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Avoiding False Positives and Optimizing Identification of True Negatives in Estrogen Receptor Binding and Agonist/Antagonist Assays

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Abstract

The potential for chemicals to affect endocrine signaling is commonly evaluated via *in vitro* receptor binding and gene activation, but these assays, especially antagonism assays, have potential artifacts that must be addressed for accurate interpretation. Results are presented from screening 94 chemicals from 54 chemical groups for estrogen receptor (ER) activation in a competitive rainbow trout ER (rtER) binding assay and a trout liver slice vitellogenin mRNA expression assay. Results from true competitive agonists and antagonists, and inactive chemicals with little or no indication of ER binding or gene activation were easily interpreted. However, results for numerous industrial chemicals were more challenging to interpret, including chemicals with: (1) apparent competitive binding curves but no gene activation, (2) apparent binding and gene inhibition with evidence of either cytotoxicity or changes in assay media pH, (3) apparent binding but non-competitive gene inhibition of unknown cause, or (4) no rtER binding and gene inhibition not due to competitive ER interaction but due to toxicity, pH change, or some unknown cause. The use of endpoints such as toxicity, pH, precipitate formation, and determination of inhibitor dissociation constants (K_i) for interpreting the results of antagonism and binding assays for diverse chemicals is presented. Of the 94 chemicals tested for antagonism only two, tamoxifen and ICI-182780, were found to be true competitive antagonists. This report highlights the use of two different concentrations of estradiol tested in combination with graded concentrations of test chemical to provide the confirmatory evidence to distinguish true competitive antagonism from apparent antagonism.

Keywords

estrogen receptor; antagonism; agonism; competitive binding; trout; vitellogenin

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INTRODUCTION

Regulatory initiatives such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) within the European Union (<http://echa.europa.eu/regulations/reach>), and those addressed by the Endocrine Disruptor Screening Program (EDSP) within the US EPA (<http://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-program-edsp-overview>) have led to increased efforts towards using *in vitro* assays to evaluate the endocrine disrupting and hazard potential of chemicals. This approach can provide increased efficiency in time and cost compared to standard whole animal toxicity testing for evaluating the activity of large numbers of chemicals. Within the context of assessing chemicals for endocrine disrupting activity, the potential for chemicals to disrupt estrogenic and androgenic activity via interaction with the respective nuclear hormone receptors has been an initial focal area.

Competitive binding assays are commonly used to identify chemicals that can directly interact with hormone receptors and potentially modulate normal endocrine hormone signaling. These assays, in combination with cell or tissue culture-based assays to determine whether the chemical-receptor interaction leads to induction of a response (agonism), or reduction of a response (antagonism), provides confirmation of the chemicals' receptor-mediated activity. Accurate interpretation of the data from these assays is essential for subsequent decisions regarding prioritization of these chemicals for further testing. When analyzing the results of *in vitro* assays there are several factors that could compromise or affect interpretation of the data. A number of these have been reported previously, such as time in assay, method of chemical addition, and cross contamination or "chemical creep", among others.¹ These are concerns common to the development of most any *in vitro* assay.

In general, interpretation of agonist type responses is relatively simple. For these assays, agonism is identified by the increase in a reporter gene or endogenous response endpoint of the receptor-mediated pathway. Assessment of agonist activity of a chemical is relatively straightforward because a series of coordinated cellular events must take place following ligand binding to the receptor, for the gene of interest to be expressed or protein to be made. This assumes there is no potential for direct chemical interference with the assay output to give a false-positive response, as could occur for example with fluorescent-based reporters and fluorescent test chemicals. A lack of response in a receptor-mediated assay indicates that the system is not being activated; presumably because the ligand is not binding to the receptor. This also assumes that there is no background toxicity or interference due to the chemical that is limiting the response of the biological system.

Receptor-mediated antagonism however, is potentially more difficult to interpret because non-receptor-mediated chemical effects could impact any of the series of cellular events necessary for gene expression. For example, a loss of cell viability or transcriptional viability would produce the same reduced mRNA expression endpoint as receptor-mediated antagonism. Identification of antagonism is usually conducted by testing the ability of a chemical to inhibit or reduce the response of the assay when stimulated with a single concentration of model agonist that will produce a near-maximal or sub-maximal response. Antagonism is then identified by a test chemical concentration-dependent decrease in

activity when the assay system is stimulated by this level of agonist. Because the antagonism assay is designed to measure a decrease in signal, there are several potential confounding factors that can obscure the true response of the system and cause the signal to decrease by mechanisms not directly related to a competitive interaction between agonist chemical or endogenous hormone, and a test chemical.²

In the present study the problem of how to detect true competitive estrogen receptor (ER) antagonism from other mechanisms leading to reduced response in an ER-mediated gene expression assay was addressed. Rainbow trout-based rtER competitive binding and precision cut liver slice Vtg mRNA expression assays were used as optimized to avoid misidentification of potentially active chemicals by testing chemicals up to the solubility limit of the chemical.³ We demonstrate how factors inherent in the properties of test chemicals or the assay system itself can result in misleading antagonism-like response curves.

Limitations of antagonism assays where chemicals were tested in combination with only one concentration of 17 β -estradiol (E2) are evaluated and contrasted to chemical dose-responses with two inducing concentrations of E2. Similarity in responses in rtER binding and Vtg mRNA expression assays in antagonism mode were used to sort the 94 chemicals into nine response categories. This type of information, along with additional data, is applicable to defining effects-based chemical categories to build an ER expert system (ERES) for predicting ER binding potential for large numbers of structurally diverse chemicals.^{4,5}

MATERIALS AND METHODS

Fish.

Immature Erwin strain rainbow trout (*Oncorhynchus mykiss*) used for hepatic cytosol ER preparation and production of liver slices, were received from the US Geological Survey, Upper Midwest Environmental Science Center (LaCrosse, WI) and maintained at US EPA in Duluth, MN as previously described.³

Chemicals.

The 94 chemicals tested for antagonist activity along with the acronyms used to identify the chemicals, their Chemical Abstract Services Registry Number (CASRN), source, and purity are listed in Table 1. These chemicals are a subset of those tested in development of the ERES in which chemicals were assigned to effects-based chemical categories based upon structural similarity and activity in trout ER competitive binding and ER-dependent trout liver slice assays.⁵ These chemicals were selected for testing for antagonism activity for a number of reasons including (1) they showed displacement in the binding assay but no agonist activity, and there was no information on antagonism for other chemicals in the ERES chemical category to which the chemicals were preliminarily assigned, (2) to verify activity when there was published data indicating they were ER antagonists, or (3) the chemical was selected as a representative of its ERES chemical category, and therefore, both ER-mediated agonist and antagonist activity were determined.

Chemicals were tested at concentrations up to those at which an effect was observed, toxicity was noted, or the apparent solubility in assay media was exceeded based on indication of precipitation by visual inspection or as detected by nephelometry (Nepheloskan Ascent, Thermo Electron Corp., Vantaa, Finland). The physiochemical property calculator SPARC (ARChem, Danielsville, GA, USA) was used to calculate pKa values of selected chemicals.

Estrogen receptor binding assay.

Rainbow trout hepatic cytosolic estrogen receptors (rtER) were prepared and used in competitive binding assays measuring displacement of [³H]-E2 as previously described.³ Each test chemical was assayed in at least two separate cytosol preparations.

Rainbow trout liver slice Vtg mRNA expression assay: Agonism and antagonism.

Precision cut liver slices from immature male rainbow trout were exposed to test chemicals for 48h at 11°C in a multi-well plate system, and assayed for expression of Vtg mRNA as previously described using quantitative real time RT-PCR technology.^{3,6}

Chemical stocks prepared in ethanol were diluted to appropriate test concentrations in phenol red-free L-15 media (Gibco, Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum, and penicillin (100 U/mL)/streptomycin (100 µg/mL) (Sigma Aldrich, Columbia, MO, USA). A test for agonist activity included exposure of individual liver slices to E2 at six concentrations with two replicates per concentration to produce a reference dose-response curve. Ethanol solvent controls and six graded concentrations of test chemicals with five replicate slices per concentration were used to determine activity of the chemical. A minimum of two agonist tests were performed on each chemical. If a test chemical lacked agonist activity it was further tested at least once for antagonist activity.

The antagonist test in single E2 concentration mode was run with parallel ethanol solvent controls, E2 positive controls, and co-treatment with graded concentrations of test chemical with a concentration of E2 that on its own would be predicted to give a sub-maximal Vtg mRNA expression response (i.e., typically -9 log M E2). In the antagonist test mode using two E2 concentrations, a sub-maximal E2 concentration (i.e. typically -9 log M E2) and an E2 concentration predicted to produce maximal Vtg mRNA expression (i.e. typically -6 log M E2) were used.

