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Complex Interactions between Dioxin-Like and Non-Dioxin-Like Compounds for *in Vitro* Cellular Responses: Implications for the Identification of Dioxin Exposure Biomarkers

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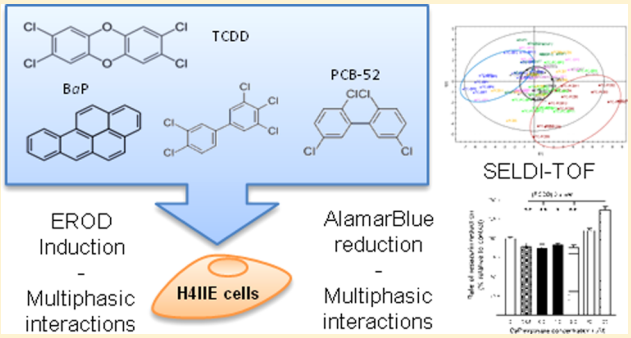
Supporting Information

ABSTRACT: Despite considerable advances in reducing the production of dioxin-like toxicants in recent years, contamination of the food chain still occasionally occurs resulting in huge losses to the agri-food sector and risk to human health through exposure. Dioxin-like toxicity is exhibited by a range of stable and bioaccumulative compounds including polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), produced by certain types of combustion, and man-made coplanar polychlorinated biphenyls (PCBs), as found in electrical transformer oils. While dioxinergic compounds act by a common mode of action making exposure detection biomarker based techniques a potentially useful tool, the influence of co-contaminating toxicants on such approaches needs to be considered. To assess the impact of possible interactions, the biological responses of H4IIE cells to challenge by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in combination with PCB-52 and benzo-*a*-pyrene (B*a*P) were evaluated by a number of methods in this study. Ethoxyresorufin-*O*-deethylase (EROD) induction in TCDD exposed cells was suppressed by increasing concentrations of PCB-52, PCB-153, or B*a*P up to 10 μM. B*a*P levels below 1 μM suppressed TCDD stimulated EROD induction, but at higher concentrations, EROD induction was greater than the maximum observed when cells were treated with TCDD alone. A similar biphasic interaction of B*a*P with TCDD co-exposure was noted in the AlamarBlue assay and to a lesser extent with PCB-52. Surface enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF) profiling of peptidomic responses of cells exposed to compound combinations was compared. Cells co-exposed to TCDD in the presence of B*a*P or PCB-52 produced the most differentiated spectra with a substantial number of non-additive interactions observed. These findings suggest that interactions between dioxin and other toxicants create novel, additive, and non-additive effects, which may be more indicative of the types of responses seen in exposed animals than those of single exposures to the individual compounds.



1. INTRODUCTION

Despite huge advances in reducing their prevalence and production in recent decades, dioxin-like toxicants remain a significant concern for public health and the environment. Human and animal exposure is predominantly via the consumption of contaminated food. Contamination of the human food chain has resulted in large losses to the agricultural sector through product recalls and reputational damage. Dioxins still commonly find their way into the food chain through contamination of animal feeds,[1−3] and methods of detection are of great importance.

The toxic mode-of-action of dioxins is typified by the compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); the most potent dioxin, and to a lesser degree by a number of other dibenzodioxin (PCDDs), dibenzofuran (PCDFs) and polychlorinated biphenyl (PCB) compounds. These are stable, persistent, lipophilic chemicals, which accumulate readily in the fatty tissues of animals that consume them, meaning very low level contaminated material fed to animals can lead to excess dioxin levels in a wide range of primary and processed foodstuffs.[4,5]

Cell-based *in vitro* methods have been devised to screen samples for dioxin-like activity and are based on the ability of dioxins to induce, in a dose-dependent manner, the cytochrome P450 1A1 (CYP1A1) enzyme system as measured by ethoxyresorufin-*O*-deethylase (EROD) activity of exposed cells[7,8] or the dioxin-responsive chemically activated luciferase gene expression (DR-CALUX) assay, which uses a luciferase transfected cell line that responds to CYP1A1 upregulation.[9,10]

Dioxins produce a myriad downstream biochemical responses in almost all tissues of an exposed organism, as demonstrated by the huge variety of toxic and biochemical end-points associated with these compounds.[11,12] The response and toxic outcome is highly dependent upon the developmental stage of the animal as well as sex, diet, stress level, and other chemical and

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environmental challenges.[13−15] The presence of other cotoxicants will, therefore, be likely to influence the biological response to intoxication. This will also determine many aspects of the toxic outcome and measurable biological indicators of exposure.

