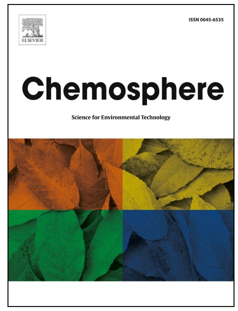

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Combined effects of environmental xeno-estrogens within multi-component mixtures: comparison of *in vitro* human- and zebrafish-based estrogenicity bioassays

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ABSTRACT

Some recent studies showed that *in vitro* bioassays based on fish or human estrogen receptor (ER) activation may have distinct responses to environmental samples, highlighting the need to better understand bioassay-specific ER response to environmental mixtures. For this purpose, we investigated a 12-compound mixture in two mixture ratios (M1 and M2) on zebrafish (zf) liver cells stably expressing zfERα (ZELHα cells) or zfERβ2 (ZELHβ2 cells) and on human ER-reporter gene (MELN) cells. The mixture included well-known ER ligands bisphenol A (BPA) and genistein (GEN), and other compounds representatives of a freshwater background contamination. In this context, the study aimed at assessing the robustness of concentration addition (CA) model and the potential confounding influence of other chemicals by testing subgroups of ER activators, ER inhibitors or ER activators and inhibitors combined. Individual chemical testing showed a higher prevalence of ER inhibitors in zebrafish than human cells (e.g. propiconazole), and some chemicals inhibited zfER but activated hER response (e.g. benzo(a)pyrene, triphenylphosphate). The estrogenic activity of M1 and M2 was well predicted by CA in MELN cells, whereas it was significantly lower than predicted in ZELHβ2 cells, contrasting with the additive effects observed for BPA and GEN binary mixtures. When testing the subgroups of ER activators and inhibitors combined, a deviation from additivity was caused by zebrafish-specific inhibiting chemicals. This study provides novel information on the ability of environmental pollutants to interfere with zfER signaling and shows that non-estrogenic chemicals can influence the response to a mixture of xeno-estrogens in a bioassay-specific manner.

KEY WORDS: estrogenicity, anti-estrogen, mixture, *in vitro* reporter gene, human, zebrafish

1. Introduction

The occurrence of numerous endocrine disrupting chemicals (EDC) in aquatic ecosystems has raised concern over their potential adverse effects in aquatic organisms, such as fish (Sumpter, 2005). Many EDCs, such as natural and synthetic hormones, pesticides or industrial chemicals, are xeno-estrogens, *i.e.* they bind the estrogen receptors (ERs) and subsequently alter the transcription of target genes involved in key physiological functions (Sumpter, 2005). *In vitro* bioassays based on ER transactivation have been used to assess the estrogenic activity of chemicals, but also of environmental samples (Jarošová et al., 2014; Snyder et al., 2001; Zacharewski, 1997). In case of environmental monitoring, they are expected to enable an integrative detection of various ER-active contaminants within complex environmental mixtures considering both known and unknown xeno-estrogens. They provide a unique quantitative response which may be summarized as estradiol-equivalent (E2-Eq, Kase et al., 2018, Jarošová et al., 2014).

To date, a large majority of *in vitro* bioassays used in environmental bio-monitoring are based on mammalian or yeast cell systems that stably express a reporter gene which expression is controlled by the human ER subtype α (hERα) (Könemann et al., 2018; Kunz et al., 2015; Leusch et al., 2010). However, the relevance of using human-based assay to assess hazard and risk for aquatic species is a question of concern in environmental assessment (Hotchkiss et al., 2008). For instance, humans express two ER subtypes, ERα and ERβ, but most teleost fish express at least three ER subtypes, ERα, ERβ1 and ERβ2 (Menuet et al., 2002; Tohyama et al., 2015). Fish and human ER have relatively low sequence homologies in their ligand binding domain (Menuet et al., 2002; Tohyama et al., 2015). These structural differences are believed to contribute to the distinct sensitivity to certain xeno-estrogens (Miyagawa et al., 2014), along with other factors linked to the cell specificities, such as cell metabolic capacities (Le Fol et al., 2015), presence/absence of transcriptional cofactors or cross-talks with other signalling pathways (Navas and Segner, 2000; Ohtake et al., 2003).

In a recent study, we reported that some surface water samples were active on a zebrafish liver cell line stably expressing zebrafish ERβ2 (zfERβ2), the ZELHβ2 cells, but not on human breast cancer MELN cells that endogenously express hERα (Sonavane et al., 2016). Similarly, some effluent extracts from sewage treatment plants produced very different *in vitro* responses in cells expressing human or medaka ERα (Ihara et al., 2014). These differences were further confirmed *in vivo* by measuring vitellogenin induction in exposed male medaka (Ihara et al., 2015). In the latter study, the estrogenic chemicals identified were not sufficient to explain the distinct response of fish bioassays. However, the authors showed that the anti-estrogenic activity measured in the samples may contribute to the different responses of medaka and human ER.

Several studies have addressed the combined effect of ER ligands in reconstituted mixtures, generally concluding on their additive effects based on concentration addition (CA) predictions (Kortenkamp, 2007). However, xeno-estrogens occur in the aquatic ecosystem together with other chemicals that have various and distinct modes of action (e.g. Escher et al., 2014; Neale et al., 2015, Busch et al., 2016). To date, few studies have investigated additive effects of xeno-estrogens in more diverse exposure scenarios, such as with non- or weak estrogenic chemicals (Evans et al., 2012) or with anti-estrogenic chemicals (Yang et al., 2015). Recently, a mixture of 12 selected environmental chemicals was tested in zebrafish and human-based bioassays as part of a larger round-robin study. The aim was to investigate whether the estrogenic activity of the ER ligands in this mixture (e.g. genistein and bisphenol A) was detectable against the background of the other environmental pollutants (Altenburger et al., 2018). This study concluded that in human MELN cells the overall estrogenic activity of the mixtures was accurately predicted by an assumed additivity of the estrogenic chemicals. However, in zebrafish ZELHβ2 cells the measured estrogenic response of the mixture was lower than expected. The reasons of this discrepancy between human and zebrafish-based ER-reporter gene assays were unknown, and therefore raised the question about potential limitations of a presumed CA additivity.

In this context, the present study was designed to investigate the different responses of zebrafish- and human-based *in vitro* reporter gene assays to chemical mixtures. We hypothesized that estrogenic chemicals within environmental mixtures have additive effects following default model of CA that are well detected by zebrafish and human-based bioassays. In such way, we investigated (1) the additivity of xeno-estrogens in zebrafish and human-based bioassays and (2) the influence of non-estrogenic chemicals of the mixtures. As in Altenburger et al. (2018), we used the same 12-compound mixture in two different mixture ratios (M1 and M2), which included xeno-estrogens (e.g. bisphenol A and genistein), and non-estrogenic chemicals representatives of a freshwater contamination background. The general experimental set-up design is outlined in Figure 1. Firstly, each chemical was tested for both estrogenic and anti-estrogenic activities in zebrafish-and human-based bioassays. Secondly, combinations of chemicals that proved to be active at M1 and M2 mixture ratios (either ER activating, ER inhibiting, or both) were tested and then discussed in relation to the outcomes from the 12-component mixture response. The concentration addition model was used to evaluate the additivity of active chemicals in each mixture scenario.

2. Material and methods

2.1 Chemical selection, mixtures design and experimental approach

Twelve environmentally relevant chemicals were selected following (1) a prioritization exercise based on occurrence, hazard and available environmental quality standard (Busch et al., 2016), and (2) a screening of prioritized contaminants through multiple bioassays (Neale et al., 2017a). As a result, two fixed-ratio mixtures of 12 chemicals with dissimilar mode of actions were designed (Table SI-1) and tested as part of a benchmarking exercise (Altenburger et al., 2018). The first mixture ratio (M1) was composed in such way that the diverse bioactivities of the individual chemicals had a chance to be detected experimentally by an array of 19 bioassays. The second mixture ratio (M2) was chosen to mimic a realistic freshwater contamination scenario. In the current study, all 12 chemicals were tested individually for their capacity to induce or inhibit ER-mediated luciferase response in different cellular assays. Based on the information on the activity of individual chemicals in each bioassay, chemicals predicted to contribute to M1 and M2 responses based on CA prediction were identified. Based on toxic unit distribution (as defined in Scholtz et al., 2017), only chemicals expected to contribute to at least 1% of the total response at non-cytotoxic concentrations were selected. Subgroup mixtures were then designed containing either only ER activators or only ER inhibitors, or both ER activators and inhibitors (Figure 1, Table 1). These mixtures were designed such that their relative concentration ratios agreed to that from the original M1 and M2 mixtures (i.e. real sub-mixtures), to allow the best possible comparison to the outcomes from the 12 compound mixtures.

2.2 Chemicals and reagents

17β-estradiol (E2, CAS#50-28-2, purity of >98%), triclosan (TCS, CAS#3380-34-5, purity of 97% - 103%), bisphenol A (BPA, CAS#80-05-7, purity of 97%), genistein (GEN, CAS#446-72-0, purity of > 98%), propiconazole (CAS#60207-90-1, purity of >98%), diclofenac (CAS#15307-79-6), diazinon (CAS#333-41-5, purity of >98%), diuron (CAS#330-54-1, purity >98%), cyprodinil (CAS#121552-61-2, purity of >98%), triphenylphosphate (TPP, CAS#115-86-6, purity >99%), benzo(a)pyrene (BaP, CAS#50-32-8, purity >96%), benzo(b)fluoranthene (BbF, CAS#205-99-2, purity of 98%), chlorophene (CAS#120-32-1, purity of 95%), hydroxy-tamoxifen (OH-TAM, CAS#68392-35-8, purity of >98%) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (France). The cell culture medium and reagents Leibovitz 15 culture medium (L-15), fetal calf serum (FCS), 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), epidermal growth factor (EGF), G418, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) and D-luciferin were purchased from Sigma Aldrich (St-Quentin Fallavier, France); Dulbecco's Modified Eagle Medium (DMEM), DMEM High Glucose (DMEM HG) powder, F-12 nutrient mixture (Ham's F12) powder, penicillin and streptomycin were purchased from Gibco (France); insulin, hygromycin B and sodium bicarbonate were purchased from Dominique Dutscher (France).

2.3 In vitro bioassays: cell lines, luciferase and cell viability assays

The zebrafish *in vitro* assays have been derived from the zebrafish liver (ZFL) cell line (Cosnefroy et al., 2012). ZFL were stably transfected, first, with an ERE-driven firefly luciferase gene, yielding the ZELH cell line, and then either with zfERα subtype, yielding the ZELHα cell line, or with zfERβ2 subtype yielding the ZELHβ2 cell line (Cosnefroy et al., 2012). Establishment of these cell models and their response to different classes of well-known xeno-estrogens have been previously described (Cosnefroy et al., 2012; Sonavane et al., 2016). The human-derived MELN cell line (Balaguer et al., 1999) was kindly provided by Dr Patrick Balaguer (INSERM Montpellier, France). It is derived from the breast cancer MCF-7 cells, which endogenously express the hERα, but no functional hERβ (P. Balaguer, *personal communication*). MELN cells were stably transfected with an ERE-driven firefly luciferase reporter gene.

Conditions for routine cell culture have been detailed previously (Balaguer et al., 1999; Cosnefroy et al., 2012). The cells used were pathogen-free and controlled on a regular basis. For exposure experiments, ZELH-derived cells were seeded in 96-well white opaque culture plates (Greiner CellStar™, Dutscher, France) at 25,000 cells per well in phenol red-free LDF-DCC medium (containing L-15 50%, DMEM HG 35%, Ham's F12 15%, HEPES 15 mM, 0.15 g/L sodium bicarbonate, 0.01 mg/mL insulin, 50 ng/mL EGF, 50 U/mL penicillin and streptomycin antibiotics, 5% *v*/v stripped serum). MELN were seeded at 80,000 cells per well in phenol red-free DMEM medium containing 5% *v*/v stripped serum. Cells were left to adhere for 24h. Then, they were exposed in triplicates to serial dilutions of test compound for either 72h at 28°C for zebrafish cells or 16h at 37°C for MELN cells. Each plate included both solvent and positive controls (in two triplicates each). E2 was used as a positive quality control for ER activation, and hydroxy-tamoxifen (OH-TAM) for ER inhibition. In addition, a serial dilution of 7 to 8 concentrations of

E2 was tested in each experiment. At the end of exposure, the culture medium was removed and replaced by 50 µL per well of medium containing 0.3 mM luciferin. The luminescence signal was measured in living cells using a microtiter plate luminometer (Synergy H4, BioTek).

The cell viability was assessed by using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). After cell exposure, the culture medium was removed and replaced by 100 µL of medium containing 0.5 mg/mL MTT. Cells were incubated for 3h. In metabolically active cells, MTT is reduced onto a blue formazan precipitate, which is dissolved by adding 100 µL of DMSO after removal of MTT-containing medium. Plates were read at 570 nm against a 640 nm reference wavelength on a microplate reader (KC-4, BioTek Instruments, France) and results are expressed as absorbance units relative to control cells.