Assay media conditions and liver slice viability.

To address potential factors that could affect this assay response, media conditions including pH and osmolality were monitored using a Radiometer Blood Gas Analyzer ABL800 Flex (Radiometer Medical, Copenhagen, Denmark). Precipitation or chemical insolubility in the slice media was determined by both visual inspection and detection of light scatter with a nephelometer. Slice viability was routinely determined by measuring leakage of lactate dehydrogenase enzyme (LDH) from the liver slices into the media. The activity of LDH in the media was measured following the 48 h incubation and compared with total slice LDH activity in the slices at 0 h as previously described.^{6,7} The mitochondrial dehydrogenase assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) as

substrate was also used to assess cytotoxicity in the liver slices with a few selected chemicals.⁸

Data analysis.

GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA) was used for curve fitting and statistical analysis. Competitive binding curves were generated and IC50 values determined using nonlinear regression curve fit and the one-site competitor equation. Vtg mRNA expression produced by chemical tested in agonist mode was compared to that of solvent control. Chemical antagonism of E2-induced Vtg mRNA expression was determined by comparison to the level of Vtg mRNA produced by the corresponding E2 concentration on its own. ANOVA analysis with a Dunnett's multiple comparison test and two-tailed t-tests were used to determine significant responses in the slice assay. When Vtg mRNA responses had unequal variances, the data were analyzed by Kruskal-Wallis and differences between treatment group and controls determined by Dunn's post hoc test.

Antagonism assay data interpretation.

A hypothetical E2 response curve is shown in Fig. 1A. Two hypothetical outcomes for chemicals tested for ER antagonism with two of the E2 concentrations selected from the E2 dose-response curve are shown in Fig. 1B and 1C. In Fig. 1B, seven concentrations of test chemical "X" from -9 to -3 log M were used in co-treatment with a low E2 (-9 log M) or with high E2 (-7 log M) concentration. Inhibition of the ER-mediated Vtg mRNA expression requires higher concentrations of chemical "X" to successfully compete with a higher E2 concentration (-7 log M) at the ER and antagonize the response, therefore the antagonism curve for chemical "X" with high E2 is shifted to the right compared to the curve produced with chemical "X" and low E2 (-9 log M). The shift in the curve is two log units which is equal to the difference in concentration of the agonist used (-7 and -9 log M E2). Thus, competitive antagonism of the ER receptor should produce characteristic response curves that are shifted when two different concentrations of E2 are tested in combination with graded concentrations of test chemical.

If the inhibition of ER-mediated Vtg mRNA expression is not due to true ER antagonism, there will not be a shift in the antagonism curves equivalent to the difference in the E2 concentrations. In Fig. 1C the inhibition of the estrogenic response occurs at the same concentration of chemical "Y", irrespective of the concentration of the co-administered E2. In this example, the loss in Vtg mRNA expression signal is not likely due to competitive displacement of E2 from the ER, because it is independent of E2 concentration.

RESULTS

An analysis of the cytosol rER competitive binding and Vtg mRNA expression assay results for the 94 diverse chemicals revealed different types of response outcomes in these paired assays. The response types fell within nine groupings shown in Table 1. Binding and Vtg mRNA expression results for representative chemicals demonstrating the response types of

each group are presented below. Results for chemicals listed in Table 1 that are not included in the examples presented below are available in a Supplemental Data file available online.

ER Binding and Vtg mRNA expression response of true competitive antagonists.

The known mammalian ER antagonists, tamoxifen (TAM) and ICI-182,780 (ICI), were used to validate the antagonism methodology. Both chemicals fully displaced [³H]-E2 from the rtER in the competitive binding assay. The slopes of the displacement curves were parallel to that of E2 indicating competition for the ER (Fig. 2A and 2C). Their relative potencies compared to E2 were different, with ICI exhibiting rtER affinity identical to E2, and TAM having over two orders of magnitude lower affinity. Both TAM and ICI were tested in the liver slice Vtg mRNA expression assay for antagonist responses. Figure 2B and 2D show results for TAM and ICI in the antagonism assays, respectively; with each tested against two concentrations of E2.

As expected (see Antagonism assay data interpretation in METHODS), higher concentrations of TAM or ICI were needed to produce an antagonist effect when tested against the higher E2 co-exposure concentration. For example, when graded concentrations of TAM were tested with $-9 \log M$ E2 a significant decrease in Vtg mRNA expression in the slices occurred at $-7 \log M$ TAM, dropping to near background levels at -6 , -5 , and $-4 \log M$ TAM. However, when TAM was tested in the presence of $-5 \log M$ E2, significant antagonism of the E2-induced Vtg expression did not occur until TAM concentration reached $-4 \log M$ or higher (Fig. 2B). Comparison of Panels B and D in Figure 2 also show that the further apart the high and low E2 concentrations are in the antagonism assays, the easier it is to discern the shift in the antagonism curves expected with true ER antagonism.

If instead, the significant decrease in Vtg expression occurred at the same TAM or ICI concentration for both -9 and $-5 \log M$ E2 co-exposures then true receptor antagonism would not be indicated (as example in Fig. 1C). Of the 94 chemicals tested in Table 1 for antagonism only TAM and ICI were found to be true competitive antagonists. Dual binding and gene activation assay response types that do not fit the profile of true antagonism with additional parameter measurements used in interpreting assay results are presented below.

Response of true ER non-binders

4-Diethylamino-azobenzene (DAA) provides an example of a negative chemical in both the cytosol rtER binding (Fig. 3A) and Vtg mRNA assays (Fig. 3B). Similar chemicals falling into the B.1 and B.2 response groups in Table 1 produced no displacement or less than 50% displacement of [³H]-E2 from the ER in the binding assay, and no induction or inhibition of Vtg mRNA expression in the slice assay run in agonism and antagonism modes. Thirty seven of the 94 chemicals tested fell into this dual assay response category.

These 37 chemicals represent significant structural diversity as members of a wide variety of chemical classes from all 8 of the ERES primary model nodes,⁵ including acyclics, chemicals with multiple cyclic rings, charged chemicals, neutrals, and a wide range of log Kow values from -6.7 to 8.2 (Table 2). Of these 37 chemicals, 26 produced 0 to 20% displacement in the liver cytosolic receptor binding assay with the other 11 chemicals producing only 20 to 50% displacement (Table 1, groups B.1 and B.2). These chemicals also

showed no indication of cytotoxicity in the slices as measured by LDH, and no significant change to the media pH (data not shown).

False positive binding with no effect on gene expression

Twelve of the 94 chemicals tested in the paired assays produced seemingly contradictory results in that they produced complete or near complete displacement in the cytosolic rtER binding assay, but no agonist or antagonist activity in the Vtg mRNA assay (Table 1, group C.1). In the case of tetrabromophenol blue (BPB) the apparent displacement occurred at concentrations in which a precipitate was observed which may have interfered with ER binding assay (Fig. 4). When the concentrations at which precipitate were noted are excluded, then only 55% displacement was achieved. This along with the lack of agonist or antagonist activity leads to the conclusion that BPB does not bind to the rtER. Precipitate formation in the cytosol is a possible explanation for the displacement seen in the cytosolic rtER binding assay for five chemicals (BPB, FPB, 5CIeth, ECD, and 43CLN) of the twelve in this group (see Supplemental Data online).

In other cases of binding displacement without altered Vtg mRNA expression, if no precipitation occurred to explain the apparent binding, then further binding experiments were carried out to determine the K_i to verify if displacement was due to competitive binding. For example, N-(3-carboxy-1-oxo-3-sulfopropyl)-N-octadecyl-DL-aspartic acid (AER) fully displaced [3 H]-E2 from the receptor (Fig. 5A) but gave no indication of activation or inhibition of Vtg mRNA expression. This suggested either a false positive binding result or AER was metabolically inactivated in the slice (Fig. 5B). If the K_i determination assay confirmed competitive binding to the ER this might suggest the chemical is metabolically inactivated in the slice to a metabolite that does not bind to the ER. Further analysis of binding to determine the K_i indicated that AER was not a competitive binder (Fig. 5C), and the lack of Vtg mRNA response in the slice assay was not due to toxicity as determined by the LDH leakage assay (Fig. 5D). Therefore, AER is classified as a non-binder.