Dioxin-like compounds tend to originate and enter the food chain from two dominant sources: residues of dioxin-generating combustion during the drying of feed ingredients[2] or from the contamination of the oil component of feed by PCBs.[16] For dioxins produced through combustion, the dioxinergic chemicals involved in exposure will include a range of dioxin and furan congeners,[17,18] but these will be present alongside a range of other well characterized classes of toxicants. (Note: The term dioxinergic is used in this article to denote a compound that exhibits a dioxin-specific mode of action in toxicological terms. It is used interchangeably with the term dioxin-like for clarity when discussion of the toxicity of different types of compounds could result in confusion.) Polynuclear aromatic hydrocarbons (PAHs) are the most toxicologically relevant due to their similarity in terms of lipophilicity and environmental persistence. In dioxin contamination associated with PCB oils, the dioxinergic contribution is principally from non-ortho coplanar PCB congeners, rather than dioxins and furans, but a large amount of mono- and diortho-substituted noncoplanar PCBs are also present.[19] PAHs are known as potent carcinogens due to the formation of reactive epoxides which form DNA and protein adducts promoting mutation.[20] *ortho*-Substituted PCBs also have distinctive neurotoxic and immunotoxic effects[21,22] not exhibited by the dioxin-like congeners. Measurable biological responses have become important tools in the detection of dioxins throughout the food chain as direct detection and quantification of all the relevant dioxinergic compounds in a sample is costly and time-consuming.[6]

While the effects of compounds with dioxin-like toxicity have been studied extensively with a view to understanding the potential for co-exposure to result in non-additive effects and to support the WHO Toxic Equivalence scheme (TEQ), the present study aimed to investigate the toxicological interactions of dioxin and potential co-contaminating non-dioxin-like toxicants. The study's aim was to demonstrate the presence and extent of the interactions, the pathways involved, and the dose−response relationships of the cotoxicants in cell culture with a view to demonstrating to what extent these issues may influence *in vivo* models of dioxin exposure, in particular, the suitability of using preparative mixtures of PCDDs, PCBs, and PAHs to simulate real exposure conditions. The H4IIE cell line is a transformed rat hepatoma cell line with an intact cytochrome P450 enzyme system, and it has been used extensively as a model system for the evaluation of AhR mediated toxicity. H4IIE cells were exposed to a wide concentration range of selected compounds in order to evaluate a range of cellular responses which could indicate interactions among TCDD, the ortho-substituted PCBs, PCB-52 and PCB-153, and the PAH, B*a*P. These compounds were chosen as prototypical effectors of the toxic mode-of-action associated with their compound class.[20,21,23] Responses to exposure were investigated using a range of techniques representing both direct, established induction pathways (EROD induction assay) and less targeted end points including the reduction of resazurin dye (AlamarBlue assay) and untargeted peptidomic profiling using SELDI-TOF mass spectrometry to measure changes in intracellular peptide abundance post-exposure.

2. EXPERIMENTAL PROCEDURES

2.1. Reagents and Consumables. TCDD, PCB-52 (2, 2′,5,5′-tetrachlorobiphenyl), and benzo-*a*-pyrene (B*a*P) were purchased as neat certified reference standards from Accustandard Inc. (New Haven, CT, USA) and dissolved in DMSO. Modified Eagle's medium, pyruvate solution, dicoumerol (3,3′-methylene-bis(4-hydroxycoumarin), PCB-153 (2,2′,4,4′,5,5′-hexachlorobiphenyl), DMSO (Biotech grade), CHAPS, urea, resorufin ethyl ether, and Bradford reagent for protein estimation were purchased from Sigma-Aldrich (St. Louis, MI, USA). Protease Inhibitor Cocktail Complete Mini was from F. Hoffman-La Roche (Basel, Switzerland). Fetal calf serum, L-glutamine, and AlamarBlue (resazurin) were from Invitrogen (San Diego, CA, USA). ProteinChip Array CM10 and ProteinChip SPA Matrix (sinapinic acid) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Pierce bicinchoninic acid (BCA) protein assay reagent kit was obtained from Thermo Fisher Scientific (Rockford, IL, USA). Cells were cultured in Nunc flasks obtained from Thermo Fisher Scientific (Rockford, IL, USA).

2.2. Cell Culture. H4IIE cells were purchased from ATCC (CRL-1548) and grown in modified Eagle's medium (Sigma M5650) with 1 mM pyruvate, 2 mM L-glutamine, and 10% fetal calf serum (FCS) (H4IIE growth medium) and grown at 37 °C with 8% CO_2. Cells were cultured in standard 25 cm^2 adherent cell culture flasks, in 10 mL of growth medium, and passed at 70−90% confluence from one flask into four. The turnover rate between passage was 2−3 days.