2.4 Testing of multi-component mixtures

The mixture compositions are given in Table SI-1, SI-2 and SI-3. The two 12-component mixtures were prepared in methanol (as part of a round robin study on bioassays, Altenburger et al., 2018). Stocks solutions and serial dilutions of single chemicals and 2-, 3-, 4- and 5-component mixtures were prepared in DMSO. The response of MELN cells to TPP and BPA using either DMSO or methanol as vehicle were similar (data not shown), thus, no significant effect of the solvent was to expect. To investigate the anti-estrogenic activity of the chemicals or mixtures, the cells were exposed in the presence of E2 at a concentration leading to 80% of maximal response, i.e. 0.1 nM in MELN and ZELHβ2 and 1 nM in ZELHα assays. The ZELH cells, that correspond to the parent cell line of ZELHα and ZELHβ2 cells but lack functional ER, were used additionally as a control for non-specific luciferase modulation. As for the other cell lines, cytotoxicity was measured in parallel in the way previously described. Final solvent concentrations in culture medium were 0.1% v/v (agonist assay) or 0.15% v/v (in case of co-exposure with E2), which do not affect luciferase expression or cell viability. Stock solutions of chemicals in DMSO and methanol were maintained at -20°C for up to three months.

2.5 Data analysis

2.5.1 Data treatment and analysis

Luciferase activity (LUC) was normalized to a response range between 0 and 1 on an experiment-to-experiment basis as follows:

$$Response = \frac{LUC_{chemical} - LUC_{control}}{LUC_{E2} - LUC_{control}} \qquad (1)$$

where $LUC_{chemical}$ is the luminescent signal induced by the tested chemical, $LUC_{control}$ is the average luminescent signal of the solvent controls and LUC_{E2} is the average luminescent signal of the E2 positive controls. Only non-cytotoxic concentrations (i.e. more than 80 % of cell viability in the MTT assay) were considered for data analysis. Concentration-effect data analysis was performed in the same way for individual compounds and mixtures. In short, a nonlinear regression model best-fit approach was used to describe pooled data sets in the best possible way (Scholze et al., 2001). If different regression functions led to similar goodness-of-fits, the logit model (which is a re-parameterised form of the Hill equation) was given preference. To account for inter-study variations we included experiments as random factor in the best-fit data analysis (nonlinear mixed effect model). A detailed description can be found in Altenburger et al. (2018).

2.5.2 Mixture prediction and uncertainty assessment

The combined response from individual substances was assumed to follow the concept of concentration addition (CA). Here we used the standard form of non-interaction, i.e.:

$$\sum_{i=1}^{n} \left(\frac{Ci}{ECxi} \right) = 1 \qquad (2)$$

where *Ci* is the concentration of the i^{th} substance in the mixture expected to produce a mixture response X, and *ECxi* the concentration of the i^{th} substance leading to the same response X as expected for the mixture.

To account for the statistical uncertainty in the CA prediction, a combination of Monte-Carlo (MC) simulations and bootstrapping nonlinear regression functions (Tibshirani and Efron, 1993) was conducted to simulate approximate 95% confidence limits around the predicted mean response of the mixture. Here the MC step is responsible for linking the data input from the single compounds (i.e. estimates about ECs or individual effects) to the mixture prediction, and the bootstrapping step is responsible for generating data information relevant for input variables (i.e. uncertainty distributions around the single substance EC's or effects). We followed a parametric bootstrap with resamples drawn from the fitted nonlinear mixed effect model. Differences between predicted and observed mixture effects (concentration) were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects (Altenburger et al., 2018). The comparative assessment was performed on mixture concentrations leading to 20% ER activation (EC20) or inhibition (IC20).

3. Results

3.1. Activation and inhibition of ER response by single chemicals

The results of ER activation and inhibition by all 12 chemicals and the reference compounds (E2 and OH-TAM) on MELN, ZELHα and ZELHβ2 cells are presented in Table 2, and the concentration-response data are provided in supplementary information (Figure SI-1 for ER activation and SI-2 for ER inhibition).

As expected, genistein and BPA were active in all cell lines, but at different sensitivity and efficacy levels. MELN cells responded to BPA with an EC20 of 0.12 µM and a maximal induction of 86% of the positive E2 control response, while ZELHα and ZELHβ2 cells showed a lower sensitivity with an EC20 of 2.1 µM and 5.0 µM, respectively, and a maximum luciferase induction around 30 % (Table 2). In case of genistein, MELN (EC20 of 0.0121 µM) and ZELHβ2 cells (EC20 of 0.015 µM) were more responsive than ZELHα cells (EC20 of 1.4 µM). BaP, TPP and diazinon weakly induced luciferase activity in MELN cells with an EC20 of 0.57 µM, 4.1 µM and 15 µM, respectively, whereas no activity was recorded at non-cytotoxic concentrations in zebrafish cells. No other chemicals showed any estrogenic response up to 30 µM in any bioassays.

The inhibition of ER response by the 12 chemicals revealed distinct response between the bioassays (Table 2). Overall, several chemicals were identified as new ER inhibitors, mainly in ZELH-zfERs cells. TPP and BaP decreased ER response in ZELHα and ZELHβ2 cells at concentrations where they did not affect cell viability or the luciferase activity in the ER-negative ZELH cells. Conversely, benzo(b)fluoranthene and propiconazole decreased E2-induced luciferase activity up to 90% in ZELHα and ZELHβ2 and in ER-negative ZELH cells. Cyprodinil decreased E2-induced luciferase activity across all the cell lines with similar sensitivity, suggesting a likely non-specific effect of this chemical on luciferase activity (Table 2, Figure SI-3).

3.2. Combined effects of xeno-estrogens in multi-component mixtures

The concentration-response curves estimated for the single chemicals were used to predict the ER activation and ER inhibition of M1 and M2 mixtures using the CA model. Since CA can describe only ER activation or ER inhibition, but not their co-occurrence, the additive response of a mixture containing both ER activators and inhibitors is predicted solely from the ER activators in case of ER activation or from the ER inhibitors in case of ER inhibition. Therefore, the chemicals expected to induce ER activation or ER inhibition in M1 and M2 mixtures were identified for each cell line based on CA prediction. They were then tested as subgroup mixtures containing either ER activating (M1_A, M2_A), ER inhibiting (M1_I, M2_I), or both ER activating and inhibiting chemicals (M1_A+I, M2_A+I) (Table 1). The relative concentration ratios were always kept in accordance to the 12-compound mixtures M1 and M2. All subgroup mixture results are presented in Figure 2 (mixture composition according to M1) and Figure 3 (mixture composition according to M2), together with the outcomes for M1 and M2 (Altenburger et al., 2018). Details about the mixture composition are given in Tables SI-1 (12-component mixtures) and in SI-2 and SI-3 (subgroup mixtures).

3.2.1 Additivity of ER activating or inhibiting chemicals

Regarding subgroup mixtures of ER activating chemicals, there was overall a good agreement between observed and predicted EC20 across all cell lines and for both mixtures M1 and M2 compositions. In MELN cells, TPP, BPA and genistein at M1 mixture ratio had additive effects very well predicted by CA model with a ratio between observed and predicted EC20 of 1.3 ($M1\_A_{MELN}$, Figure 2A, Table 3). In comparison, the measured estrogenic activity of BPA and genistein in $M2\_A_{MELN}$ was below the predicted response, although not statistically significant ($M2\_A_{MELN}$, Figure 3A, Table 4). BPA and genistein were the only two identified estrogenic chemicals in ZELHα and ZELHβ2 cells. Their binary mixture induced an estrogenic response in a good agreement with CA prediction at M1 and M2 concentration ratios in ZELHα (Figure 2E and 3E) and ZELHβ2 cells (Figure 2I and 3I). The ratio of observed against predicted EC20 was of 0.40 and 0.55 in ZELHα cells, and 0.71 and 0.73 in ZELHβ2 cells for M1 and M2, respectively.

As observed for single chemicals, ER inhibiting chemicals were more prevalent in ZELHα and ZELHβ2 cells than in MELN cells. In MELN cells, cyprodinil was predicted to inhibit E2 response in M1, but only at high concentrations ($M1\_I_{MELN}$, Figure 2B), and no inhibiting chemical was identified for M2. In contrast, TPP, chlorophene and propiconazole were identified as ER inhibiting chemicals of M1 in ZELHα and ZELHβ2 cells. In subgroup mixtures, they induced a strong ER inhibition in ZELHα ($M1\_I_{ZELH\alpha}$, Figure 2F) and ZELHβ2 cells ($M1\_I_{ZELH\beta2}$, Figure 2J), well predicted by the CA model (EC20 ratio of 0.87 and 0.83, respectively). Similarly, the subgroup mixtures of ER inhibitors based on M2 mixture ratio induced a strong inhibition, well predicted by CA model ($M2\_I_{ZELH\alpha}$, figure 3F and $M2\_I_{ZELH\beta2}$, Figure 3J, respectively).

Overall, the combined effects of ER activating or ER inhibiting chemicals were in good agreement with CA predictions for both M1 and M2 mixture ratios and across all cell lines.

3.2.2 Estrogenic response to the 12-component mixtures: influence of inhibiting chemicals

For each cell line, the combined effects of activator and inhibitor subgroup mixtures (M1_A+I and M2_A+I) were determined and compared to the results of the 12 component mixtures M1 and M2 (Figures 2 and 3, right part). The observed and predicted EC20 or IC20 of each mixture are presented in Tables 3 (M1) and 4 (M2).

In MELN cells, the estrogenic activity of M1_A+I_{MELN} (Figure 2C) was well predicted by CA, and this accuracy was not impacted negatively by the presence of 9 other environmental substances (M1, Figure 2D). No active ER inhibitors were present at non-cytotoxic concentration in the mixture M2, and therefore a mixture of activators and inhibitors was not tested. Nevertheless, the mixture effect of all 12 substances was well explained by the additivity of the only two estrogenic chemicals identified, BPA and genistein (M2, Figure 3D).

In zebrafish ZELHα cells, M1 was not expected to induce any estrogenic response in the range of tested concentrations, and indeed no estrogenic response was observed neither with the 5-component mixture (M1_A+$I_{ZELH\alpha}$, Figure 2G) nor with the 12-component mixture M1 (Figure 2H). Conversely, a strong ER inhibiting response was measured (up to 80% inhibition) for both the 5- and 12-component mixtures, which was well predicted by the CA model (IC20 ratio of 0.74 and 0.95, respectively). Thus, the ER inhibition measured remained unaffected by addition of estrogenic and inactive chemicals, including ER inhibiting chemicals present at non-effective concentrations (e.g. cyprodinil). In case of M2, the estrogenic activity of ER activating and inhibiting chemicals was correctly predicted by CA model (Figures 3G and 3H). However, the estrogenic activity measured was lower than that of BPA and genistein binary mixture results (Figure 3E), suggesting an influence of ER inhibiting compounds.

In zebrafish ZELHβ2 cells, an estrogenic response was expected according to CA for the mixture of activators and inhibitors, as supported by the additive outcomes from the binary mixture of BPA and genistein (M1_A$_{ZELH\beta2}$, Figure 2I). However, M1_A+I$_{ZELH\beta2}$ did not induce any estrogenic response at test concentrations (Figure 2K). Instead, a strong inhibition of ER response was measured, which was in line with the M1_I$_{ZELH\beta2}$ results and CA prediction (Figure 2J). As observed for the subgroup mixture of ER activating and inhibiting chemicals (M1_A+I$_{ZELH\beta2}$), M1 mixture did not induce any estrogenic activity but inhibited E2-induced response (Figure 2H). Hence, these results indicate that inhibiting chemicals in M1 indeed influenced ER response in ZELHβ2 cells. Compared with M1, the estrogenic activity measured for the subgroup mixture of ER activators and inhibitors corresponding to M2 mixture ratio was well predicted by CA model (M2_A+ I$_{ZELH\beta2}$, Figure 3K), although the maximal efficacy observed was well below the one of the BPA and genistein binary mixture (M2_A$_{ZELH\beta2}$, Figure 3I). When ER activating and inhibiting chemicals were grouped with inactive chemicals in M2, the estrogenic activity was well predicted by CA up to 20% (Figure 3L), but the maximal estrogenic response remained lower than expected based on the M2_A$_{ZELH\beta2}$ mixture results (Figure 3I). In comparison, the inhibition of ER response was well predicted by CA for both M2_A+I$_{ZELH\beta2}$ (Figure 3K) and M2 (figure 3L). The results of the 4-component mixture M2_A+I$_{ZELH\beta2}$ on ZELHβ2 cells are very similar to M2 results, considering both ER activation and inhibition (Figure 3K and 3L).

4. DISCUSSION

The current study investigated the distinct responses of zebrafish ZELHα and ZELHβ2 and human MELN cells ER reporter gene bioassays to 12-component mixtures composed of xeno-estrogens and other environmental relevant chemicals (Altenburger et al., 2018). By using a stepwise experimental approach from individual chemicals to subgroup mixture testing, we were able to explain the distinct response of human and zebrafish bioassays to the same 12-component mixtures.

4.1. Distinct responses of human and zebrafish cell lines to individual chemicals

BPA and genistein are well-known ER agonist ligands and were indeed active in all ER-based bioassays, in agreement with previous studies using the same cellular models (Balaguer et al., 1999; Cosnefroy et al., 2012; Le Fol et al., 2017; Sonavane et al., 2016). Apart from these two compounds, the screening of individual chemicals highlighted some marked differences between cell assays for some of the 10 chemicals.