AER along with three other structurally similar chemicals (DBS, SOS, and DSS; see Supplemental Data) would contain a net charge at assay pH and temperature based upon their calculated pKa (0.30, 0.83, and 0.0 at 11°C for DBS, SOS, and DSS, respectively). As charged chemicals they can non-competitively disrupt the normal E2-ER interaction resulting in the displacement of [3 H]-E2 from the receptor. For three other chemicals in this group (octyl-beta-d-glucopyranoside, 1-decanol, and alpha-terpineol) that produced apparent binding with no changes in Vtg mRNA expression, the very steep binding curves suggest something other than competitive binding was responsible for displacement of [3 H]-E2 from the receptor.

Inhibition of gene expression due to toxicity.

Chemical-induced cytotoxicity resulted in the inhibition of Vtg mRNA expression in the liver slices for 18 of the 94 chemicals tested in the antagonist mode. Within this group there were seven chemicals that exhibited minor to full displacement in the cytosolic rtER binding assay (Table 1, group C.2) and the other 11 showed no displacement (Table 1, group D.1).

For some of these chemicals an antagonist test using a single E2 concentration was sufficient to reach a conclusion regarding activity. For example, chemicals that produced little or no E2 displacement in the cytosolic rER binding assay and inhibited E2-induced Vtg mRNA expression at a concentration of test chemical that also produced toxicity, was indication that the decreased Vtg mRNA expression was due to toxicity and not true antagonism.

More often chemicals tested against a single E2 concentration in antagonist mode needed additional testing such as with the time- and reagent-intensive K_i analysis to reach a conclusion about activity. An example is octylbenzenesulfonic acid (OBS). OBS fully displaced [^3H]-E2 from the receptor (Fig. 6). In the liver slice Vtg mRNA assay it did not induce Vtg mRNA expression when tested for agonist activity, but when tested in the presence of $-9 \log \text{ M E2}$ there was apparent antagonism at $-3 \log \text{ M OBS}$. The LDH assay indicated cytotoxicity at this concentration, so the decreased Vtg mRNA expression at $-3 \log \text{ M OBS}$ in presence of $-9 \log \text{ M E2}$ was likely due to toxicity in the slices and not competitive antagonism by OBS. To verify this conclusion OBS was tested to determine the K_i . The results indicated that OBS did not competitively bind to the trout ER (Fig. 6C). OBS is also an example of a charged chemical at the assay pH, similar to AER presented above.

It became apparent that using two concentrations of E2 in the antagonist experiments was an effective and efficient tool for confirming true competitive antagonism from other processes that inhibited Vtg mRNA expression. For example, chlorhexidine diacetate (CDS) produced supra-maximal displacement curves in the cytosolic rER binding assay, however, the four highest concentrations caused a visible precipitate to form in the ER binding assay cytosol/buffer mix (Fig. 7A). CDS produced no agonist activity in the liver slice Vtg mRNA induction assay when tested alone, but did inhibit gene expression when tested in the antagonist mode. Significant inhibition of the Vtg mRNA response occurred with the four highest CDS concentrations when co-exposed to either -9 or $-5 \log \text{ M E2}$; therefore, the inhibition was not due to an E2-dependent mechanism. Significant toxicity was observed at the two highest concentrations of CDS tested in the antagonist mode. It was concluded that CDS was not competitively binding to the rER because the displacement observed in the binding assay was the result of precipitation, and the inhibition in the antagonist assay was likely due to cytotoxicity.

Of the seven chemicals that had complete binding curves and produced toxicity in the liver slice Vtg mRNA assay, two (HOI and EAD) showed precipitation in the binding assay at concentrations that caused displacement, three (OBS, DBS, and ODA) were charged chemicals, and for PAS and DiPrP the cause of apparent binding was unknown (see Supplemental Data online).

Another example of a seemingly conflicting result was that of diiodomethyl-p-tolysulfone (DTS) which had very little, to no displacement in the binding assay, but reduced Vtg mRNA expression in the slice assay (Fig. 8). The inhibition of Vtg expression was independent of the E2 concentration and solely dependent on the DTS concentration, indicating the apparent inhibition was not an ER-mediated mechanism (Fig. 8B). Measurement of LDH leakage from the slices revealed toxicity at the same concentration at which Vtg expression was reduced; therefore, the inhibition of gene expression was not ER

antagonism, but more likely due to cytotoxicity or other DTS-related effects. DTS and ten other chemicals had this response profile (Table 1, group D.1). Three chemicals (BHP, BCY, and EAD) showed no indication of toxicity measured by the LDH assay but were found to be toxic using the MTT assay with liver slices from the same experiments (see Supplemental Data).

Test chemical mediated pH changes.

Another assay parameter that can affect binding and gene expression is media pH. As concentration of chemical increases, the capacity of the assay medium to buffer the pH of the solution can become overwhelmed resulting in a change in the pH of the assay. To determine the acceptable pH range of the binding and vitellogenin slice assays HCl or NaOH were added to the assays to adjust the pH. There is a fairly narrow pH range over which the ER binding and Vtg mRNA expression assays are operable. When the pH of the binding assay is outside of the pH range of pH 7 – 9 the [³H]-E2 begins to dissociate from the receptor (Fig. 9B). This occurs in the absence of any competing ligand. The decrease in pH also resulted in observed precipitates in the binding assay at HCl concentrations of –1.7, –2.0 and –2.3 log M (Fig. 9A). These points corresponded to pH values of 2.9, 4.6, and 6.3, respectively (Fig. 9B).

The expression of Vtg mRNA in the liver slices was as sensitive, and perhaps, even slightly more sensitive to decreased pH than the ER binding assay. Figure 10 displays how decreasing the media pH with HCl or increasing it with NaOH affects E2-responsive Vtg mRNA expression. The pH of the media used in the trout liver slice assay is normally near pH 7.6. There was a substantial decrease in Vtg mRNA expression with only a modest decrease in pH, whereas there was no decrease in signal when pH was increased to pH 8.6. Thus, a test chemical that alters the pH of the slice media to slightly more acidic pH could be interpreted falsely as antagonism due to the pH-dependent decrease in E2-induced Vtg mRNA expression. Furthermore, this demonstrates why determination of pH was critical to accurately interpret the Vtg mRNA response when antagonism was suspected.

Ten of the 94 chemicals tested for antagonism were found to have significantly lowered the pH of the rtER binding and liver slice Vtg mRNA expression assays which adversely affected assay performance (Table 1, group D.2). The apparent competitive binding curve of 2,6-pyridinedicarboxylic acid (PDC) in Fig. 11A and apparent antagonism in the liver slice assay in Fig. 11B would at first glance suggest that PDC was capable of interacting with the rtER to displace [³H]-E2 and antagonize the activation by E2 in the slice assay. However, measurement of the pH in binding and slice assay media indicated significant acidification when the PDC concentration approached –3 log M. These same PDC concentrations produced displacement of [³H]-E2 from the receptor in the binding assay and decreased vitellogenin mRNA induced by –8 log M E2 in the slice assay. Thus the apparent ER binding and antagonism by PDC was an artifact of low pH and likely due to pH-associated protein denaturation or cytotoxicity.

For some of the chemicals that lowered the pH of the assay this could be anticipated because these chemicals are acids; for example, phosphoric acid diphenyl ester (PHOS), propylphosphonic acid (PPA), heptafluorobutyric acid (PFBA) and the carboxylic acid PDC

described above. The calculated pKa of these chemicals at the assay temperature of 11°C provides an alert to this potential to lower the pH. The pKa of PDC is 3.92, PHOS is -0.37, PPA is 2.75, and PFBA is -0.11. The two nitrophenols that also lowered the pH, 2-methyl-4,6-dinitrophenol (MDP) and 4-nitrophenol (4NP), had pKa values of 4.52 and 6.74 respectively. At the pH of the liver slice assay 100% of MDP and 90% of the 4NP in solution are calculated to be in ionized form. Two other chemicals in this group do not have a calculated pKa, but in aqueous solution hydrolyze to produce acids: 2,6-dimethyl-1,3-dioxan-4-ol acetate (DDA) hydrolyzes to acetic acid and the corresponding alcohol,⁹ and 1,3-dichloro-5,5-dimethylhydantoin (DDH) hydrolyzes to produce hypochlorous acid.¹⁰

Unknown reason for decrease but not true ER interaction

Fifteen of the 94 chemicals tested for antagonism against two E2 concentrations had a decrease in Vtg mRNA expression but the decrease occurred at the same test chemical concentration regardless of the E2 concentration; therefore, the decrease in Vtg mRNA induction was likely not mediated by competitive interaction with the ER (Table 1, groups C.3 and D.3). No apparent explanation for the inhibition was determined because no precipitation was present, the media pH was within the acceptable range and no toxicity was detected by LDH analysis. However, use of a different cell cytotoxicity assay may have shown decreased viability (e.g., MTT) at lower concentrations than that indicated by LDH which requires cell membrane leakage to be detected. These chemicals may have also interfered with Vtg mRNA expression via some mechanism that would not be detected by a cytotoxicity assay.