2.3. AlamarBlue Cell Viability Assay. H4IIE cells were seeded into 96 well cell culture plates at a density of 1×10^4 cells per well. After 24 h of incubation at 37 °C and 8% CO_2, the media from each well were removed and replaced with 100 μL of spiked media containing various combinations of toxicants in 0.3% (v/v) final DMSO concentration. Over a number of experiments, toxicants were tested in the following concentration range: TCDD, 5×10^{-9} M to 1×10^{-6} M; PCB-52, 1×10^{-8} M to 1×10^{-5} M; and B*a*P, 1×10^{-8} M to 2×10^{-5} M along with negative controls. The concentration range is higher than that often associated with dioxinergic response in cell culture as it was designed to show overt cell lethality. After further incubation for 24 h, 10 μL of AlamarBlue solution was added to each well, incubated at 37 °C with absorbance measurements taken at 562 nm at a number of time points from 30 min to 8 h using a Tecan SAFIRE II plate reader. Statistical significance of differences were determined on raw data using ANOVA with Bonferroni's post-hoc multiple comparison test.

2.4. EROD Assays. *2.4.1. EROD Induction Activity Assay.* EROD activity was measured according to a modification of the method of Peters et al.[24] Briefly, H4IIE cells were seeded at a density of 1×10^4 cells in 100 μL of medium into 96-well, white walled, clear bottomed, cell culture plates (Nunc, Thermo Fisher Inc.). Cells were incubated for 24 h after which growth medium was removed and replaced with 100 μL of exposure medium per well. The exposure compounds were diluted in DMSO, then spiked into normal H4IIE growth medium in a range of concentrations over a number of experiments in binary combinations of TCDD from 6×10^{-14} to 1×10^{-9} M; PCB-52 from 1×10^{-5} to 2×10^{-11} M; B*a*P from 2×10^{-5} to 2×10^{-11} M; and PCB-153 from 1×10^{-5} to 2×10^{-11} M with a final DMSO concentration of 0.3% (v/v) in all wells. PCB-153 was added into this part of the study as another non-dioxin-like PCB, to give additional insight into the interaction between TCDD and these compounds. Cells were incubated in this medium at 37 °C and 8% CO_2 for 48 h after which the medium in each well was replaced with 100 μL of EROD substrate medium (H4IIE growth medium without FCS, containing 10 μM dicoumerol, 5 mM $MgCl_2$, 5 μM ethoxyresorufin, and 0.1% DMSO). Plates were incubated at 37 °C and 8% CO_2, and formation of resorufin was followed using fluorescence measured at time points from 10 min to 1 h using a Tecan SAFIRE2 plate reader; excitation λ, 530 nm (20 nm bandwidth); emission λ, 590 nm (20 nm bandwidth). The protein content of each well was determined by BCA protein assay (Pierce) using a 4-parameter calibration with BSA, following lysis of cells by five successive rounds of freezing (−80 °C) and thawing plates with 100 μL of 0.2 M phosphate buffer (pH 7.0) in

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each well. Statistical significance of differences were determined on raw data using ANOVA with Bonferroni's post-hoc multiple comparison test.

2.4.2. Inhibition of EROD Enzyme Activity. To assess if tested chemicals interacted only at the activation level or exhibited a direct inhibitory effect on the kinetics of the EROD enzyme itself, cells were activated with 1×10^{-9} M TCDD to give maximum uniform induction of enzyme activity. After 48 h of incubation at 37 °C and 8% CO_2, the media were exchanged with EROD substrate medium containing 0.3125 μM to 5 μM ethoxyresorufin to which toxicants had been added in the following concentrations: PCB-52, 1×10^{-4} to 1×10^{-5} M; B*a*P, 2×10^{-4} to 2×10^{-6} M; and PCB-153, 1×10^{-4} to 1×10^{-5} M, or a DMSO control. The final DMSO concentration for all wells was 0.3% (v/v). An additional group treated with 1 nM TCDD was included for comparison. Formation of resorufin was monitored as described in 2.4.1. Conversion of substrate to resorufin by the EROD enzyme was followed fluorimetrically. Data were normalized to the highest reading in each plate and fitted into a Michaelis–Menten plot using $Y = V_{max} \cdot X/(K_m + X)$. K_m and V_{max} values were obtained for each inhibited and uninhibited set of data, allowing the calculation of apparent K_i values for compounds as shown in Table 2. The mean K_i for each compound was calculated using the following equation for each inhibiting concentration: $K_i = [K_m][I]/K_{m_{app}} - K_m$.