One major outcome relates to the higher prevalence of chemicals inhibiting E2-induced luciferase activity in ZELH-zfERs cells than in MELN cells (Table 2). Some chemicals had opposite responses in zebrafish and human cells. For instance, BaP -a known AhR-ligand- and TPP were estrogenic in MELN cells but decreased E2-induced response in ZELHα and ZELHβ2 cells. The mechanistic interaction between AhR and ER signalling pathways has been documented in human (Matthews and Gustafsson, 2006; Ohtake et al., 2003) and in fish (e.g. Navas and Segner, 2000). The prototypical AhR ligand TCDD was shown to induce a weak estrogenic response in MELN cells (Balaguer et al., 1999) while it decreased E2 response in all ZELH-zfER cells (Sonavane, 2015). The distinct responses to BaP in ZELH-zfERs and MELN cells might thus be explained, at least partially, by AhR-ER interactions. In comparison, less information is available on the ability of TPP to interact with ER signalling. Previous studies have reported a weak agonist effect on hERα transactivation (Kojima et al., 2013), as observed in the current study in MELN cells, while some TPP metabolites are reported to have an anti-estrogenic activity on hERβ transactivation (Kojima et al., 2016). However, TPP was unable to induce the ER-regulated brain aromatase expression gene in transgenic cyp19a1b-GFP zebrafish embryos (Neale et al., 2017a). Considering the anti-estrogenic activity of TPP evidenced in zebrafish liver cells, further research would be warranted to assess whether TPP (or metabolites) either binds directly zfERs or alters zfER transactivation through cross-talk(s) with other signaling pathways.

Other chemicals, such as propiconazole and cyprodinil, decreased E2-induced estrogenic activity in an ER non-specific manner, i.e. they decreased firefly luciferase also in the parent cell line ZELH that does not express functional zfER (Table 2, Figure SI-5). Such inhibition may reflect either a direct effect on luciferase enzyme or an indirect effect on baseline transcriptional machinery in the promoter region of the reporter gene, irrespectively of ER activity. Despite a weak estrogenic activity on hERα reported *in vitro* (Medjakovic et al., 2014; Schlotz et al., 2017), cyprodinil decreased firefly luciferase activity in all cells, irrespectively of E2 addition. The structural similarities of cyprodinil with known firefly luciferase inhibitor (Auld and Inglese, 2004) and its capacity to interfere with ATP production (Coleman et al., 2012) suggest a possible effect on the reporter gene system. In case of propiconazole, a weak hERα agonist activity was reported in the high µM range in MVLN cells (Kjeldsen et al., 2013) and anti-proliferative effects measured in MCF-7 cells (Kjaerstad et al., 2010). In fish, interference of propiconazole with estrogen signalling pathway has been reported *in vivo* (Skolness et al., 2013) but no information on ER agonist or antagonist activity is available. Thus, additional assays would be warranted to assess the specific activity of propiconazole and cyprodinil on ER-signalling pathway in zebrafish.

4.2. Deciphering cell-specific response to xeno-estrogen mixtures

BPA and genistein were the main drivers for ER agonistic response in M1 and M2. When combined as binary mixture, they induced in all zebrafish and human-based bioassays responses that were in good agreement with CA predictions. This additivity is consistent with several previous studies which reported additive effects of selected estrogens on different biological models such as mammalian cells (Ghisari and Bonefeld-Jorgensen, 2009; Heneweer et al., 2005) or *in vitro* fish cells (Le Page et al., 2006; Petersen and Tollefsen, 2011) and *in vivo* in fish (Brian et al., 2005; Brion et al., 2012). Furthermore, our results demonstrate for the first time the suitability of the ZELH-zfER cell line to investigate mixture effects of ER agonists at the receptor level in a zebrafish cell context.

The screening for anti-estrogenic activity showed that some inhibiting chemicals active on ZELH-zfER cells were present at effective concentrations in M1 and M2, e.g. TPP and propiconazole. Although the underlying mechanism of ER inhibition remains unclear, the subgroup mixtures of inhibiting chemicals had additive effects in ZELHα and ZELHβ2 cells, in all co-exposure scenario, i.e. with inactive and/or estrogenic chemicals. In case of M1, a decreased luciferase activity was also observed in ZELH cells, well predicted by the additive effects of TPP and propiconazole (Figure SI-4). These results indicate that the inhibition observed in ZELH-zfERs cells for M1 may involve non-ER specific luciferase inhibition.

Interestingly, we observed in ZELHβ2 cells that the addition of the inhibiting chemicals to the binary mixture of BPA and genistein resulted in a decrease in the expected estrogenic response to a similar level as observed in the 12-component mixtures M1 and M2. In case of M1, the presence of inhibiting chemicals silenced entirely the estrogenic activity expected, whereas in M2, only the efficacy of the response was decreased. To a lesser extent, a similar trend was observed for M2 in ZELHα cells. The experimental approach consisting of testing ER activating and inhibiting chemicals separately and then together allowed us to evidence the role of inhibiting chemicals in the deviation from expected additivity of genistein and BPA in ZELHβ2 cells. The experimental results from the stepwise testing approach demonstrate that the response to the 12-chemical mixtures in each bioassay can be explained knowing the individual responses of the 12 chemicals.

4.3. Differences between zebrafish and human-based bioassay responses

Our results highlight marked differences between human and zebrafish cells responses. Each cell line displays cell-specific features, such as co-activator recruitment or metabolic capacities. For instance, ZELH cells originate from zebrafish liver cells and have retained some metabolic capacities qualitatively similar to zebrafish hepatocytes but distinct from MELN cells (Le Fol et al., 2015), which may have played a role in the specific response to inhibiting chemicals in our study. Indeed, metabolism has been previously suggested to negatively influence the response to xeno-estrogen mixtures in rainbow trout hepatocytes (Petersen and Tollefsen, 2011) and in the E-SCREEN assay (Evans et al., 2012). The characterization of internal concentrations of chemicals in MELN and ZELH-zfER cells would be needed to estimate the influence of metabolism on the xeno-estrogen response.

To further investigate the relevance of the estrogenic mixture response in fish, both M1 and M2 were tested on transgenic zebrafish embryos expressing GFP under control of cyp19a1b promoter in radial glial cells in the EASZY assay (Brion et al., 2012). Indeed, in previous studies, we showed that ZELH-zfER response profile to individual chemicals or environmental samples was better correlated than the MELN assay with *in vivo* estrogenic activity measured in the EASZY assay (Neale et al., 2017b; Sonavane et al., 2016). As a result, no estrogenic activity was measured for both M1 and M2 mixtures because of a high embryo mortality, especially for M1 (Altenburger et al., 2018). Thus, we could not confirm *in vitro* combined effects in zebrafish *in vivo*.

4.4. Implication for quantifying the estrogenic activity of samples

A consistent body of literature exist regarding the assessment of additivity of xeno-estrogens according to CA. However, very few studies investigated the robustness and validity of CA model in more complex and realistic mixture scenarios. In the current study, the main factors differentiating zebrafish and human ER response to M1 and M2 was the presence of inhibiting chemicals that had higher influence on zfER activation in zebrafish cells. This agrees well with the findings of Ihara et al. (2014) that evidenced that anti-estrogenic activity in wastewater treatment plant extracts was a key factor to explain the different estrogenic activity measured in human and medaka ERα transactivation *in vitro*.

The 12-component mixtures were designed to mimic a simplified scenario of environmental surface water contamination. To assess whether the mixture context would have influenced the quantification of estrogenic activity mediated by xeno-estrogens, the mixture results were used to quantify estradiol-equivalents (E2-Eq) in each bioassay (Table SI-4). Overall, M2 was predicted to be more estrogenic (mean E2-Eq > 10 µM) than M1 (mean E2-Eq < 1 µM). In MELN cells, the estrogenicity of M1 and M2 was almost not affected by the mixture context: the ratio of observed to predicted E2-Eq was close to 1 for both mixtures. In contrast, ZELHα and ZELHβ2 responses to xeno-estrogens in this specific mixture scenario were more susceptible to co-occurrence of inhibiting chemicals: the estrogenic activity was underestimated in M1 and M2, whenever quantified. In case of ZELHβ2 cells, similar IC20 were derived for both M1 and M2, however, the inhibiting chemicals abolished the estrogenic response in case of M1, while they only partially decreased the maximal efficacy level in case of M2, without altering significantly the EC20 measured. These results suggest the presence of a balance between estrogenic and ER inhibiting chemicals which can influence the detection, and thus the quantification, of xeno-estrogens in ZELHβ2 cells.

5. CONCLUSION

In summary, this study demonstrates that BPA and genistein had additive effects *in vitro* in zebrafish bioassays, comforting their use to assess combined effects of xeno-estrogens. In addition, we show that the distinct responses of zebrafish and human-based bioassays to a 12-component mixture were due to newly identified ER inhibiting chemicals selectively active in ZELHα and ZELHβ2 cells (e.g. TPP, propiconazole) and altering zfER response to xeno-estrogens. In the context of water bio-monitoring, this study illustrates the need for a mindful consideration of the bioassay specificities (e.g. fish *vs* human ER, cell context) to ensure a proper interpretation of results, as environmental chemicals may interfere with ER response, positively or negatively, in a cell-specific manner. In future works, comparative assessment of real water samples using human and zebrafish bioassays will help further documenting the environmental relevance of such cross-species differential effects to complex mixtures.

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DECLARATIONS OF INTEREST

The authors declare that no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS:

H.S., M.S., R.A., W.B., H.B., F.B and S.A conceived and designed the experiments; H.S. has performed the experiments; H.S and M.S. analysed the data; H.S., M.S. and S.A. have written the manuscript; all authors have read and approved the final manuscript.

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TABLES AND FIGURES

Table 1: Overview of mixtures and their abbreviations tested on four different cell lines. More details about the composition of the mixtures are provided in the Supplementary Information (Tables SI 1-3). (1) published in Altenburger et al; (2) corresponds to cyprodinil which was the only ER inhibitor.

	ER activation		ER inhibition	
mixture	M1	M2	M1	M2
MELN				
activators	M1_A$_{MELN}$	M2_A$_{MELN}$	-	-
inhibitors	-	-	M1_I$_{MELN}$ (2)	-
activators + inhibitors	M1_A+I$_{MELN}$	-	M1_A+I$_{MELN}$	-
activators + inhibitors + inactives	M1 (1)	M2 (1)	M1	-
ZELHα				
activators	M1_A$_{ZELH\alpha}$	M2_A$_{ZEsLH\alpha}$	-	-
inhibitors	-	-	M1_I$_{ZELH\alpha}$	M2_I$_{ZELH\alpha}$
activators + inhibitors	M1_A+I$_{ZELH\alpha}$	M2_A+I$_{ZELH\alpha}$	M1_A+I$_{ZELH\alpha}$	M2_A+I$_{ZELH\alpha}$
activators + inhibitors + inactives	M1 (1)	M2 (1)	M1	M2
ZELHβ2				
activators	M1_A$_{ZELH\beta2}$	M2_A$_{ZELH\beta2}$	-	-
inhibitors	-	-	M1_I$_{ZELH\beta2}$	M2_I$_{ZELH\beta2}$
activators + inhibitors	M1_A+I$_{ZELH\beta2}$	M2_A+I$_{ZELH\beta2}$	M1_A+I$_{ZELH\beta2}$	M2_A+I$_{ZELH\beta2}$
activators + inhibitors + inactives	M1 (1)	M2 (1)	M1	M2
ZELH				
inhibitors	-	-	M1_I$_{ZELH}$	M2_I$_{ZELH}$
inhibitors + inactives	-	-	M1	M2

Table 2: ER activation (EC20) and inhibition (IC20) of 12 test substances in MELN, ZELHα, ZELHβ2 and ZELH cells. Results are expressed in EC20 (activation) or IC20 (inhibition) are expressed in M concentration. E2 and OH-TAM were the positive control substances for ER activation and inhibition, respectively. Data originate from at least 2 independent experiments done in triplicates. Chemicals were tested in the 0.01 – 30×10^{-6} M range, except for genistein (from 10^{-9} M). All concentration-response data are presented in SI-1 and SI-2.