Five chemicals that produced non-competitive Vtg mRNA inhibition exhibited apparent binding with greater than 50% displacement binding curves (Table 1, group C.3). Two of these chemicals, CNA and DBM, only produced 55 and 60% displacement, respectively, prior to precipitation occurring with increasing concentration. Therefore, the observed displacement may have been related to factors leading to precipitate formation. Based upon the pKa of BRO, it was charged at assay pH, which likely contributed to the apparent displacement of E2 from the receptor if protein denaturation of the ER occurred. The other two chemicals in this set, DMO and pMxBA, produced 70 and 75% displacement curves, respectively, but the cause of the displacement of E2 from the ER was unclear.

Ten of the chemicals in this group had 0 to 50% displacement in the binding assay (Table 1, group D.3). One of these chemicals, benzotriazole (BZOL), was found by Harris et al. to be an ER antagonist in the *in vitro* recombinant yeast estrogen assay using a single E2 concentration method but they observed no antagonist activity when tested *in vivo* in the fathead minnow (*Pimephales promelas*).¹¹ We found BZOL to produce only 45% displacement of E2 from the cyto rtER (Fig. 12A) suggesting BZOL does not bind the rtER; but in light of the Harris et al. report we also tested BZOL in the Vtg mRNA expression assay.

No agonist activity was observed in the trout liver slice Vtg mRNA induction assay but a decrease in Vtg mRNA expression was observed (Fig. 12B). This inhibition occurred at the same concentrations of BZOL when tested with either -9 or -6 log M E2, therefore it was interpreted not to be true competitive antagonism. The media pH was within an acceptable

range, no precipitation was noted in the media, and no toxicity was apparent from LDH analysis. In this case the reason for the inhibition is unknown although it is possible toxicity did occur but was not detected using the LDH method. Three chemicals, BHP, BCY, and EAD, had no indication of toxicity measured by the LDH assay but were found to be toxic using the MTT assay with liver slices from the same experiments.

DISCUSSION

Relatively few chemicals have been determined to be true ER antagonists outside of pharmaceuticals or others specifically designed for this activity. The chemical features often associated with antagonism of the ER such as a structural bulky side-chain,¹² suggests that the number of chemicals that would have the potential to act as antagonists would be limited. Therefore, in the course of testing a diverse group of chemicals to develop a rule-based expert system using the rainbow trout ER-based competitive binding and trout liver slice Vtg mRNA induction assay,⁵ it was surprising to find a relatively high proportion of chemicals that inhibited E2-induced Vtg mRNA expression.

Of the 267 chemicals tested for agonist activity in the trout liver slice assay 94 underwent further testing for antagonist activity, and of these there were 45 that significantly decreased the E2-induced Vtg mRNA expression at one or more concentrations of test chemical. However, only two of these chemicals, the known ER antagonists TAM and ICI,^{13,14} produced a true competitive inhibition response in the liver slice assay and therefore were identified as antagonists. The remaining 43 chemicals did not produce a true antagonist response in the liver slice assay.

Thirty-one of these 43 that inhibited Vtg mRNA expression produced no binding activity in the cytosol rER binding assay which provided strong evidence that the decreased Vtg mRNA induction was not due to competition between parent chemical and E2 for binding to the ER. For nine of the remaining twelve chemicals that did produce ER binding displacement curves, the apparent binding could be attributed to precipitation, altered pH, or the chemical had an ionic charge at assay pH. Of the 94 chemicals tested for antagonism, there were 7 classified as “Ionic acyclics” from Node I of the ERES and 10 classified as “Belongs to a known charged group” from Node II (Table 2). None of these bound ER, supporting the hypothesis that ionic characteristics of chemicals can be incompatible with ER binding and activation.

For the remaining five chemicals which exhibited some binding displacement and inhibition of E2-induced Vtg mRNA expression, the decreased Vtg expression occurred at the same concentration(s) of test chemical regardless of the concentration of E2 used in the assay. This again indicated the inhibition of E2-induced Vtg mRNA expression was not true competitive antagonism. But unlike the seven chemicals previously mentioned, the decreased gene expression for these five chemicals was unexplained because it was not associated with other factors such as precipitate formation, an unacceptable shift in pH, or LDH leakage from the liver slice indicative of cytotoxicity. Thus, in our assessment of this relatively large set of structurally diverse chemicals, the only two that could be confidently

considered as true ER antagonists were TAM and ICI, suggesting that true competitive antagonism of the rtER is uncommon.

Making a positive identification of a true loss of signal response, whether from a competitive binding assay, enzyme inhibition assay, or antagonism assay can be supported by additional assays to rule out non-receptor mediated activities that would result in a decrease in signal. For example, a determination of K_i when conducting competitive binding assays does provide verification of competitive binding activity where it is in question,¹⁵ but it is a very intensive exercise in both the amount of receptor consumed and the time required. Assessing parameters such as changes in pH or observations of precipitation can provide information that eliminates the need for K_i determinations. Although in some cases as shown above for AER, when potential artifacts cannot be identified to explain apparent binding or apparent antagonism, an experiment to determine K_i may be required to obtain a definitive answer.

Reports in the literature of assessment of ER antagonist activity of industrial or commercial use chemicals of environmental interest similarly indicate that only a limited number of chemicals appear to exhibit this activity. Only 3 percent of 200 pesticides tested by Kojima et al. exhibited antagonism activity using a human ER α - and ER β -activated reporter gene in a Chinese hamster ovary (CHO) cell line.¹⁶ Additionally Takeuchi tested 100 hydroxylated polychlorinated biphenyls in reporter gene assay using CHO cell line and found 12% exhibited antagonist activity toward hER α or hER β .¹⁷ Work by Kramer et al. on the estrogenic and anti-estrogenic activity of hydroxylated polychlorinated biphenyls (PCBs) showed that of those that were apparent anti-estrogens, most were cytotoxic at the concentrations that inhibited the estrogen response, and only two produced antagonism responses that did not occur at concentrations also producing cytotoxicity.¹⁸ In an assay of a small set of phenolic-based chemicals Yamasaki found only one of the 10 showed antagonist activity in the hER α -based reporter gene assay.¹⁹ High-throughput screening of the Tox21 library of 10,000 chemicals using two different cell-based assays identified approximately 4% of the tested chemicals as true antagonists.²⁰ Additional chemicals were identified as potential antagonists but they were assigned as inconclusive antagonists due to potential assay artifact interferences or cytotoxicity.

Yet there are reports of greater proportions of chemicals analyzed acting as ER antagonists. For example, Kolle et al tested 88 chemicals, including 28 literature negatives and 60 literature agonists/antagonists in a validation exercise using the yeast estrogenic screening (YES) assay.²¹ Their literature review identified 34% (30 of 88) of the chemicals as estrogen antagonists and experimentally determined 26% (23 of 88) were antagonists. In light of the reported low incidence of antagonism from multiple reports and knowledge of potential artifacts that can lead to false identification of antagonism, a high incidence of antagonist activity should be viewed with caution. In the case of all of these studies in which antagonism activity was assessed at only a single concentration of E2, further confidence in the classification of a chemical as an antagonist could be obtained by testing against a second concentration of E2.

Testing chemicals for antagonist activity against dual E2 concentrations can provide an additional level of confidence for categorizing the activity of a chemical as an ER antagonist.

The use of two concentrations of positive control ligands, whether 5 α -dihydrotestosterone or estradiol when assessing chemicals for androgen receptor (AR) or ER antagonism, respectively, has been incorporated in experimental designs by others;^{22,23,24} however, it is far from common in the published literature. For gene expression assays additional cytotoxicity assays could be included to monitor different cytosolic functions. These include detection of cellular ATP or MTT assays that can identify mitochondrial functional integrity;^{9,25} or assays such as the propidium iodide assay that like LDH measures membrane integrity;²⁶ or those that determine cytosolic enzyme activity such as the calcein-based cytosolic esterase activity assays.²⁷ With any of these cytotoxicity assays there is always some uncertainty whether the endpoint of the assay will capture the type of toxicity or off-target mechanism targeted by the test chemical that would produce the loss of signal response.