2.5. Surface Enhanced Laser Desorption/Ionization-Time of Flight (SELDI-TOF) Mass Spectrometry Analysis. H4IIE cells were grown to 70% confluence in eight 75 cm^2 cell culture flasks (Nunc, Thermo Fisher) from a single passage. The medium in each flask was then replaced with 20 mL of normal H4IIE growth medium containing TCDD, PCB-52, and/or B*a*P from stock solutions prepared in cell culture grade DMSO. The labels and final concentrations of each treatment flask were as follows: "CTRL" (DMSO only)," TCDD" (2 nM TCDD), "TCDD+PCB" (2 nM TCDD + 15 nM PCB-52), "TCDD+PCB+BaP" (2 nM TCDD + 15 nM PCB-52 + 20 μM B*a*P), "PCB" (15 nM PCB-52), "PCB+BaP" (15 μM PCB-52 + 20 μM B*a*P), "BaP" (20 μM B*a*P), and "TCDD+BaP" (2 nM TCDD + 20 μM B*a*P). These labels are used throughout the study, unless otherwise indicated. The final concentration of DMSO in all media was adjusted to 0.3% (v/v). Flasks were incubated for 24 h at 37 °C and 8% CO_2, and after removal of exposure media, cells were washed twice with 10 mL of cold PBS at pH 7.4. The cells were then scraped off and rinsed with 1 mL of PBS into a centrifuge tube, and 1 mL of SELDI-compatible lysis buffer (PBS, pH 7.4, containing 8 M urea, 4% CHAPS, and protease inhibitor cocktail) was added. After 10 min on ice, the cell suspensions were centrifuged at 15,700*g* for 15 min at 4 °C, and the supernatant removed and frozen in 100 μL aliquots at −80 °C overnight until analysis. Protein content was estimated using the Bradford reagent microassay, read at 595 nm after 20 min of incubation at 37 °C, and all lysates were adjusted to a protein concentration of 200 μg/mL by the addition of lysis buffer. Fifty microliters of adjusted cell lysate (equivalent to 10 μg protein) was then added to 200 μL of 5 mM sodium acetate buffer at pH 4.5 and applied to the surface of a single spot on each of 12 BIO-RAD ProteinChip CM10 arrays for 10 min at 37 °C with shaking, using the supplied bioreactor array device, then washed with 3 rounds of phosphate buffered saline (PBS) at pH 7.4. Sinapinic acid was then applied as an energy absorbing molecule and allowed to dry, according to the manufacturer's instructions. Analysis was performed using a Ciphergen SELDI-TOF ProteinChip instrument operating Ciphergen ProteinChip Software (Ciphergen Biosystems, version 3.2.1.1216) at the following settings: mass deflector, 2000 Da; optimized, 2000 to 15000 Da; laser intensity, 170 (2 warming shots at 175, not included); detector sensitivity, 8; acquisition mode, SELDI quantitation; and positions, every 5 from 20–80 for each spot. Peak height data was exported to SIMCA-P+ (version 12.0.1) software for multivariate statistical analysis.

3. RESULTS

3.1. Cytoxicity and Cell Viability. H4IIE cells were exposed to TCDD, PCB-52, and B*a*P for 24 h in a range of concentrations. The cells were then exposed to AlamarBlue reagent (resazurin), and the rate of reduction to resorufin was measured spectrophotometrically. The concentration of resorufin formed following all treatments increased in a sigmoidal progression, with a plateau reached between 4.5 to 6 h (data not shown). Data acquired from this time frame were used to perform comparisons in responses between cell treatment groups as shown in Figure 1. Significant differences by *t* test ($n = 6$) in the rate of AlamarBlue reduction were observed between groups exposed to binary mixtures of TCDD between