	ER activation (EC20)			**ER inhibition (IC20)**			
	MELN mean (95% CI)	**ZELHα** mean (95% CI)	**ZELHβ2** mean (95% CI)	**MELN** mean (95% CI)	**ZELHα** mean (95% CI)	**ZELHβ2** mean (95% CI)	**ZELH** mean (95% CI)
E2	3.4×10^{-12} ($2.6\,\mathrm{x}10^{-12}$ - $4.3\,\mathrm{x}10^{-12}$)	1.3×10^{-10} (1.1×10^{-10} - 1.6×10^{-10})	6.0×10^{-12} (4.74×10^{-12} - 7.7×10^{-12})	-	-	-	-
OH-TAM	-	-	-	5.2×10^{-9} (4.5×10^{-9} - 6.0×10^{-9})	1.8×10^{-9} (9.4×10^{-10} - 3.4×10^{-9})	1.9×10^{-9} (1.4×10^{-9} - 2.8×10^{-9})	$> 3\times10^{-5}$
Bisphenol A	1.2×10^{-7} (8.2×10^{-8} -$1.7\,\mathrm{x}10^{-7}$)	2.1×10^{-6} (1.3×10^{-6} - 3.6×10^{-6})	5.0×10^{-6} (2.4×10^{-6} - 6.1×10^{-6})	$> 3\times10^{-5}$	2.02×10^{-5} (1.1×10^{-5} - 3.6×10^{-5})	8.8×10^{-6} (8.7×10^{-7} - 1.3×10^{-5})	$> 3\times10^{-5}$
Genistein	1.21×10^{-8} (6.0×10^{-9} - 2.9×10^{-8})	1.4×10^{-06} (9.5×10^{-7} - 1.9×10^{-6})	1.5×10^{-8} (6.9×10^{-9} - 3.1×10^{-8})	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$
Diazinon	1.5×10^{-5} (1.2×10^{-5} - 1.9×10^{-5})	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 1\times10^{-5}$	$> 3\times10^{-5}$
Triphenylphosphate	4.1×10^{-6} (2.9×10^{-6} - 5.7×10^{-6})	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	8.0×10^{-6} (3.2×10^{-7} - 1.3×10^{-5})	1.7×10^{-6} (8.3×10^{-7} - 3.5×10^{-6})	1.1×10^{-5} (3.0×10^{-7} - 1.3×10^{-5})
Benzo(a)pyrene	5.7×10^{-7} (4.6×10^{-7} - 7.2×10^{-7})	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	4.2×10^{-6} (2.5×10^{-6} - 7.3×10^{-6})	1.4×10^{-6} (7.7×10^{-7} - 2.4×10^{-6})	$> 3\times10^{-5}$
Benzo(b)fluorantene	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	1.95×10^{-6} (1.1×10^{-6} - 3.4×10^{-6})	1.5×10^{-6} (5.4×10^{-7} - 4.1×10^{-6})	1.8×10^{-6} (7.2×10^{-7} - 4.4×10^{-6})
Chlorophene	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	1.0×10^{-5} (2.6×10^{-6} - 1.7×10^{-5})	6.2×10^{-6} (3.4×10^{-6} - 9.8×10^{-6})	$>1\times10^{-5}$
Propiconazole	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	8.1×10^{-6} (3.1×10^{-6} - 1.9×10^{-5})	4.4×10^{-6} (2.6×10^{-6} - 7.7×10^{-6})	2.4×10^{-6} (3.7×10^{-7} - 1.4×10^{-5})
Cyprodinil	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	4.9×10^{-6} (3.0×10^{-6} - 8.1×10^{-6})	2.0×10^{-6} (1.2×10^{-6} - 3.4×10^{-6})	4.2×10^{-6} (1.4×10^{-6} - 1.3×10^{-5})	4.1×10^{-6} (2.6×10^{-6} - 1.6×10^{-5})
Triclosan	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$
Diuron	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$
Diclofenac	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$	$> 3\times10^{-5}$

Table 3: Observed and predicted ER activation and inhibition for mixture M1 and its subgroups in MELN, ZELHα and ZELHβ2 cells. All concentrations are in M. (-) not tested as none of the individual compounds showed activity below its cytotoxic concentration range. (n.a.): the calculation is not applicable. Star indicates statistical significance ($p<0.05$). [a] re-calculated from Altenburger et al., 2018; [b] corresponds to cyprodinil which was the only ER inhibitor; [c] above cytotoxic concentration range.

		ER activation (EC20)			**ER inhibition (IC20)**		
		Observed	**Predicted**	**Ratio**	**Observed**	**Predicted**	**Ratio**
Cell line	Mixture (name)	Mean (95% CI)	Mean (95% CI)	obs/pred	Mean (95% CI)	Mean (95% CI)	obs/pred
MELN	$M1\_A_{MELN}$	1.2×10^{-6} (9.3×10^{-7} - 1.6×10^{-6})	8.9×10^{-7} (5.9×10^{-7} - 1.3×10^{-6})	1.3	-	-	-
	$M1\_I_{MELN}$	-	-	-	4.9×10^{-6} [b] (3.0×10^{-6} - 8.2×10^{-6})	4.9×10^{-6} [b] (3.0×10^{-6} - 8.2×10^{-6})	1
	$M1\_A{+}I_{MELN}$	2.1×10^{-6} (1.5×10^{-6} - 2.9×10^{-6})	2.6×10^{-6} (1.7×10^{-6} - 3.8×10^{-6})	0.81	$> 2 \times 10^{-5}$ [4]	8.3×10^{-5} (5.0×10^{-5} - 1.4×10^{-4})	n.a.
	M1	6.1×10^{-6} [a] (3.9×10^{-6} - 9.2×10^{-6})	6.7×10^{-6} [a] (4.4×10^{-6} - 9.5×10^{-6})	0.91	3.4×10^{-5} (1.1×10^{-5} - 1.0×10^{-4})	5.9×10^{-4} [c] (3.6×10^{-4} - 9.8×10^{-4})	0.058*
ZELHα	$M1\_A_{ZELH\alpha}$	8.2×10^{-7} (6.5×10^{-7} - 1.6×10^{-6})	2.0×10^{-6} (1.0×10^{-6} - 3.0×10^{-6})	0.41	-	-	-
	$M1\_I_{ZELH\alpha}$	-	-	-	2.7×10^{-6} (1.9×10^{-6} - 3.6×10^{-6})	3.1×10^{-6} (1.2×10^{-6} - 1.2×10^{-5})	0.87
	$M1\_A{+}I_{ZELH\alpha}$	$> 4 \times 10^{-5}$ [c]	2.1×10^{-4} (1.3×10^{-4} - 3.2×10^{-4})	n.a.	4.2×10^{-6} (1.9×10^{-6} - 9.5×10^{-6})	5.7×10^{-6} (2.4×10^{-6} - 2.3×10^{-5})	0.74
	M1	$> 10^{-5}$ [c]	3.0×10^{-4} (1.8×10^{-4} - 4.6×10^{-4})	n.a.	4.2×10^{-6} (2.0×10^{-6} - 8.7×10^{-6})	4.4×10^{-6} (1.7×10^{-6} - 1.7×10^{-5})	0.95
ZELHβ2	$M1\_A_{ZELH\beta2}$	8.6×10^{-8} (3.7×10^{-8} - 1.8×10^{-7})	1.2×10^{-7} ($5.5 \times^{-8}$ - 2.4×10^{-7})	0.71	-	-	-
	$M1\_I_{ZELH\beta2}$	-	-	-	2.9×10^{-6} (2.0×10^{-6} - 4.0×10^{-6})	3.5×10^{-6} (2.1×10^{-6} - 5.1×10^{-6})	0.83
	$M1\_A{+}I_{ZELH\beta2}$	$> 2 \times 10^{-5}$ [c]	1.3×10^{-5} (5.8×10^{-6} - 2.5×10^{-5})	n.a.	4.4×10^{-6} (3.0×10^{-6} - 6.3×10^{-6})	6.4×10^{-6} (4.0×10^{-6} - 9.0×10^{-6})	0.69
	M1	$> 3 \times 10^{-5}$ [c]	1.8×10^{-5} (8.1×10^{-6} - 3.5×10^{-5})	n.a.	3.7×10^{-6} (2.1×10^{-6} - 6.3×10^{-6})	5.0×10^{-6} (3.0×10^{-6} - 7.1×10^{-6})	0.74

Table 4: Observed and predicted ER activation and inhibition for mixture M2 and its subgroups. All concentrations are in M. (-) not tested as none of the individual compounds showed activity below its cytotoxic concentration range. (n.a.): the calculation is not applicable. Star indicates statistical significance ($p<0.05$). [(a)] re-calculated from Altenburger et al., 2018; [(b)] maximal induction measured below 20%.

		ER activation (EC20)			**ER inhibition (IC20)**		
		Observed	**Predicted**	**Ratio**	**Observed**	**Predicted**	**Ratio**
Cell line	Mixture (name)	Mean (95% CI)	Mean (95% CI)	obs/pred	Mean (95% CI)	Mean (95% CI)	obs/pred
MELN	$M2\_A_{MELN}$	1.6×10^{-7} (8.2×10^{-8} - 2.9×10^{-7})	6.4×10^{-8} (4.0×10^{-8} - 9.5×10^{-8})	2.5	-	-	-
	M2	1.5×10^{-7} [(a)] (6.8×10^{-8} - 2.8×10^{-7})	2.08×10^{-7} [(a)] (1.3×10^{-7} - 3.3×10^{-7})	0.72	-	-	-
ZELHα	$M2\_A_{ZELH\alpha}$	1.1×10^{-6} (7.4×10^{-7} - 1.7×10^{-6})	2.0×10^{-6} (1.2×10^{-6} - 3.1×10^{-6})	0.55	-	-	-
	$M2\_I_{ZELH\alpha}$	-	-	-	6.7×10^{-6} (2.9×10^{-6} - 1.3×10^{-5})	6.1×10^{-6} (2.2×10^{-6} - 1.1×10^{-5})	1.1
	$M2\_A{+}I_{ZELH\alpha}$	1.5×10^{-6} (7.8×10^{-7} - 2.8×10^{-6})	4.9×10^{-6} (3.0×10^{-6} - 7.5×10^{-6})	**0.31***	7.6×10^{-6} (5.3×10^{-6} - 1.0×10^{-5})	1.0×10^{-5} (3.7×10^{-6} - 1.7×10^{-5})	0.76
	M2	$> 1.5 \times 10^{-7}$ [(b)]	6.6×10^{-6} (4.0×10^{-6} - 1.0×10^{-5})	n.a.	8.3×10^{-6} (6.0×10^{-6} - 1.1×10^{-5})	1.4×10^{-5} (5.3×10^{-6} - 2.4×10^{-5})	0.59
ZELHβ2	$M2\_A_{ZELH\beta2}$	1.1×10^{-7} (3.3×10^{-8} - 3.2×10^{-7})	1.5×10^{-7} (7.0×10^{-8} - 3.0×10^{-7})	0.73	-	-	-
	$M2\_I_{ZELH\beta2}$	-	-	-	7.5×10^{-6} (5.3×10^{-6} - 1.0×10^{-5})	6.6×10^{-6} (1.7×10^{-6} - 8.2×10^{-6})	1.1
	$M2\_A{+}I_{ZELH\beta2}$	1.2×10^{-6} (2.9×10^{-7} - 4.5×10^{-6})	3.7×10^{-7} (1.7×10^{-7} - 7.3×10^{-7})	3.2	7.7×10^{-6} (2.1×10^{-6} - 1.8×10^{-5})	6.8×10^{-6} (1.8×10^{-6} - 8.6×10^{-6})	1.1
	M2	1.8×10^{-6} (3.2×10^{-7} - 6.6×10^{-6})	5.0×10^{-7} (2.3×10^{-7} - 9.8×10^{-7})	3.6	4.1×10^{-6} (3.2×10^{-6} - 5.1×10^{-6})	9.2×10^{-6} (2.4×10^{-6} - 1.2×10^{-5})	0.44

Figure 1: Experimental approach selected to study the combined effects of ER activating and inhibiting chemicals within the 12-component mixtures M1 and M2. Subgroups of activators and inhibitors were designed with the chemicals predicted to contribute to the 12-component mixture responses at M1 and M2 mixture ratios.

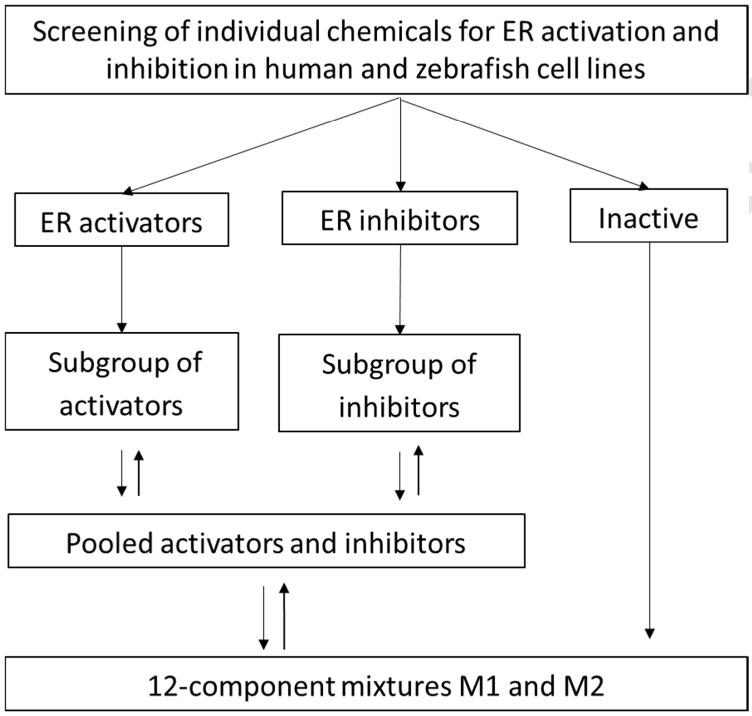


Figure 2: Predicted and measured effects of multi-component mixtures based on M1 concentration ratios. Data represent the mean (+/- SD) of a minimum of 3 independent experiments done in triplicates and pooled together. The green line represents CA prediction for ER activation and the orange line ER inhibition, and their respective dotted line represent the 95% CI belt. Cytotoxic concentrations (measured by MTT) were removed.