Alternatively, selecting a suite of cytotoxicity assays may provide greater confidence in confirming or ruling out cytotoxicity, however there can be uncertainty in knowing how many or which suite of cytotoxicity assays are sufficient to have confidence that a loss in signal is due to cytotoxicity. Other approaches for identifying antagonists in high throughput screening have included developing network models of estrogen activity from a suite of *in vitro* assays and assessing the activity patterns across all of the assays to identify potential effects not likely mediated via the ER.²⁸ In any of these efforts to assess chemicals for potential antagonist activity in hormone receptor systems, utilization of a second concentration of model agonist could help verify antagonist activity of test chemicals.

CONCLUSIONS

The data presented above are the results of efforts to address the potential for industrial and commercial use chemicals to have estrogenic activity via binding to, and subsequent activation or inhibition of E2-mediated gene expression via the rainbow trout ER. In this project in which a structure-activity approach was taken to assign chemicals into effects-based chemical categories and develop a rule-based expert system,^{3,5} correctly interpreting the apparent antagonistic responses was critical for assigning chemicals as ER Binders or Non-binders. The quality of the resulting data using this approach was recommended by an EPA Science Advisory Panel as a “gold-standard” dataset for chemicals prioritization of the defined EPA industrial chemical lists under study.²⁹

The use of multiple lines of evidence to indicate when toxicity, chemical precipitation, media pH, or other factors resulted in displacement of the [³H]-E2 from the receptor and/or inhibition of Vtg mRNA expression which was not ER-mediated, is essential to avoid incorrect labeling of chemicals as ER Binders. Using two concentrations of E2 tested in combination with graded concentrations of test chemical was critical for an accurate assessment of apparent antagonist activity for many chemicals. Separation of the two E2 concentrations by at least 2 log M units is recommended to provide sufficient separation in E2 activation response to confidently identify a shift in antagonist response curves.

Rainbow trout livers were the source material for the estrogen receptor (ER) binding and estrogen-responsive gene expression assays but the concepts are applicable to *in vitro* or *ex*

vivo assays utilizing any freshly isolated tissues or cultured cells. Utilizing the methods described above can help to avoid false positives due to potential artifacts inherent in the test system and increase confidence in negative determinations. Thus, as the focus on in vitro gene activation assays increases as a tool for screening chemicals for potential antagonist activity these approaches can go a long way toward optimizing the use of in vitro results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Curves depicting theoretical outcomes from ER-mediated Vtg mRNA expression assay conducted in antagonist mode showing test chemical concentration-response in the presence of a low or a high E2 agonist concentration. Panel A: The E2 concentration-response curve for Vtg expression ($\blacksquare$) is shown with open symbols highlighting the low E2 (Δ ; -9 log M) and high E2 ($\square$; -7 log M) concentrations used in the antagonist assays in panel B and C. The bar from -9 to -7 log M indicates the two log unit dose separation between the low and high E2 concentrations used in the antagonist assays. Panel B: The expected concentration-response curves when a chemical "X" exhibiting true competitive antagonism is tested with low (Δ) and high ($\circ$) E2 concentrations. Declining Vtg mRNA expression curves are separated by the same two log units of concentration difference (bar with dotted line), as the difference in concentration range between the low and high E2 levels, indicating true chemical competitive antagonism at the ER. Panel C: Concentration-response curves are shown for chemical "Y" which produces decreasing Vtg mRNA expression via some mechanism that is not true competitive antagonism. The declining E2-induced Vtg mRNA expression for chemical "Y" with low E2 (Δ) and "Y" with high E2 ($\circ$) occurs at the same concentration of "Y" (-6 log M in this example) and is independent of the E2 co-treatment level; thus the decreased expression is due to a cell/tissue slice response to an alternative mechanism from "Y" competing with E2 to bind and inactivate ER-mediated Vtg expression.

Figure 2.

Activity of the two known ER antagonists, tamoxifen (TAM) and ICI, in rtER competitive binding and trout liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for estradiol (■) and TAM (▲). Symbols represent mean & SEM of three replicate binding determinations. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve is shown by solid symbols with dashed line; mean & SD of n=2 technical replicates for E2), and graded concentrations of TAM with -9 log M E2 (◇) or -5 log M E2 (▽) (mean & SD, n=3 technical replicates for TAM).

Circled symbol on TAM with $-9 \log M$ E2 curve ($\diamond$) is the data point at which Vtg mRNA expression is significantly reduced compared to $-9 \log M$ E2 alone (circled symbol on E2 curve). Boxed symbol on TAM with $-5 \log M$ E2 curve (∇) is data point at which Vtg mRNA expression is significantly reduced compared to $-5 \log M$ E2 alone (boxed symbol on E2 curve). Panel C: Competitive binding curves for E2 ($\blacksquare$) and ICI ($\blacktriangle$). Symbols represent mean & SEM of three replicate binding determinations. Panel D: Vtg mRNA expression in slices exposed to ethanol solvent control ($\blacktriangle$), E2 alone is shown by solid symbols and dotted line, and graded concentrations of ICI with $-8 \log M$ E2 ($\triangle$), or $-7 \log M$ E2 ($\circ$) (mean & SD of $n=4$ technical replicates). Circled and boxed symbols highlight ICI with E2 data points where Vtg mRNA is significantly reduced compared to the same concentration of E2 alone, as similarly described above for panel B.

Figure 3.

Activity of 4-diethylamino-azobenzene (DAA) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for E2 positive control (■) or DAA (◆). Symbols represent mean and SEM of two replicate binding runs. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●) or DAA alone (◆) in ER agonism test, and for the antagonist test solvent control (○), E2 alone (concentration-response curve shown as solid symbols with dashed line), and graded concentrations of DAA with -6 log M E2 (▽) or with -9 log M E2 (△). Panel C: Determination of LDH leakage into media from slices exposed to ethanol (●) or DAA (◆). LDH was determined in media from the same test wells as Vtg mRNA expression antagonism runs in panel B.

Figure 4.

Activity of tetrabromophenol blue (BPB) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for E2 positive control (■) or BPB (%). Symbols represent mean and SEM of three replicate binding runs. Vertical dashed line indicates concentrations at and above which precipitates were observed. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve shown by solid symbols with dashed line), BPB alone tested for agonism response (%), or graded concentrations of BPB with -5 log M E2 (◆) or graded concentrations of BPB with -9 log M E2 (▽). Panel C: Determination of LDH leakage into media from slices exposed to ethanol (●), E2 (■), or BPB (▲). LDH was determined in media from same test wells as Vtg mRNA expression antagonism run in panel B.

Figure 5.

Activity of DL-aspartic acid, N-(3-carboxy-1-oxo-3-sulfopropyl)-N-octadecyl-, tetrasodium salt (AER) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for E2 positive control (■) or AER (◆). Symbols represent mean & SEM of three replicate binding runs. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve shown as solid symbols with dashed line), AER alone (%), or graded concentrations of AER with $-8 \log M$ E2 (Δ). Symbols represent mean and SD (n=5 technical reps) from one experimental run. Panel C: Ki assay slope replots did not exhibit a linear relationship

characteristic of competitive binding. Symbols represent means of technical duplicates from one Ki assay. Panel D: Cytotoxicity determination of LDH leakage into media from slices exposed to ethanol (●) or AER (◆). LDH was determined in media from same test wells as AER Vtg mRNA expression antagonism run in panel B.

Figure 6.

Activity of octylbenzenesulfonic acid (OBS) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for E2 positive control (■) or OBS (◆). Symbols represent mean & SEM of two replicate binding runs. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve shown as solid symbols and dashed line), OBS alone tested in agonist mode (◆), or antagonism test with graded concentrations of OBS with -9 log M E2 (▽). Symbols represent mean and SD of n=5 technical replicates from one experiment.

Panel C: K_i determination slope replot showing non-linear relationship indicating non-competitive binding. Panel D: Determination of LDH leakage into media from slices exposed to solvent control (●) or OBS (◆).

Figure 7.

Activity of chlorhexidine diacetate salt, dihydrate (CDS) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for E2 positive control (■) or CDS (◆). Symbols represent mean & SEM of two replicate binding runs. Vertical dashed line indicates concentrations at and above which precipitates were observed. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve is indicated by solid symbols and dashed line), CDS alone in agonist mode (◆), and graded concentrations of CDS with -5 log M E2 (Δ) or with -9 log M E2 (▽). Symbols represent mean and SD of n=5 technical replicates from single experiments. Panel C: Determination of LDH leakage into media from slices exposed to ethanol (●) or CDS (◆). LDH was determined in media from same test as Vtg mRNA expression antagonism runs in panel B.

