. Development of sensitive esterase assays based on alpha-cyano-containing esters. Anal Chem 73:54–62.

Silliman CC, Wang M. 2006. The merits of in vitro versus in vivo modeling in investigation of the immune system. Environ Toxicol Pharmacol 21:123–134.

Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben-Jonathan N, et al. 1996. Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. Cancer Res 56:5403–5409.

Tingle MD, Heisby NA. 2006. Can in vitro drug metabolism studies with human tissue replace in vivo animal studies? Environ Toxicol Pharmacol 21:184–190.

U.S. Environmental Protection Agency. 1998. High Production Volume (HPV) Challenge Program. Available: http://www.epa.gov/HPV/ [accessed 8 June 2009].

Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. 2008. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. Environ Sci Technol 42:75–95.

Vandenberg LN, Silva MH, Hammock BD. 1988. Affinity purification of cytosolic epoxide hydrolase using derivatized epoxy-activated Sepharose gels. Anal Chem 60:71–80.

Yang D, Kim KH, Phimister A, Bastetetter A, Ward T, Stackman R, et al. 2009. Developmental exposure to polychlorinated biphenyls (PCBs) interferes with experience-dependent dendritic plasticity and tyrosine receptor expression in weaning rats. Environ Health Perspect 117:426–435.

Zhang J, Chung TDY, Oldenburg KR. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen 4:67–73.