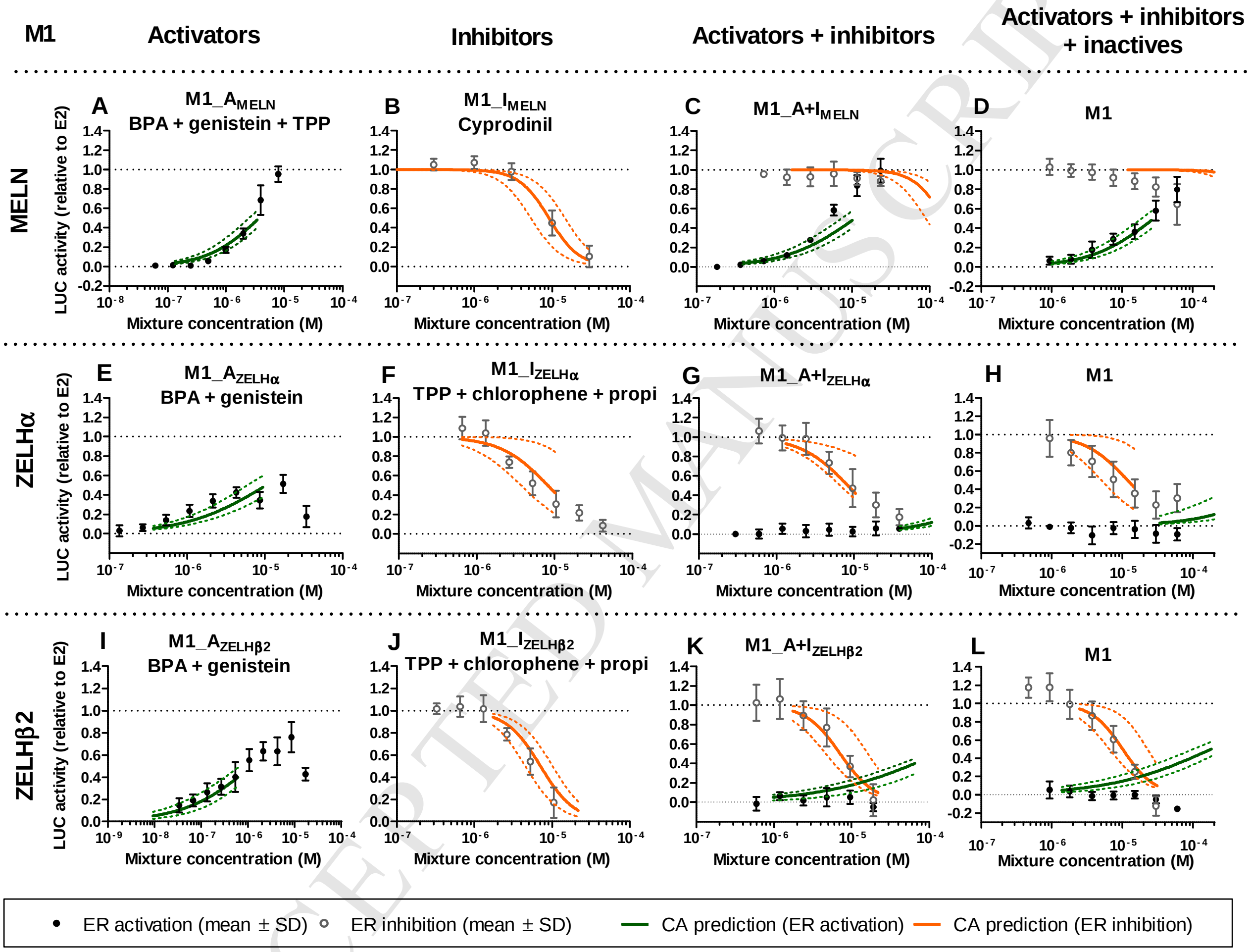
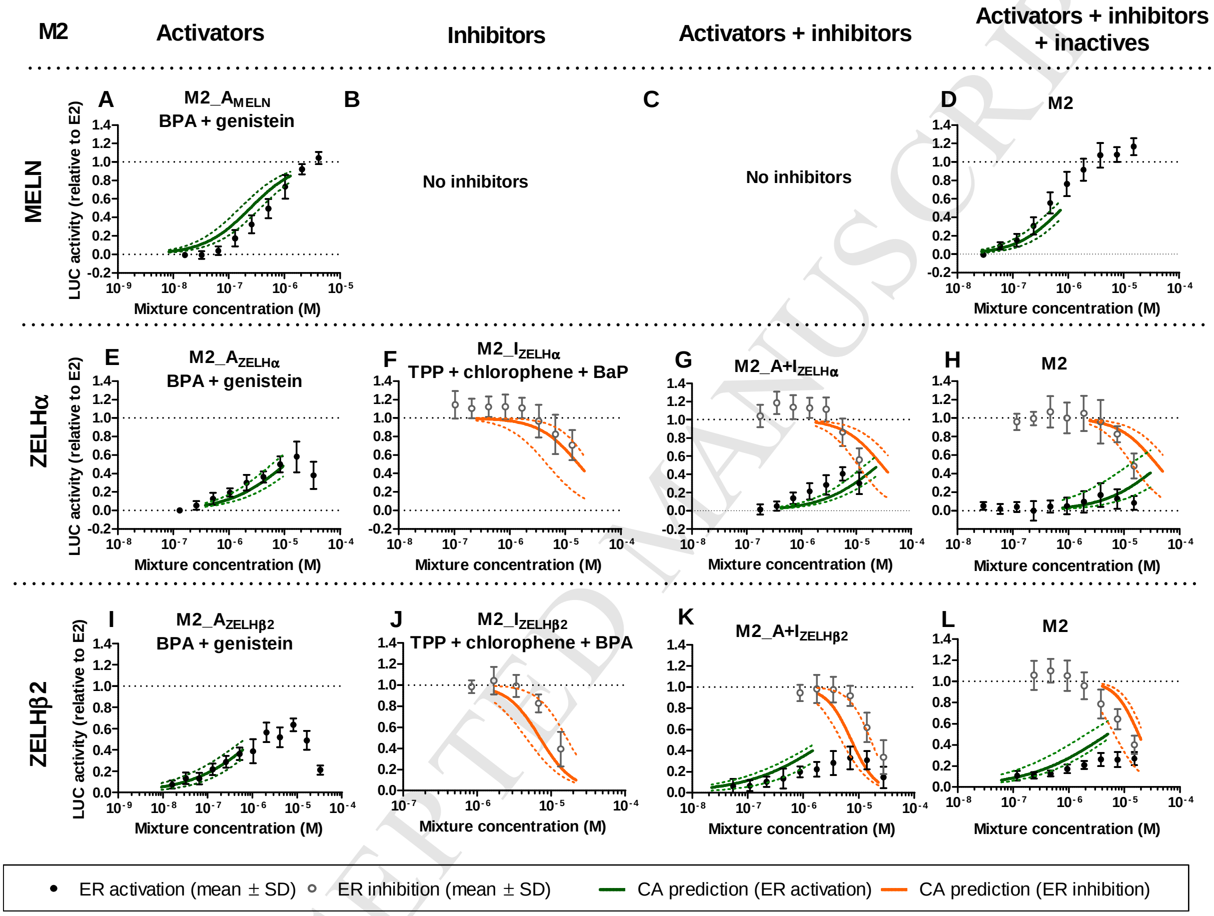


Figure 3: Predicted and measured effects of multi-component mixtures based on M2 concentration ratios. Data represent the mean (+/- SD) of a minimum of 3 independent experiments done in triplicates and pooled together. The green line represents CA prediction for ER activation and the orange line ER inhibition, inhibition, and their respective dotted line represent the 95% CI belt. Cytotoxic concentrations (measured by MTT) were removed.



SUPPLEMENTARY INFORMATION

Combined effects of environmental xeno-estrogens within multi-component mixtures: comparison of *in vitro* human- and zebrafish-based estrogenicity bioassays

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Content:

Table SI 1: Composition of the 12 compound mixtures M1 and M2 and the highest substance concentration tested *in vitro*.

	M1		M2	
	Concentration (M)	proportion[1)]	Concentration (M)	proportion[1)]
Benzo(a)pyrene	6E-08	0.05%	9.47E-09	0.06%
Benzo(b)fluorantene	1E-07	0.08%	9.51E-09	0.06%
Bisphenol A	7E-07	0.58%	4.17E-06	27.70%
Chlorophene	9E-06	7.50%	6.40E-06	42.51%
Cyprodinil	1E-06	0.83%	1.87E-07	1.24%
Diazinon	6E-09	0.00%	1.96E-08	0.13%
Diclofenac	3E-05	24.99%	2.90E-06	19.26%
Diuron	6E-07	0.50%	2.08E-07	1.38%
Genistein	1E-07	0.08%	4.47E-07	2.97%
Propiconazole	6E-05	49.97%	8.48E-08	0.56%
Triphenylphosphate	1.5E-05	12.49%	2.32E-07	1.54%
Triclosan	3.5E-06	2.92%	3.89E-07	2.58%
Mixture	1.2E-04	100%	1.51E-5	100%

[1)] mixture composition according to Altenburger et al., (2018)

Table SI 2: Composition of mixtures of ER activator (M1_A), ER inhibitors (M1_I) or combined ER activators and inhibitors (M1_A+I) tested in MELN, ZELHα, ZELHβ2 and ZELH cells. The mixture composition is based on their relative proportion in the 12-compound mixture M1 (Table SI 1).

	MELN			**ZELHα / ZELHβ2**			**ZELH**
Type of mixture	Activators	Inhibitors	Inhibitors + activators	Activators	Inhibitors	Inhibitors + activators	Inhibitors
Mixture name	$M1\_A_{MELN}$	$M1\_I_{MELN}$	$M1\_A{+}I_{MELN}$	$M1\_A_{ZELH\alpha}$, $M1\_A_{ZELH\beta2}$	$M1\_I_{ZELH\alpha}$, $M1\_I_{ZELH\beta2}$	$M1\_I{+}A_{ZELH\alpha}$, $M1\_I{+}A_{ZELH\beta2}$	$M1\_I_{ZELH}$
Genistein	1%	-	1%	13%	-	0.2%	-
Bisphenol A	4%	-	4%	87%	-	0.8%	-
Triphenylphosphate	95%	-	89%	-	18%	17.7%	20%
Cyprodinil	-	100%	6%	-	-	-	1%
Diclofenac	-	-	-	-	-	-	-
Chlorophene	-	-	-	-	11%	10.6%	-
Propiconazole	-	-	-	-	71%	70.8%	79%
Total	100%	100%	100%	100%	100%	100%	100%

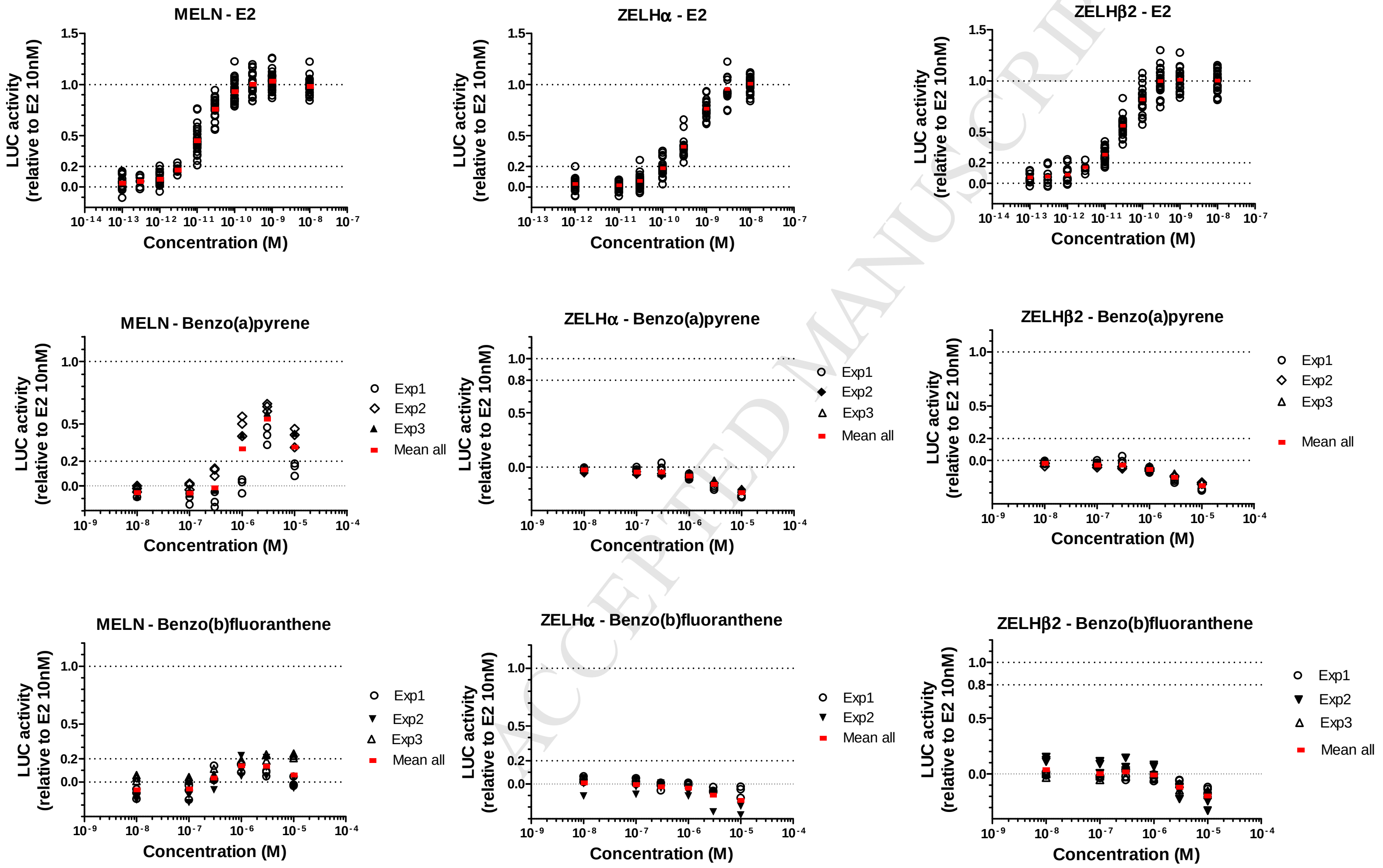
Table SI 3: Composition of mixtures of ER activator (M2_A), ER inhibitors (M2_I) or combined ER activators and inhibitors (M2_A+I) tested in MELN, ZELHα, ZELHβ2 and ZELH cells. The mixture composition is based on their relative proportion in the 12-compound mixture M2 (Table SI 1).