Figure 8.

Activity of diiodomethyl-p-tolysulfone (DTS) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curves for E2 positive control (■) or DTS (◆). Symbols represent mean & SEM of three replicate binding runs. Vertical dashed line indicates concentration at and above which precipitates were observed. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve is shown by solid symbols and dashed line), DTS alone in agonism mode (◆), and graded concentrations of DTS in antagonism mode with $-5 \log \text{M}$ E2 (Δ) or with $-9 \log \text{M}$ E2 (▽). Symbols represent mean and SD of $n=5$ technical replicates from single experiments. Panel C: Determination of LDH leakage into media from slices exposed to ethanol (●) or DTS (◆). LDH was determined in media from same test as antagonism runs in panel B.

Figure 9.

Effect of changing pH on estradiol binding to cytosolic rtER. Panel A: Competitive binding curve for E2 positive control at normal test pH of 7.4 (■) and apparent binding curves with addition of HCl (▲) or NaOH (◇). Panel B: Effect of decreased pH (HCl addition) or increased pH (NaOH addition) on dissociation of [³H]-E2 from the rtER. Un-shaded area between pH 7 and 8.8 indicates acceptable pH range of the competitive binding assay.

Figure 10.

Effect of pH on Vtg mRNA expression in liver slices. Liver slices were treated for 48h with $-6 \log M$ E2 ($1 \mu M$) to achieve maximum Vtg mRNA induction. The responses from two separate experiments are shown. Slices were exposed to solvent control (●) at normal assay pH, or to $-6 \log M$ E2 at normal assay media pH (Δ), or pH adjusted with HCl or NaOH ($\blacktriangle$).

Figure 11.

Activity of 2,6-pyridinedicarboxylic acid (PDC) in cytosol competitive binding and Vtg mRNA expression assays. Panel A: Competitive binding curve for E2 (■) and PDC apparent binding curve (◆). Symbols represent mean & SEM of two replicate binding runs. Vertical dashed line indicates concentration at and above which precipitates were observed. Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve shown by solid symbols and dashed line), PDC alone (◆) in agonist mode, or graded concentrations of PDC tested in antagonist mode with -8 log M E2 (Δ). Symbols represent mean and SD of n=5 technical replicates from one experiment. Panel C: pH of binding assay matrix at 20h is shown for ethanol solvent control (●) and PDC (▲). Panel D: pH of slice assay media at 0h for ethanol solvent control (●) and PDC (▲).

Figure 12.

Activity of benzotriazole (BZOL) in cytosol competitive binding and liver slice Vtg mRNA expression assays. Panel A: Competitive binding curve for E2 (■) and BZOL apparent binding curve (◆) (mean and SEM of $n=2$ experiments). Panel B: Vtg mRNA expression in slices exposed to ethanol solvent control (●), E2 alone (concentration-response curve shown with solid symbols and dashed line), graded concentrations of BZOL tested in antagonism mode with $-6 \log \text{ M E2}$ (Δ) or graded concentrations of BZOL with $-9 \log \text{ M E2}$ (▽); (mean and SD of $n=5$ technical reps from one experiment). Panel C: Determination of LDH leakage into media from slices exposed to ethanol (●) or BZOL (▲). LDH was determined in media from same test as Vtg mRNA expression antagonism runs in panel B.

Table 1.

Chemicals tested in antagonism assays sorted by their similarity in responses in the rtER competitive binding and liver slice Vtg mRNA expression assay.

Chemical Name	Acronym	Fig. ^a	CASRN	Source ^b	% ^c
A.1 True Antagonists: Complete binding, E2 sensitive antagonism					
Tamoxifen	TAM	Fig. 2	10540291	SA	99
ICI-182,780	ICI	Fig. 2	129453618	TOC	99
B.1 Non-Binder: 0–20% binding displacement, no effect on Vtg mRNA expression					
4-Diethylamino-azobenzene	DAA	Fig. 3	2481949	AA	98
Dehydroacetic acid	DHAA		520456	SA	98
Fluorescein sodium salt	FSS		518478	SA	98
1,2,4-Tribromobenzene	124TBB		615543	SA	95
Methyl 2-methylbenzoate	MMB		89714	SA	99
N-Cyclohexyl-2,2-dichloro-n-methylacetamide	NCD		39085182	SA	NR
6-O-Palmitoyl-L-ascorbic acid	PAA		137666	SA	99
2-Ethylhexyl 4 (dimethylamino) benzoate	EDAB		21245023	SA	98
D-Gluconic acid delta-lactone	GAGL		90802	SA	99
2,4-Hexadienoic acid	SBA		110441	SA	99
2-Diethylaminoethyl –4 aminobenzoate hydrochloride	ABH		51058	AA	99
5-tert-Butyl-4-hydroxy-2-methylphenyl sulfide	BHS		96695	SA	96
Piperonal	PIP		120570	SA	99
4-Methylbenzaldehyde	pMBA		104870	SA	97
2,4,6-Tris(dimethylaminomethyl) phenol	TDAP		90722	SA	95
N-Ethyl-p-toluenesulfonamide	NEMB		80397	SA	98
Acid yellow 23	AYE		1934210	SA	90
Trimethylborate	BOR		121437	SA	98
n-Hexanophenone	HXP		942927	SA	99
Isoamylbenzoate	IAB		94462	SA	98
Sodium 2-ethylhexylsulfate	SHE		126921	SA	50
Ethyl phenylglycidate	EPG		121391	SA	92
Mefenpyr-diethyl	MFD		135590919	SA	100
6-Hydroxy-2-naphthyl disulfide	NTH		6088513	SA	98
4-Aminobenzoic acid	PABA		150130	SA	99
Salicylic acid	SAA		69727	SA	99
B.2 Non-Binder: 20–50% binding displacement, no effect on Vtg mRNA expression					
Isophthalic acid	IPHA		121915	SA	99
2-Bromoethyl-benzene	2BB		103639	SA	98
Naphthalene	NAFT		91203	SA	99
2-Amino-4-nitroaniline	25AN		121880	TCI	98

Chemical Name	Acronym	Fig. ^a	CASRN	Source ^b	% ^c
Acetophenone	ACP		98862	SA	99
4-Chloropropylbenzene	CRB		52944340	M	NR
2-Ethyl-1-hexanol	EHX		104767	SA	99
2-Ethylhexyl 2-cyano-3,3-diphenylacrylate	ECDA		6197304	SA	97
Phloxine B	POB		18472872	SA	80
p-Propyl anisole	pPrA		104450	SA	99
2,4,6-Tribromophenol	TBROH		118796	SA	99
<u>C.1 Apparent binding: with no effect on Vtg mRNA expression</u>					
Tetrabromophenol blue	BPB	Fig. 4	115399	SA	95
DL-Aspartic acid, N-(3-carboxy-1-oxo-3-sulfopropyl)-N-octadecyl-, tetrasodium salt	AER	Fig. 5	38916426	SA	NR
Octyl beta-d-glucopyranoside	ObGP		29836268	TCI	98
2-Dodecen-1-yl succinic acid anhydride	DSA		19780111	SA	95
1-Decanol	DL		112301	SA	99
4-Pentyl fluorobenzene	FPB		28593148	S	99
Sodium octylsulfate	SOS		142314	SA	95
alpha-Terpineol	AT		98555	A	99
Pentachloroethane	5ClEt		76017	TCI	96
Ethylenediaminetetraacetic acid copper (II)	ECD		39208156	SA	97
2-Chloro-5-(trifluoromethyl)nitrobenzene	43CLN		121175	TCI	97
Dicyclohexyl sulfosuccinate sodium salt	DSS		23386529	SA	98
<u>C.2 Apparent binding: Vtg expression inhibition due to toxicity</u>					
4-Octylbenzenesulfonic acid sodium salt	OBS	Fig. 6	6149037	SA	97
4-Dodecylbenzenesulfonic acid	DBS		27176870	SA	70
Octadecanoic acid, 9(or 10)-(sulfoxy)-, 1-butyl ester, sodium salt	ODA		42808366	MFG	NR
1,10-Phenanthroline, anhydrous	PAS		66717	AA	99
2,6-Diisopropylphenol	DiPrP		2078548	SA	97
1H-Imidazole-1-ethanol, 2-(8-heptadecenyl)-4,5-dihydro-	HOI		95385	CS	93
5-Ethyl-1-aza-3,7-dioxabicyclo(3.3.0)-octane	EAD		7747355	SA	97
<u>C.3 Apparent binding: Vtg expression inhibition due to unknown cause</u>					
4,4-Dimethyloxazolidine	DMO		51200874	SA	NR
4-Methoxybenzaldehyde	pMxBA		123115	SA	98
Cinnamyl alcohol	CNA		104541	SA	98
4,4'-Isopropylidenebis (2,6-dibromophenol)	BRO		79947	SA	97
Dimethyl benzylidenemalonate	DBM		6626842	SA	NR
<u>D.1 No binding: Vtg expression inhibition due to toxicity</u>					
Chlorhexidine diacetate salt, dihydrate	CDS	Fig. 7	56951	SA	NR
Diiodomethyl-p-tolysulfone	DTS	Fig. 8	20018091	D	NR
8-Hydroxyquinoline hemisulfate salt	8HQ		207386912	SA	98
Benzethonium chloride	BEZ		121540	SA	99