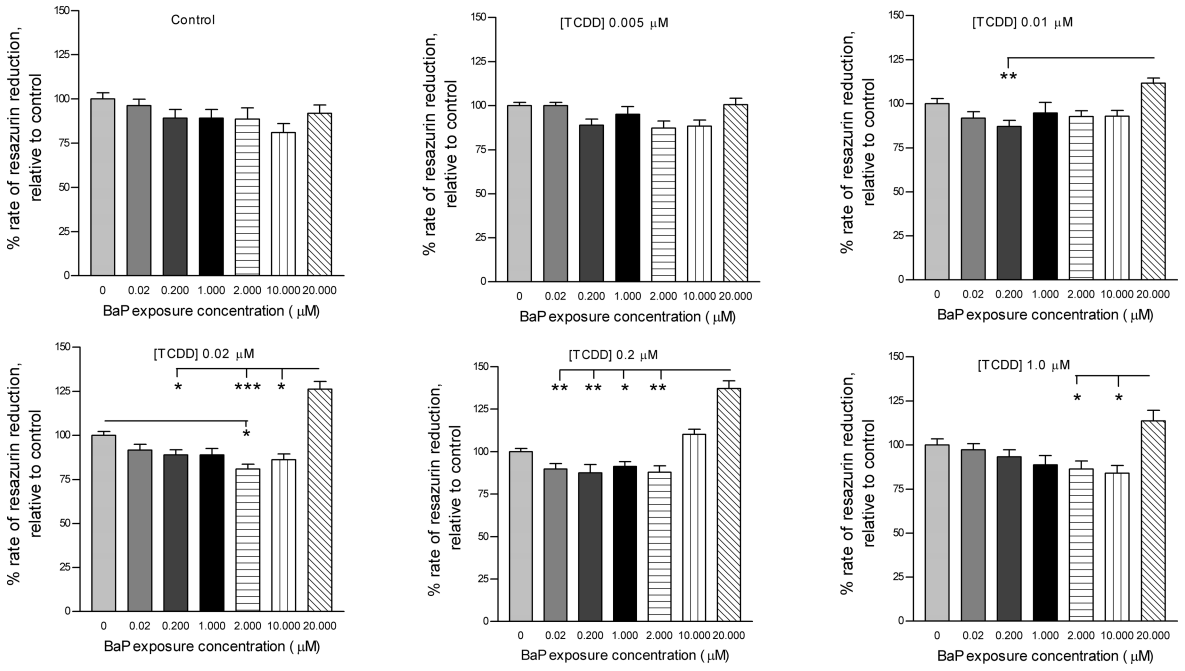

Figure 1. Effect of combined exposure to TCDD and B*a*P on H4IIE cell viability as determined by the AlamarBlue assay. Significance by ANOVA with Bonferroni's post hoc multiple comparison test indicated by asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$).

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Table 1. Key Parameters from Sigmoidal Fitting (4-Parameter Equation) of EROD Activity Induced by TCDD Concentrations from 1×10^{-14} to 1×10^{-9} in H4IIE Cells Exposed for 48 h, with Increasing B*a*P, PCB-52, or PCB-153 Concentration (n = 6, Replicate Plates)

B*a*P (nM)	10000	1000	100	10	1	0.1	0.01	0
Hillslope	0.905	0.994	1.56	1.44	0.754	1.45	1.51	1.48
EC50 (M)	7.36×10^{-12}	5.86×10^{-12}	5.44×10^{-12}	7.76×10^{-12}	6.66×10^{-12}	7.82×10^{-12}	7.19×10^{-12}	8.89×10^{-12}
bottom	43.40	17.26	8.586	7.633	8.448	7.735	7.385	7.596
top	83.08	92.94	95.66	88.47	93.03	86.66	81.59	89.87
PCB52 (nM)	10000	1000	200	20	2	0.2	0.02	0
Hillslope	0.8269	1.265	1.090	0.8456	1.232	1.171	1.493	1.359
EC50 (M)	4.83×10^{-12}	8.16×10^{-12}	1.53×10^{-11}	1.18×10^{-11}	1.04×10^{-11}	1.36×10^{-11}	9.94×10^{-12}	1.01×10^{-11}
bottom	25.61	5.895	5.321	5.113	5.196	5.126	5.188	5.134
top	99.09	82.13	86.91	75.79	81.15	79.66	73.90	76.00
PCB153 (nM)	10000	2000	200	20	2	0.2	0.02	0
Hillslope	1.723	1.522	1.966	1.484	1.534	1.534	1.538	1.376
EC50 (M)	1.11×10^{-11}	8.33×10^{-12}	8.16×10^{-12}	8.24×10^{-12}	7.56×10^{-12}	1.01×10^{-11}	8.33×10^{-12}	8.95×10^{-12}
bottom	7.256	7.165	7.236	7.060	7.208	7.591	7.010	6.732
top	86.87	82.18	76.52	79.42	77.91	78.83	82.56	95.57

0.005 μM and 1 μM and PCB-52 or B*a*P within the range 0.02−20 μM. However, no clear dose dependent relationship was discernible from these interactions, and no significant change with exposure to any concentration of TCDD used in the absence of another compound was observed. With increasing levels of B*a*P, there was an apparent "scooped" response profile as seen in Figure 1, with a significant decrease in rate compared to that of the control noted at intermediate concentrations but not at the highest. This was most evident between 0.01 and 1 μM TCDD, where treatment with lower concentrations of B*a*P resulted in a decreased rate of AlamarBlue reduction. In contrast at 2−20 μM B*a*P, there was an apparent increase in the rate of reduction with increasing TCDD concentrations. Treatment of cells with PCB-52 produced a similar but less significant effect than that observed following B*a*P and TCDD co-exposure as shown in Figure S1 (Supporting Information).