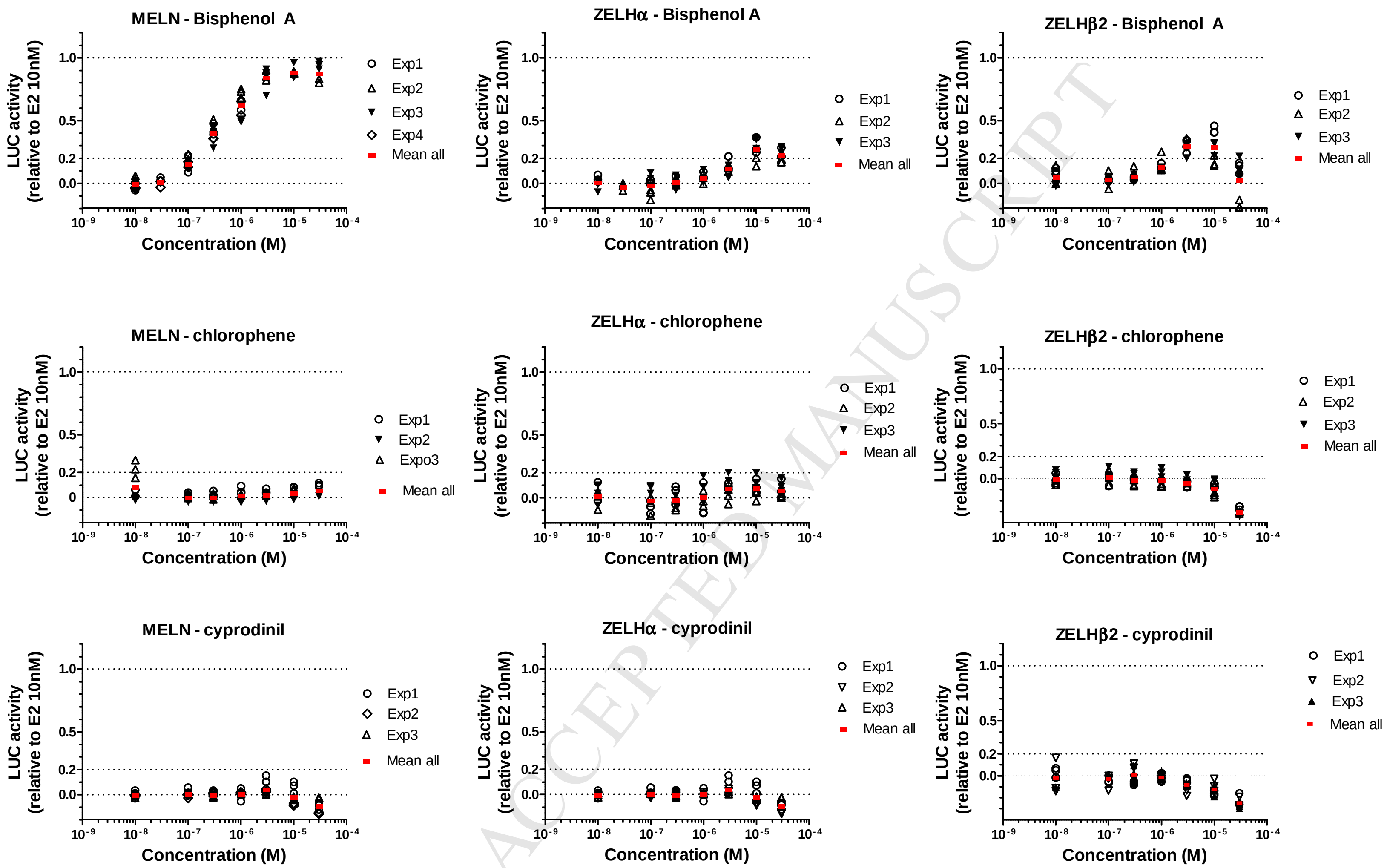
	MELN	**ZELHα**			**ZELHβ2**			**ZELH**
Type	Activators	Activators	Inhibitors	Inhibitors + activators	Activators	Inhibitors	Inhibitors + activators	Inhibitors
Name	$M2\_A_{MELN}$	$M2\_A_{ZELH\alpha}$	$M2\_I_{ZELH\alpha}$	$M2\_I{+}A_{ZELH\alpha}$	$M2\_A_{ZELH\beta 2}$	$M2\_I_{ZELH\beta 2}$	$M2\_I{+}A_{ZELH\beta 2}$	$M2\_I_{ZELH}$
Genistein	10%	10%		4.0%	10%		4.0%	
Bisphenol A	90%	90%		37.2%	90%	38.6%	37.2%	
Triphenylphosphate			3.5%	2.0%		2.1%	2.1%	77%
Chlorophene			96.4%	56.6%		59.3%	57.1%	
Propiconazole								23%
Benzo(a)pyrene			0.14%	0.08%				
Total	100%	100%	100%	100%	100%	100%	100%	100%

Table SI 4: Estrogenic activity of the M1 and M2 expressed in estradiol-equivalents (E2-Eq). E2-Eq (expressed in µM) were calculated for the 12-component mixtures on the basis of their predicted and observed EC20s (reported in Tables 3 and 4) in relation to the EC20 of E2 (in Table 2). The E2-Eq (observed) is the ratio between the EC20(E2) and the measured EC20(mixture), and E2-Eq(predicted) is the ratio between the EC20(E2) and the CA predicted EC20(mixture). n.a.: not applicable (no estrogenic activity measured).

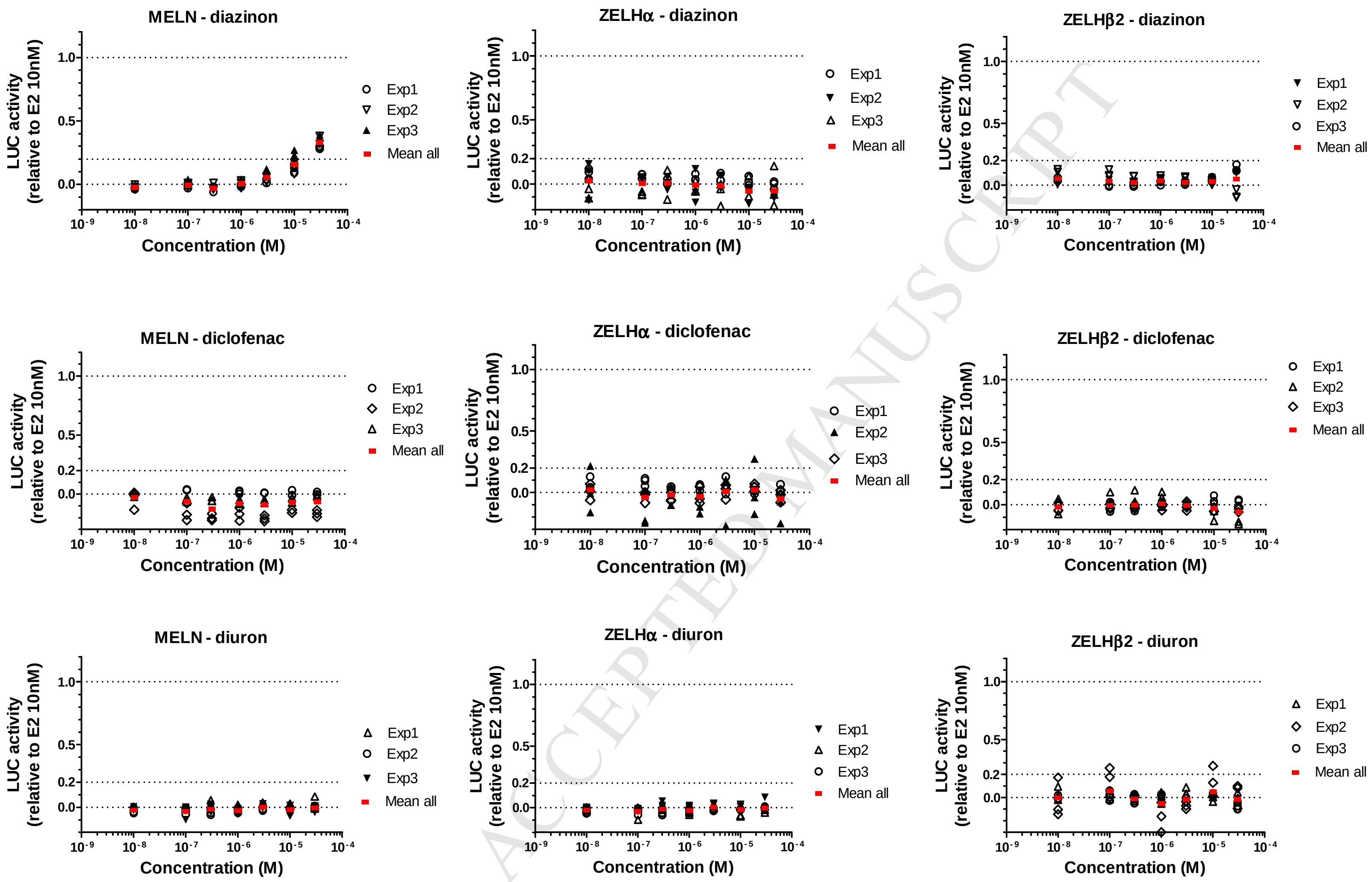
	M1 E2-Eq			**M2 E2-Eq**		
	Observed (µM)	**Predicted** (µM)	**Observed/Predicted** (%)	**Observed** (µM)	**Predicted** (µM)	**Observed/Predicted** (%)
MELN	0.56	0.51	110	22.7	16.3	139
ZELHα	n.a.	0.43	n.a.	n.a.	19.7	n.a.
ZELHβ2	n.a.	0.33	n.a.	3.33	12	28

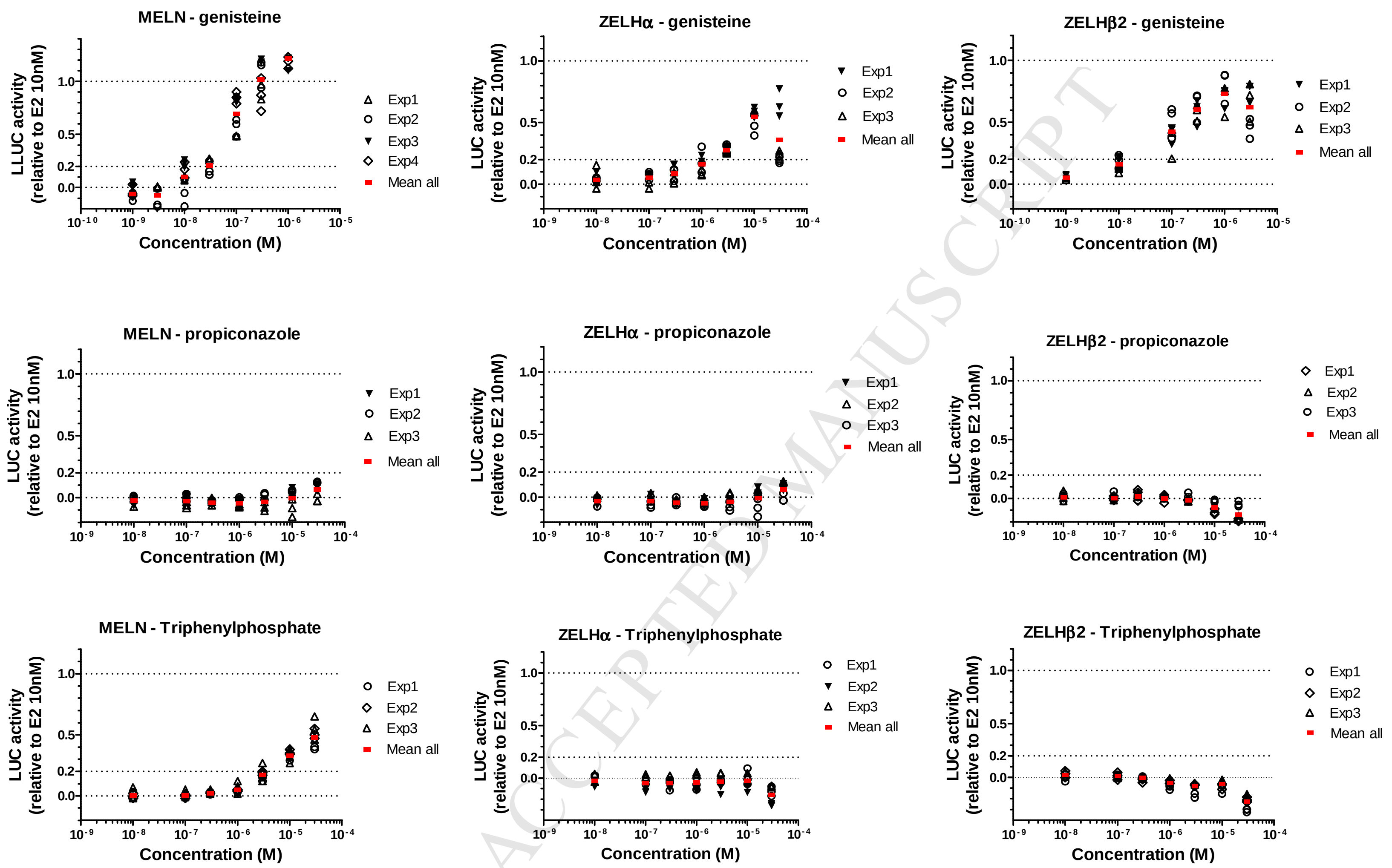
Figure SI 1: Response of the 12 chemicals on ER activation in MELN, ZELHα and ZELHβ2 cells. Data represent each replicate and their mean (red dash) of at least 2 independent experiments done in triplicates. Chemicals were tested in the 10 nM - 30 µM range, except for genistein (from 1 nM). 17β-estradiol (E2) was used as positive control. The horizontal dotted line at 20% figures the threshold of effect.