Chemical Name	Acronym	Fig. ^a	CASRN	Source ^b	% ^c
Disulfiram (tetraethylthiuram disulfide)	DSF		97778	SA	97
2-Thiocyanomethylthio benzothiazole	BCY		21564170	CG	NR
4-Chloroaniline	CLAN		106478	SA	99
Tributyl tin benzoate	TBB		4342363	SA	98
1-Phenyl-2-nitropropene	TNS		705602	SA	99
N-Octylpyrrolidone	NOP		2687947	SA	98
2-Bromo-4'-hydroxyacetophenone	BHP		2491385	IFC	97
D.2 No binding: Vtg expression inhibition due to pH change					
2,6-Pyridinedicarboxylic acid	PDC	Fig. 11	499832	SA	99
Phosphoric acid, diphenyl ester	PHOS		838857	SA	99
Pentachlorophenol	PCP		87865	SA	98
2-Methyl-4,6-dinitrophenol	MDP		534521	SA	99
2,6-Dimethyl-1,3-dioxan-4-ol acetate	DDA		828002	SA	85
Propylphosphonic acid	PPA		4672382	SA	95
Perfluorobutyric acid	PFBA		375224	SA	98
1,3-Dichloro-5,5-dimethylhydantoin	DDH		118525	SA	98
4-Nitrophenol	4NP		100027	SA	99
3,5-Dimethylpyrazole-1-methanol	DPM		85264331	SA	99
D.3 No binding: Vtg expression inhibition due to unknown cause					
Benzotriazole	BZOL	Fig. 12	95147	SA	99
Benzyl bromoacetate	BBA		5437456	AA	99
n-Dodecyl gallate	DDG		1166525	AA	98
Isobornyl acetate	IBA		125122	SA	90
2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid	BHMB		4065456	SA	97
Dibutyl fumarate	DF		105759	TCI	98
2(3h)-Furanone, dihydro-5-pentyl-	GNL		104610	SA	98
1-Amino-2-chloro-4-nitrobenzene	24CN		121879	TCI	98
N,N'-Bis(salicylidene)-1,2-propanediamine	PDA		94917	TCI	98
1,3-Diphenyl-1,3-propanedione	DPD		120467	SA	98

^a Figure location in manuscript showing the response of this chemical as an example.

^b SA=Sigma Aldrich, PO Box 14508, St. Louis, MO 63178, USA; TCI= Tokyo Chemical Industry, 9211 North Harborage Street, Portland, OR, 97203, USA; AA=Alfa Aesar, Shore Road, Port of Heysham Industrial Park, Lancashire, LA3 2XY, Heysham, Lancashire, United Kingdom; TOC= Tocris Bioscience, Tocris House, IO Centre, Moored Farm Avenue, Bristol, BS11 0QL, United Kingdom; IFC=Ivy Fine Chemicals, 1879 Old Cuthbert Road, Suite 23, Cherry Hill, NJ 0803, USA; CG= Chemos GmbH & Co. KG, Werner-von-Siemens Str. 3, D-93128 Regensburg, Germany; D= Dow Chemical Co., 2030 Dow Center, Midland, MI, 48674, USA; CS= Chem Service, Inc., 660 Tower Lane, PO Box 599, West Chester, PA 19381-0599, USA; MFG= MFG Chemical Inc., 1804 Kimberly Park Drive, Dalton, GA 30720, USA; A=Acros Organics, Geel West Zone 2, B-2440, Geel, Belgium; S= SynQuest Laboratories, Inc., PO Box 309, Alachua, FL 32616-0309, USA; M= MatrixChemical Inc., 5960 W Parker Rd. Suite 278, #235 Plano, TX 75093, USA

^c Chemical purity % as reported by supplier. NR = not reported.

Table 2.

Chemicals in trout ER binding and Vig mRNA expression activity groups sorted by ER Expert System (ERES) Node, Sub-node, Chemical Group and Sub-groups.

ERES Node & Subnode ^a	ERES Chemical Group	ERES Chemical Sub-group	Chemical Acronym ^b	rtER Binding/Vig mRNA Response Type ^c	Log Kow
I. Acyclics.					
Ionic Acyclics					
	Ionic Non-Perfluoro Acyclics	Acyclic Sulfates	SHE	B.1	-0.35
	Ionic Non-Perfluoro Acyclics	Acyclic Sulfates	SOS	C.1	-0.27
	Ionic Non-Perfluoro Acyclics	Acyclic Sulfates	ODA	C.2	5.09
	Ionic Non-Perfluoro Acyclics	Acyclic Phosphates	PPA	D.2	0.28
	Ionic Non-Perfluoro Acyclics	Acyclic Carboxylic Acids	SBA	B.1	1.62
	Ionic Non-Perfluoro Acyclics	Acyclic Carboxylic Acids	AER	C.1	2.37
	Ionic Perfluoro Acyclics	PerfluoroCarboxylic Acid [LogKow<3.5]	PFBA	D.2	2.43
Non-Ionic Acyclics					
	Acyclic Alcohols	Acyclic Alcohols	EHX	B.2	2.73
	Acyclic Alcohols	Acyclic Alcohols	DL	C.1	3.79
	Acyclic Halogenated	Acyclic Chlorinated	5ClEt	C.1	3.11
	Acyclic Fumarates & Maleates	Acyclic Fumarates	DF	D.3	4.16
	Acyclic Others	Acyclic Others	BOR	B.1	-1.90
	Acyclic Others	Acyclic Others	DSF	D.1	3.67
II. Contains a Charge.					
Belongs to Known Charged Group					
	Anhydrides	Anhydrides	DSA	C.1	6.41
	Charged Functional Groups	Carboxylic Acid Functional Group	PDC	D.2	0.57
	Charged Functional Groups	Carboxylic Acid Functional Group	IPHA	B.2	1.76
	Charged Functional Groups	Carboxylic Acid Functional Group	SAA	B.1	2.24
	Charged Functional Groups	Quat Amine Functional Group	BEZ	D.1	4.00
	Charged Functional Groups	Quat Amine Functional Group	CDS	D.1	4.85
	Alkylaromatic Sulfonic Acids	Alkylaromatic Sulfonic Acids	OBS	C.2	1.03