3.2. EROD Assays. *3.2.1. Induction of EROD Activity.* Again, H4IIE cells were exposed to a range of concentrations and combinations of TCDD, PCB-52, and B*a*P for 24 h. The EROD activity of the cells was then assessed by adding ethoxyresorufin, and the formation of resorufin was measured fluorimetrically at 10 min intervals after the addition of substrate for up to 60 min. The progression of resorufin formation within this time frame was shown to be linear where the induction of the enzyme was sufficient for measurement as illustrated in Figure S2 (Supporting Information). The rate of formation was also affected by the presence of the other toxicants, though in a complex multiphasic manner. The results are presented in units of nmol resorufin/mg protein/min, which ensures that the increase in activity is specific to the CYP1A1 enzyme itself and not as an increase in overall protein production by another pathway.

CYP1A1 induction within cells was measured through the increased EROD activity of cells to treatment with increasing concentrations of TCDD with initial induction observed at approximately 1×10^{-12} M and reaching an apparent maximum at 1×10^{-9} M. B*a*P, a known inducer of EROD, was found to induce activity in the concentration range from 1×10^{-7} to 1×10^{-5} M. PCB-52 produced no effect on EROD activity up to 1×10^{-5} M when cells were treated with individual compounds. Plotting log TCDD concentration against EROD activity produced a sigmoid logistical function with variable slope (4 parameter equation). The same concentrations of TCDD were applied to cells in the presence of increasing concentrations of B*a*P or PCB-52. The effects of co-exposure to other chemicals on the normal EROD inductive ability of TCDD was thereby robustly evaluated, by their influences on the fitting of the sigmoidal function to the TCDD data, as summarized in Table 1. The effects of PCB-52 and B*a*P were non-additive when compared to the effects of the individual compounds at the same concentrations, and the values were significantly different by ANOVA (p = <0.05).

Table 1 demonstrates that there is agreement in terms of all four parameters between curves from cells treated with TCDD only (PCB or B*a*P 0 nM) in each experiment, indicating good reproducibility between assays on different days. The TCDD-B*a*P coexposed curves show a clear influence of co-exposure, which is broadly in line with the additive induction by B*a*P alone at 10000 and 1000 nM and displays an elevated value for the bottom of the curve without significantly changing other reported parameters. Treatment of cells with 10000 nM PCB-52 produced an unusually elevated curve across the whole TCDD concentration range (6×10^{-14} to 1×10^{-9} M), and cells exposed to lower concentrations of PCB-52 produced TCDD induction curves which differed from the control, but there was no progression of this effect with increasing PCB-52. It appears to be a noncontinuous effect observed with exposure to 10000 nM PCB-52 only.

Additional experiments were performed focusing on the effect of cotoxicants at three levels of induction. Cells were exposed to TCDD at 1×10^{-10} M, 1×10^{-11} M, and 1×10^{-12} M, representing a high level, midpoint, and low level of EROD induction by TCDD. These results are illustrated in Figure 2 with B*a*P showing an initial suppression of induction due to TCDD alone, at both 1×10^{-10} M and 1×10^{-11} M TCDD. However, the higher levels of B*a*P increased the level of induction above that shown for the highest level of TCDD alone at 2000 to 10000 nM B*a*P at 1×10^{-10} M TCDD and 200−1000 nM B*a*P at 1×10^{-11} nM TCDD. Variability of EROD measurements also significantly increased at the highest level of induction. PCB-52 suppresses EROD induction levels at concentrations as low as 2×10^{-11} M with a gradual increase in suppression up to 2×10^{-9} M at all induction levels. At induction with 1×10^{-10} M TCDD, the suppressive effect of PCB-52 on induction levels produced by TCDD alone lessened