MELN - Bisphenol A
ZELHα - Bisphenol A
ZELHβ2 - Bisphenol A
MELN - chlorophene
ZELHα - chlorophene
ZELHβ2 - chlorophene
MELN - cyprodinil
ZELHα - cyprodinil
ZELHβ2 - cyprodinil
LUC activity (relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Exp4
Expo3
Mean all


MELN - diazinon
ZELHα - diazinon
ZELHβ2 - diazinon
MELN - diclofenac
ZELHα - diclofenac
ZELHβ2 - diclofenac
MELN - diuron
ZELHα - diuron
ZELHβ2 - diuron
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all


MELN - genisteine
LLUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Exp4
Mean all
ZELHα - genisteine
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
ZELHβ2 - genisteine
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
MELN - propiconazole
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
ZELHα - propiconazole
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
ZELHβ2 - propiconazole
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
MELN - Triphenylphosphate
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
ZELHα - Triphenylphosphate
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all
ZELHβ2 - Triphenylphosphate
LUC activity
(relative to E2 10nM)
Concentration (M)
Exp1
Exp2
Exp3
Mean all

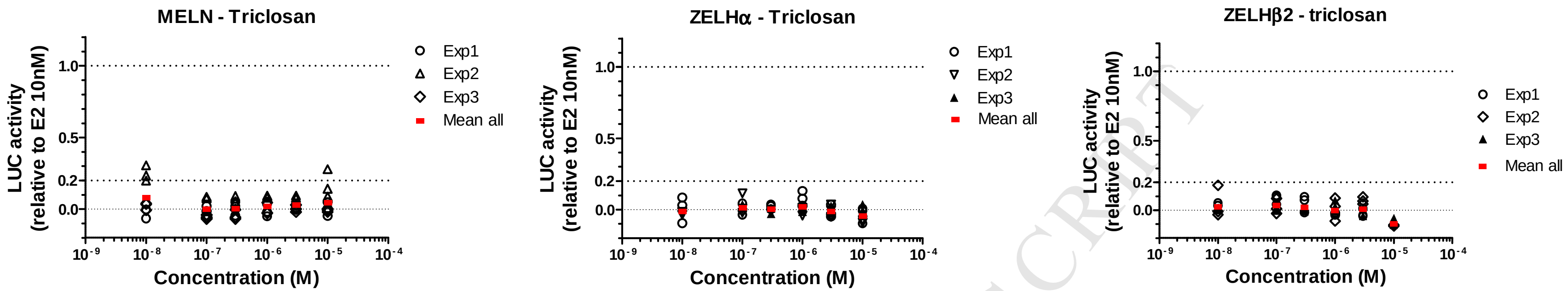
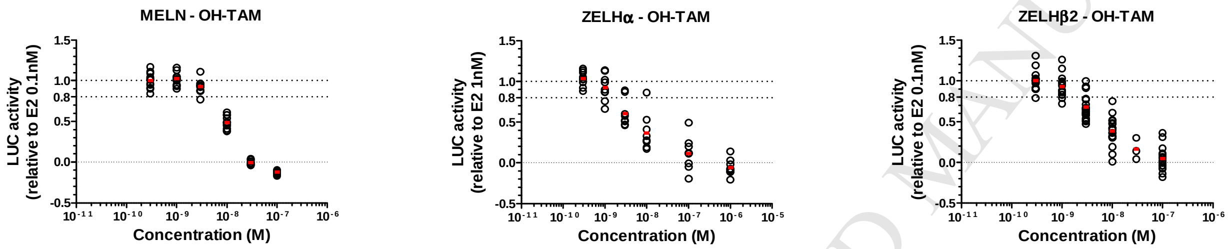
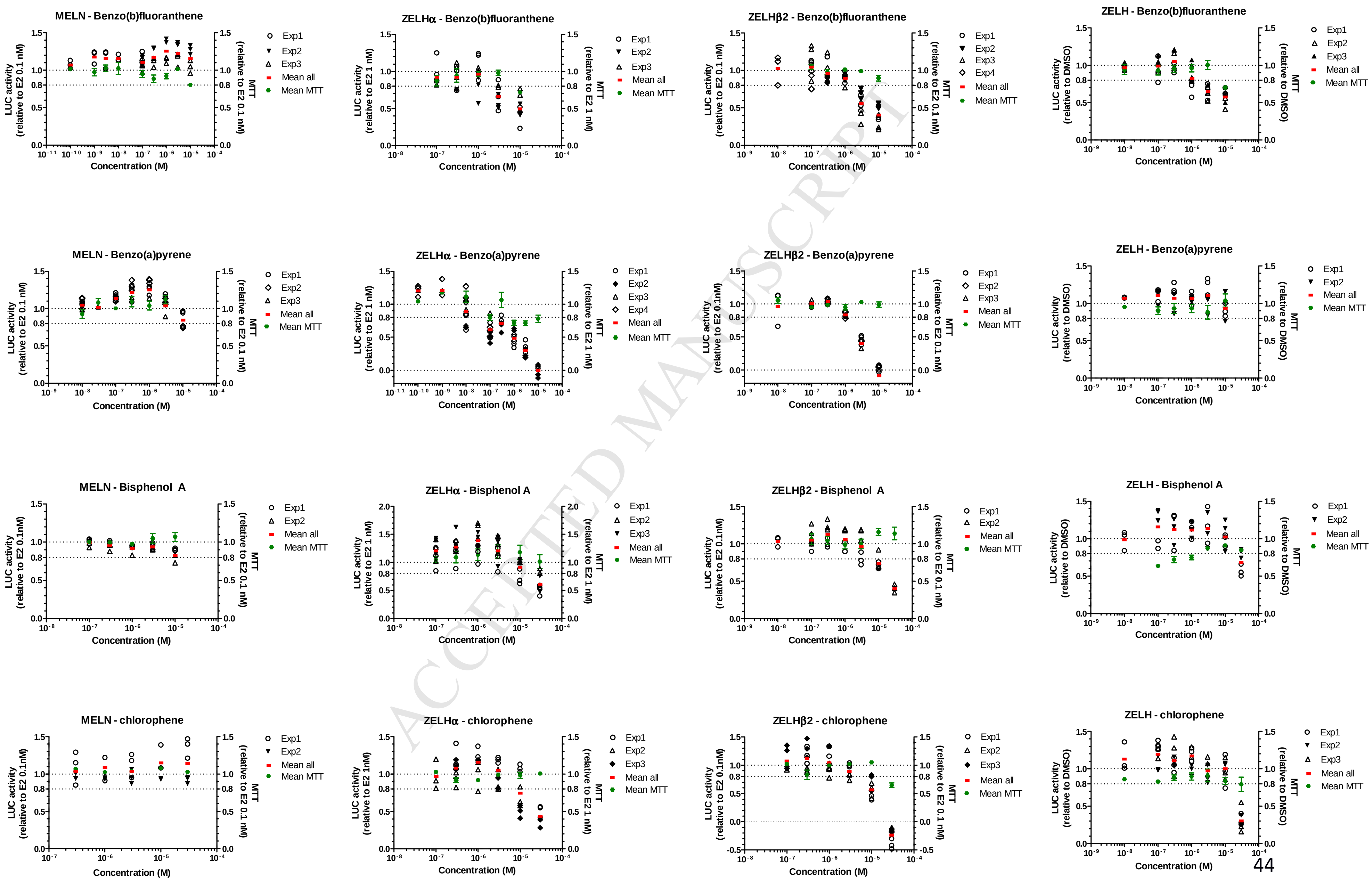
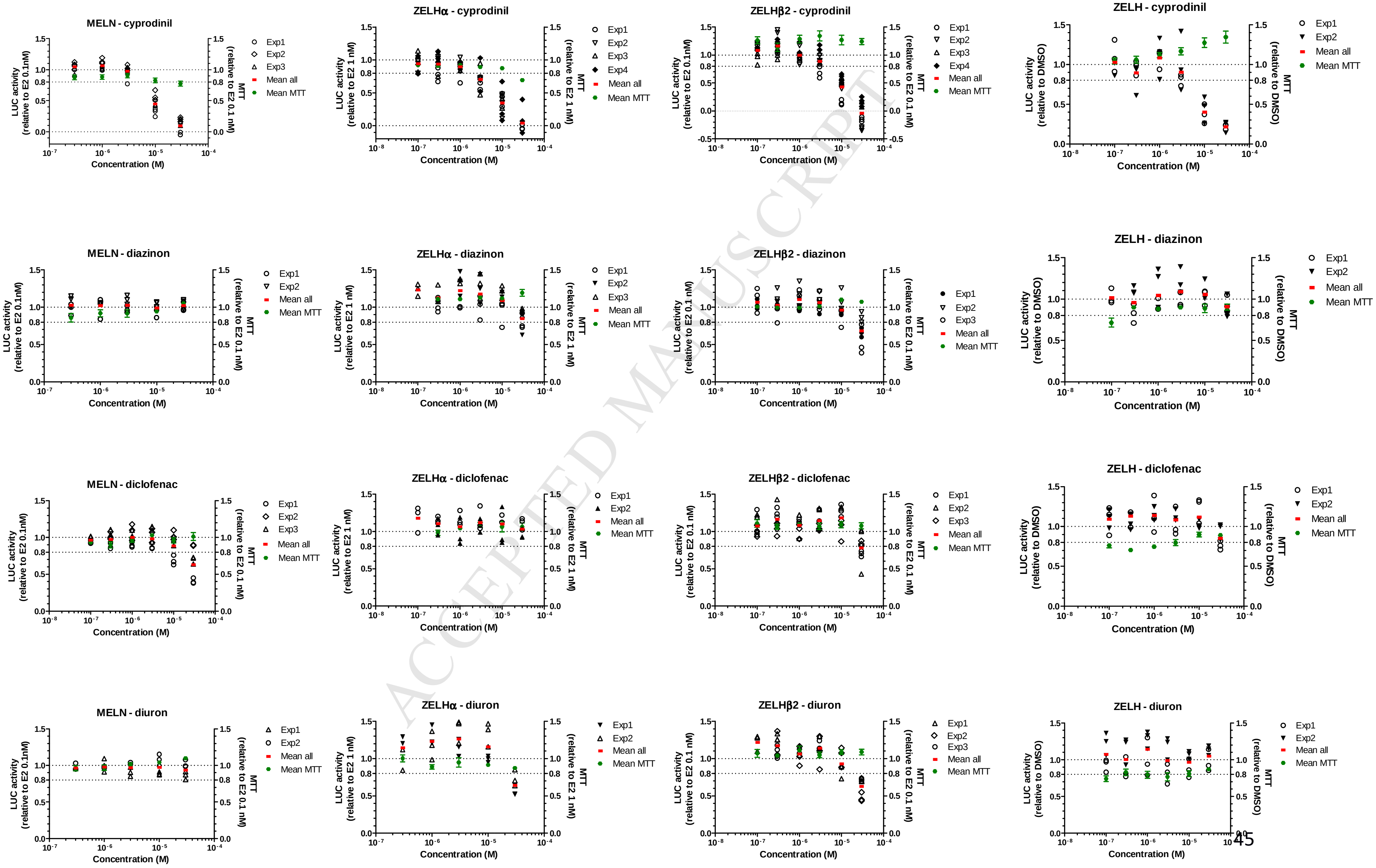

MELN - Triclosan
LUC activity
(relative to E2 10nM)
1.0
0.5
0.2
0.0
10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶ 10⁻⁵ 10⁻⁴
Concentration (M)
Exp1
Exp2
Exp3
Mean all
ZELHα - Triclosan
Exp1
Exp2
Exp3
Mean all
ZELHβ2 - triclosan
Exp1
Exp2
Exp3
Mean all

Figure SI 2: Response of the 12 chemicals on E2-induced ER inhibition in MELN, ZELHα, ZELHβ2 and ZELH cells. Data represent each replicate and the mean (red dash) of at least 2 independent experiments done in triplicates. Chemicals were tested in the 10 nM - 30 µM range. MELN and ZELHβ2 cells were co-exposed with 0.1 nM E2, and ZELHα and ZELH cells with 1 nM E2. Cell viability (MTT) was measured for at least one experiment and is represented in green full circles (mean +/- SD) on the right Y axis. The horizontal dotted line at 80% figures the threshold of effect (IC20). Hydroxy-tamoxifen (OH-TAM) was used as positive control.