ERES Node & Subnode ^a	ERES Chemical Group	ERES Chemical Sub-group	Chemical Acronym ^b	rtER Binding/Vtg mRNA Response Type ^c	Log Kow
	Alkylaromatic Sulfonic Acids	Alkylaromatic Sulfonic Acids	DBS	C.2	5.77
	Charged Functional Groups	Sulfonic Acid Functional Group	DSS	C.1	1.76
	Sulfonic Acid Dyes	Sulfonic Acid Dyes	AYE	B.1	-6.74
III. Known Inactive Class:					
Belongs to Known Inactive Group					
	2,6-Dialkylphenols	2,6-Dialkylphenols	DiPrP	C.2	3.57
	4-Alkylfluorobenzenes	4-Alkylfluorobenzene [LogKow 4.8]	FPB	C.1	4.71
	Acetals	Acetals [LogKow 5.5]	DDA	D.2	0.49
	Alcohols	Alcohols [LogKow 3.4]	CNA	C.3	1.84
	Alcohols	Alcohols [LogKow 3.4]	AT	C.1	3.33
	Benzoates (non-ring Subst)	Benzoates (non-ring Subst)[3.3<LogKow<4.3]	IAB	B.1	3.72
	Benzothiazoles	Benzothiazoles [1.9<LogKow<3.2]	BCY	D.1	3.12
	Chloro Nitrobenzenes	Chloro Nitrobenzenes [1.6<LogKow<3.5]	24CN	D.3	2.12
	Chloro Nitrobenzenes	Chloro Nitrobenzenes [1.6<LogKow<3.5]	43CLIN	C.1	3.42
	Cyano Compounds	Cyano Compounds [1.8<LogKow<6.9]	ECDA	B.2	6.88
	Dichloro Acetamide	Dichloro Acetamide [2.3<LogKow<3.2]	NCD	B.1	2.86
	Dimethylamino Phenols	Dimethylamino Phenol [LogKow 1.4]	TDAP	B.1	0.77
	Epoxides	Epoxides [LogKow 3.9]	EPG	B.1	2.55
	Hydrofurans	Hydrofurans [LogKow 2.1]	GNL	D.3	2.08
	Imidazolidines	Imidazolidines [LogKow 0]	DDH	D.2	-0.94
	N,N-Dimethylanilines	N,N-Dimethylanilines [2.7<LogKow<5.8]	EDAB	B.1	5.77
	Nitro Anilines	Nitro Anilines [LogKow 2.2]	25AN	B.2	0.99
	Oxazoles	Oxazoles [LogKow 0.4]	EAD	C.2	0.40
	Phenones (n-alkyl)	Phenones (n-alkyl)[LogKow 4.2]	ACP	B.2	1.67
	Phenones (n-alkyl)	Phenones (n-alkyl)[LogKow 4.2]	HXP	B.1	3.64
	Pyrrolidiones	Pyrrolidiones [LogKow 3.4]	NOP	D.1	3.33
	Quinolines	Quinolines [LogKow 5.3]	8HQ	D.1	-1.50
	Quinolines	Quinolines [LogKow 5.3]	PAS	C.2	2.29

ERES Node & Subnode ^a	ERES Chemical Group	ERES Chemical Sub-group	Chemical Acronym ^b	rtER Binding/Vtg mRNA Response Type ^c	Log Kow
	Sugars	Sugars [LogKow 3.0]	GAGL	B.1	-1.98
	Sugars	Sugars [LogKow 3.0]	ObGP	C.1	2.93
IV. Phenolic OH + 2nd -OH or =O spec. dist					
# of Nucleophilic Sites = 2					
	A_B-type	A_B-type [Atten.]	NTH	B.1	5.70
	A_B-type	A_B-type [Atten.]	BHS	B.1	8.24
	A_C-type	A_C-type [Atten. - Steric Hind.]	BRO	C.3	7.20
	A_B_C-type	A_B_C-type - Antagonist	ICI	A.1	9.09
V. Special Rules Groups					
Tamoxifen-Like Selection Rules					
	Tamoxifen-Like	Tamoxifen-Like [RBA>0.00001%] ^d	TAM	A.1	6.30
Alkyl Halobenzenes Selection Rules					
	4-Alkylchlorobenzenes	Alkylchlorobenzene [LogKow<4.5]	CRB	B.2	4.17
	Bromo- and Alkylbromobenzenes	Bromobenzenes [3.4<LogKow<4.7]	124TBB	B.1	4.66
Anisole Selection Rules					
	Anisoles (a)	Anisoles [RBA<0.00001%]	pMxBA	C.3	1.79
	Anisoles	Anisoles [RBA<0.00001%]	pPrA	B.2	3.60
Benzodioxolane Selection Rules					
	Benzodioxolane	Benzodioxolane (Exact Match) [RBA<0.00001%]	PIP	B.1	1.77
Multicyclic Hydrocarbons Selection Rules					
	Multicyclic Hydrocarbons	Multicyclic Hydrocarbons (Exact Match) [RBA<0.00001%]	NAFT	B.2	3.17
Organometallic Selection Rules					
	Organometallics	Organometallics [LogKow<1.3]	ECD	C.1	-10.42
	Organometallics	Organometallics (Exact Match) [RBA<0.00001%]	TBB	D.1	4.69
Phosphoric Acid Ester Selection Rules					
	Phosphoric Acid Esters	Phosphoric Acid Esters [RBA<0.00001%]	PHOS	D.2	2.88
Sulfonamides Selection Rules					
	Sulfonamides	Sulfonamides (ring subst.)	NEMB	B.1	1.87

ERES Node & Subnode ^a	ERES Chemical Group	ERES Chemical Sub-group	Chemical Acronym ^b	rtER Binding/Vtg mRNA Response Type ^c	Log Kow
VI. Site A, Contains Phenol Fragment					
Belongs to known Active sub-class					
	2-Hydroxy Benzophenones	2-Hydroxy Benzophenones [Atten. - Charged]	BHMB	D.3	0.37
	Fluoresceins	Fluorescein [LogKow<1.3]	FSS	B.1	-4.25
	Fluoresceins	Fluorescein [Atten.- Charged]	POB	B.2	5.47
	Halophenols	Halophenols [# of Halogens 3]	TBROH	B.2	4.18
	Halophenols	Halophenols [# of Halogens 3]	PCP	D.2	4.74
	Gallates	Gallates (Exact Match) [RBA<0.00001%]	DDG	D.3	6.21
	Phenolsulfonphthaleins	Phenolsulfonphthaleins [Atten. - Steric Hind.]	BPB	C.1	6.77
Not a known Active sub-class					
	Mixed Phenol	Mixed Phenol (Exact Match) [RBA<0.00001%]	BHP	D.1	1.54
	Mixed Phenol	Mixed Phenol (Exact Match) [RBA<0.00001%]	4NP	D.2	1.91
	Mixed Phenol	Mixed Phenol (Exact Match) [RBA<0.00001%]	MDP	D.2	2.27
	Mixed Phenol	Mixed Phenol (Exact Match) [RBA<0.00001%]	PDA	D.3	2.52
VII. Site 'B', Contains Specified Fragment					
Belongs to known Active sub-class					
	Mixed Anilines	Mixed Anilines [LogKow<1.3]	ABH	B.1	-0.84
	Aminobenzoates	Aminobenzoates [LogKow<1.3]	PABA	B.1	0.96
	Chloroanilines	Chloroanilines [LogKow<2.3]	CLAN	D.1	1.72
	Aldehydes	Aldehydes [LogKow<2.3]	pMBA	B.1	2.26
	Benzoic Acid, methyl or ethyl ester (ring CH3).	Benzoic Acid, methyl ester (ring CH3)	MMB	B.1	2.38
VIII. Mixed Organics					
Not a known Active sub-class					
	Mixed Organics	Mixed Organics [LogKow<1.3]	DHAA	B.1	-1.31
	Mixed Organics	Mixed Organics [LogKow<1.3]	DMO	C.3	-0.08
	Mixed Organics	Mixed Organics [LogKow<1.3]	DPM	D.2	0.79
	Mixed Organics	Mixed Organics [LogKow<1.3]	BZOL	D.3	1.17

ERES Node & Subnode ^a	ERES Chemical Group	ERES Chemical Sub-group	Chemical Acronym ^b	rtER Binding/Vtg mRNA Response Type ^c	Log Kow
	Mixed Organics	Mixed Organics - Nitro Aromatics - (Exact Match) [RBA<0.00001%]	TNS	D.1	2.49
	Mixed Organics	Mixed Organics - Carboxy Esters - (Exact Match) [RBA<0.00001%]	DBM	C.3	1.95
	Mixed Organics	Mixed Organics - Carboxy Esters - (Exact Match) [RBA<0.00001%]	BBA	D.3	2.42
	Mixed Organics	Mixed Organics - Carboxy Esters - (Exact Match) [RBA<0.00001%]	IBA	D.3	3.86
	Mixed Organics	Mixed Organics - Carboxy Esters - (Exact Match) [RBA<0.00001%]	MFD	B.1	4.82
	Mixed Organics	Mixed Organics (Exact Match) [RBA<0.00001%]	DPD	D.3	2.51
	Mixed Organics	Mixed Organics (Exact Match) [RBA<0.00001%]	2BB	B.2	3.37
	Mixed Organics	Mixed Organics (Exact Match) [RBA<0.00001%]	DTS	D.1	4.03
	Mixed Organics	Mixed Organics (Exact Match) [RBA<0.00001%]	DAA	B.1	5.27
	Mixed Organics	Mixed Organics (Exact Match) [RBA<0.00001%]	PAA	B.1	6.00
	Mixed Organics	Mixed Organics (Exact Match) [RBA<0.00001%]	HOI	C.2	7.51

^aERES = ER Expert System nodes as described in Schmieder et al. 2014 [5].

^bChemical names for these acronyms can be found in Table 1.

^cThese activity groups are defined in this paper and can be found in Table 1.

^dRBA is the relative binding affinity calculated as 100 x (IC50 E2 / IC50 Chemical)