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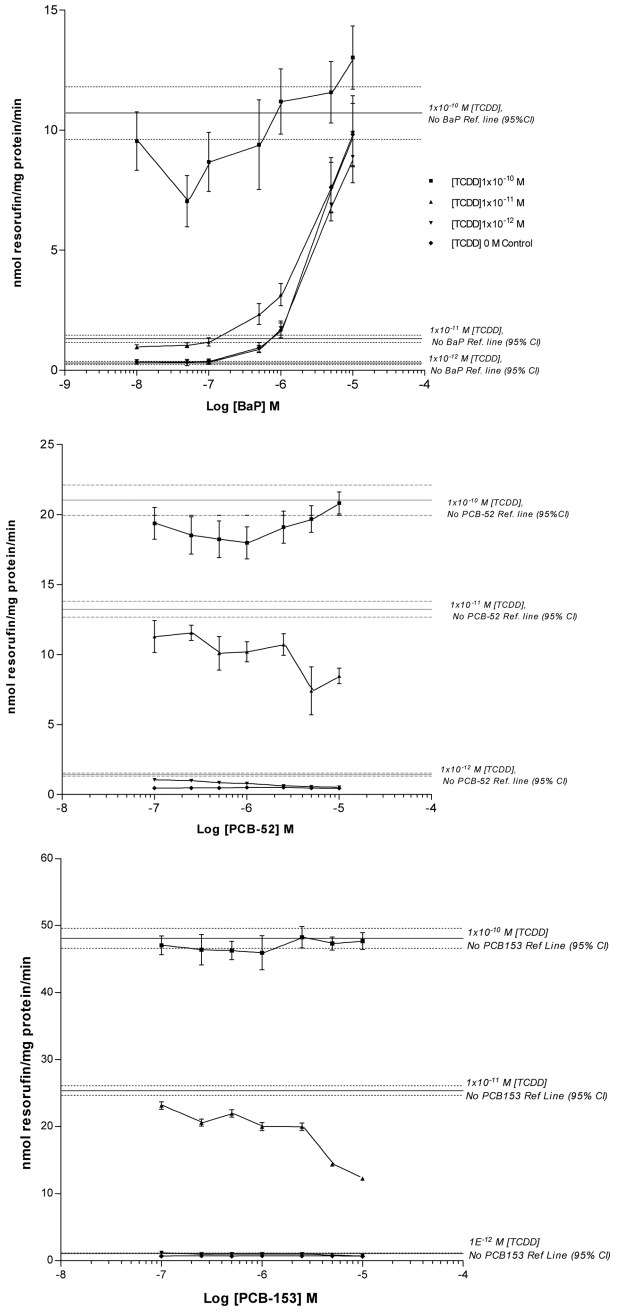

Figure 2. Effects of co-exposure with PCB-52 PCB-153 and B*a*P on EROD activity in H4IIE cells induced by exposure to TCDD at 1 × 10−10 M (ν), 1 × 10−11 M (π), and 1 × 10−12 M (□) for 48 h. Horizontal lines indicate the mean induction level of TCDD alone (n = 6), with 95% confidence intervals (CI) shown by dotted lines. Error bars indicate 95% CI for data (n = 9, 3 wells in each of 3 plates).

sharply at 1000 nM PCB-52 and reached normal levels of induction at 10000 nM PCB-52. At the 1 × 10^{-11} M TCDD induction level, the suppressive effect was more apparent, lowering the induction level from ∼55% to ∼35% as the concentration of PCB-52 increased. PCB-153 co-exposure showed no significant difference from the cells exposed only to TCDD at the highest TCDD exposure (1 × 10^{-10} M TCDD); however, at 1 × 10^{-11} M increasing PCB-153 concentration had a clear and significant dose dependent decrease on EROD activity. The effect responsible for the raised curve profile at 10000 nM PCB-52 as illustrated in Table 1 and described above is not apparent from these results.

3.2.2. EROD Inhibition. H4IIE cells were exposed to TCDD at 1 × 10^{-9} M for 48 h to induce maximum production of EROD activity and then treated with a range of ethoxyresorufin concentrations in the presence of B*a*P, PCB-52, or PCB-153, and the results are presented in Table 2. B*a*P, PCB-52, and

Table 2. Michaelis−Menten Derived Kinetic Parameters for EROD Activity in H4IIE Cells Exposed to 1 nM TCDD for 48 h, with Substrate Measurements Taken in the Presence of B*a*P, PCB-52, and PCB-153 (n = 3)

compound	treatment during EROD substrate addition		K_i value (μM)	
[B*a*P]	10000 nM	1000 nM	0	7.16
V_{max}	98.36	96.49	97.61	
K_m (nM)	0.7904	0.8133	0.4482	
[PCB-52]	10000 nM	1000 nM	0	14.0
V_{max}	96.45	51.39	108	
K_m (nM)	0.7496	0.1988	0.5603	
[PCB-153]	10000 nM	1000 nM	0	20.6
V_{max}	100.7	95.73	97.2	
K_m (nM)	0.9436	1.622	0.7558	
		mean uninhibited K_m for EROD	0.5881 μM	

PCB-153 were each shown to have a significant and distinctive influence on the kinetics of the EROD enzyme itself as shown in Figure S3 (Supporting Information). PCB-52 behaved primarily as a classical competitive inhibitor, suppressing V_{max} and increasing the Michaelis constant (K_m); other compounds showed somewhat different behavior, which does not conform to the Michaelis−Menten model, as demonstrated by R^2 values for the fitting falling below 95% for the inhibited data. The presence of either PCB-153 or B*a*P at any concentration used, resulted in a significant lowering of rate of resorufin production by EROD at 2.5 μM substrate concentration, relative to the uninhibited rate and the rate in the presence of 1 nM TCDD. In contrast, the rate of product formation at 5 μM substrate was increased relative to the controls, and the profile of these measurements suggests that V_{max} had not been reached. This departure is masked somewhat by the application of the Michaelis−Menten equation to the fitting of the data; hence, both this fitting and a point-to-point fitting are shown in Figure S3 (Supporting Information) for direct comparison.