MELN - Benzo(b)fluoranthene
ZELHα - Benzo(b)fluoranthene
ZELHβ2 - Benzo(b)fluoranthene
ZELH - Benzo(b)fluoranthene
MELN - Benzo(a)pyrene
ZELHα - Benzo(a)pyrene
ZELHβ2 - Benzo(a)pyrene
ZELH - Benzo(a)pyrene
MELN - Bisphenol A
ZELHα - Bisphenol A
ZELHβ2 - Bisphenol A
ZELH - Bisphenol A
MELN - chlorophene
ZELHα - chlorophene
ZELHβ2 - chlorophene
ZELH - chlorophene
LUC activity (relative to E2 0.1 nM)
LUC activity (relative to E2 1 nM)
LUC activity (relative to DMSO)
MTT (relative to E2 0.1 nM)
MTT (relative to E2 1 nM)
MTT (relative to DMSO)
Concentration (M)
Exp1
Exp2
Exp3
Exp4
Mean all
Mean MTT


MELN - cyprodinil
ZELHα - cyprodinil
ZELHβ2 - cyprodinil
ZELH - cyprodinil
MELN - diazinon
ZELHα - diazinon
ZELHβ2 - diazinon
ZELH - diazinon
MELN - diclofenac
ZELHα - diclofenac
ZELHβ2 - diclofenac
ZELH - diclofenac
MELN - diuron
ZELHα - diuron
ZELHβ2 - diuron
ZELH - diuron
LUC activity (relative to E2 0.1nM)
LUC activity (relative to E2 1 nM)
LUC activity (relative to E2 0.1 nM)
LUC activity (relative to DMSO)
MTT (relative to E2 0.1 nM)
MTT (relative to E2 1 nM)
MTT (relative to DMSO)
Concentration (M)
Exp1
Exp2
Exp3
Exp4
Mean all
Mean MTT

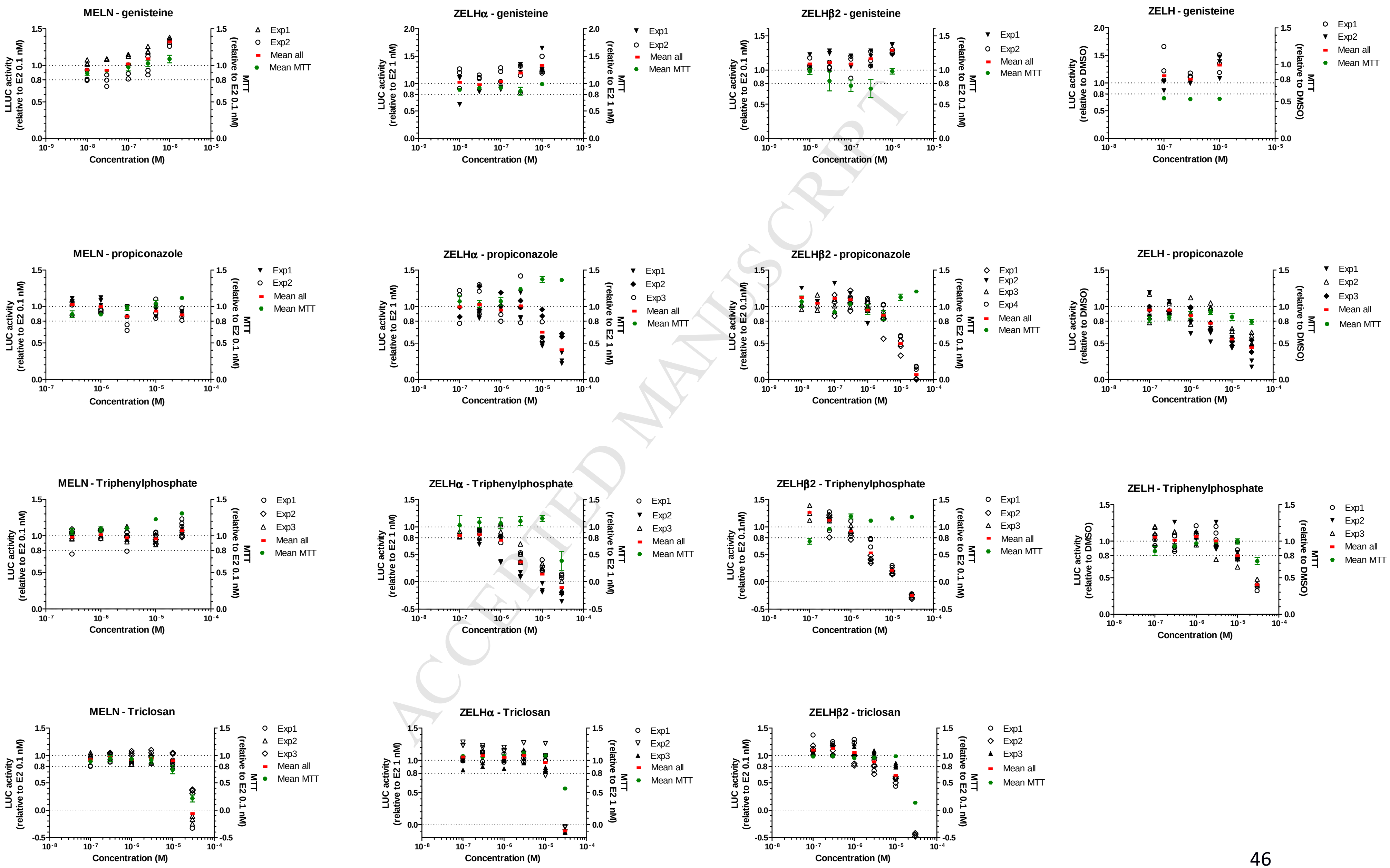

MELN - genisteine
ZELHα - genisteine
ZELHβ2 - genisteine
ZELH - genisteine
MELN - propiconazole
ZELHα - propiconazole
ZELHβ2 - propiconazole
ZELH - propiconazole
MELN - Triphenylphosphate
ZELHα - Triphenylphosphate
ZELHβ2 - Triphenylphosphate
ZELH - Triphenylphosphate
MELN - Triclosan
ZELHα - Triclosan
ZELHβ2 - triclosan
LUC activity (relative to E2 0.1 nM)
LUC activity (relative to E2 1 nM)
LUC activity (relative to DMSO)
MTT (relative to E2 0.1 nM)
MTT (relative to E2 1 nM)
MTT (relative to DMSO)
Concentration (M)
Exp1
Exp2
Exp3
Exp4
Mean all
Mean MTT

Figure SI 3: Cyprodinil response in MELN, ZELHα, ZELHβ2 and ZELH cells. The response was measured with cyprodinil alone (ER, luciferase induction relative to DMSO control) or in presence of E2 (antiER, luciferase induction relative to E2 positive control). Data represent the mean (+/- SD) of a minimum of 2 independent experiments done in triplicates and pooled together.

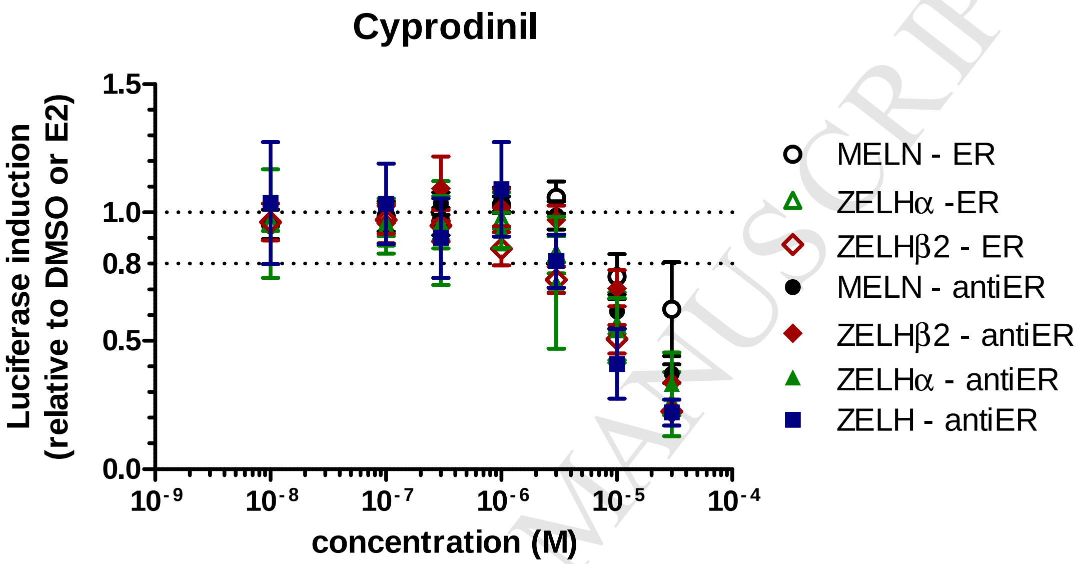
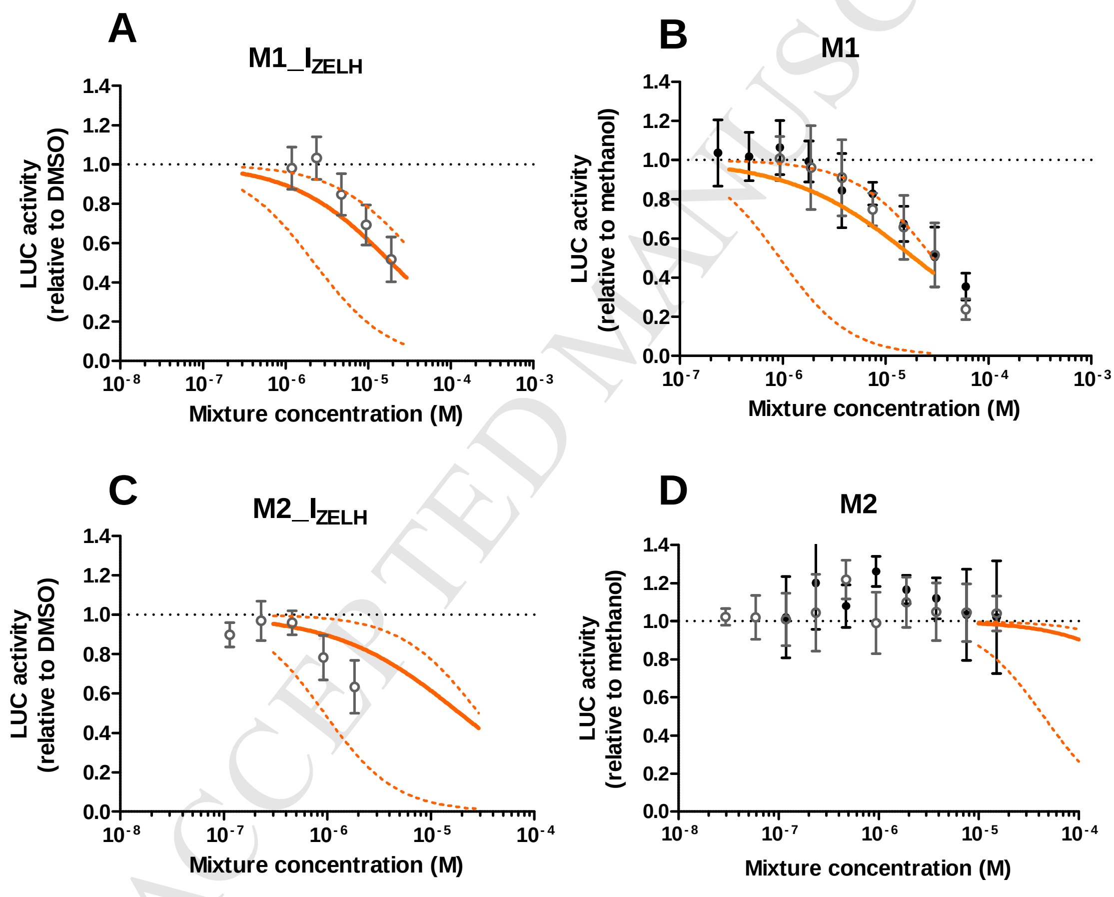


Figure SI 4: Predicted and observed effects of inhibiting chemicals on ZELH cells. Results of subgroup mixtures M1_I_{ZELH} (A), M2_I_{ZELH} (B), and 12-component mixtures M1 (B) and M2 (D). Mixture effects were predicted according to CA model (orange line, 95% CI belt). Luciferase (LUC) activity was measured in absence (black circles) or in presence of E2 (co-exposure with E2 at 1 nM, grey open circles). The data (mean +/- SD) originate from at least 2 independent experiments done in triplicates and pooled together. Cytotoxic concentrations (measured by MTT) were removed.



Highlights:

- 12-chemical mixtures including xenoestrogens were tested in ER-reporter gene assays
- Human and zebrafish cells had distinct estrogenic response to the mixtures
- Several ER inhibitors were identified but in zebrafish cells only
- Inhibitors decreased the ER response in zebrafish cells compared with expected CA
- Non-estrogenic chemicals influenced ER mixture response in a cell-specific manner