3.3. SELDI-TOF Data. H4IIE cells were incubated for 24 h in the presence of combinations of TCDD, PCB-52, and B*a*P and the resulting cell lysates analyzed by SELDI-TOF. Comparable spectra of peaks with a mass to charge ratio (m/z) of 2000−15000 were obtained from each cell treatment group. Figure S4 (Supporting Information) shows an example of a typical spectra obtained. Changes in peak height at a particular m/z value indicated the relative abundance of the corresponding peptide in the original sample. A number of control unexposed cell (CTRL) spectra possessed a large anomalous peak in the 11,000 m/z region of 5 of the spectra, which was absent in all other spectra. These spectra were excluded to minimize skewing of subsequent analysis. Then, 198 peaks were manually selected from the SELDI spectra, and 103 were shown to have a p-value of <0.05 for difference between treatment groups as determined by the Biomarker Wizard software function of the Ciphergen ProteinChip software package. The cluster information was imported to SIMCA (v13.0) software as a table of relative

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intensity data for each biomarker cluster and analyzed using principle component analysis (PCA) to show unsupervised relationships (R2x, 0.802; Q2x, 0.603). Orthogonal partial least squares−discriminant analysis (OPLS-DA) was then used to demonstrate the difference in spectra influenced by the conditions in each group. The scores plot of this analysis (Figure 3, top)

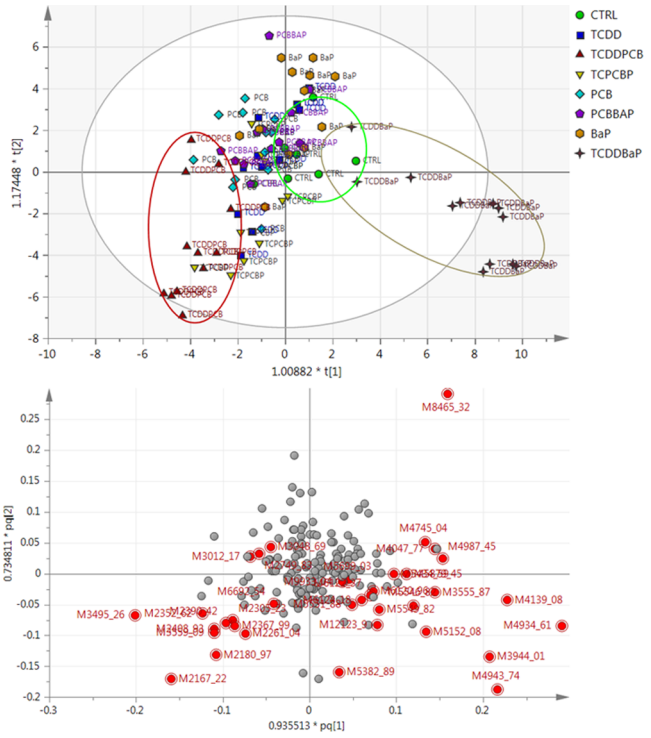

Figure 3. (Top) OPLS-DA scores plot of SELDI-TOF peptidomic profile data from H4IIE cells following exposure to combinations of TCDD, PCB, and B*a*P for 24 h. Ellipses are included to highlight the separation of the groups exposed to TCDD and PCB-52 together (red triangle "TCDDPCB" left, red ellipse) and TCDD and B*a*P together (brown diamond, right, brown ellipse), in addition to the control, unexposed group shown in green (green circles, CTRL, center-left, green ellipse). (Bottom) Loadings plot showing variables which contribute to the separation of the group. Variables significantly influenced by interactions between TCDD and the other toxicants (included in Table 3) are labeled showing their juxtaposition with the scores plot clusters for the co-exposed cells above.

indicates some separation of observations by treatment group. Of these, the clearest separation is between the spectra generated from the cells exposed to B*a*P or PCB-52 in the presence of TCDD as indicated by the ellipses in the figure. The group co-exposed to TCDD and PCB-52 is also the only group to show clear separation from the unexposed control cells (CTRL). The classification rate of the OPLS-DA analysis is calculated at 86.81% as shown in Table S1 (Supporting Information). Fisher's probability could not, however, be calculated on this analysis. A shared and unique structures (SUS) plot was also constructed (Figure 3, bottom) by comparing the models generated by OPLS-DA analysis of 2 subsets of the data comprising cells exposed to TCDD (groups TCDD, TCDD + PCB-52, TCDD + B*a*P, and TCDD + PCB-52 + B*a*P) treated as a single class and cells exposed to any of the toxicant combinations excluding TCDD (groups PCB52, B*a*P, and PCB52 + B*a*P) as a single class versus unexposed cells. The peptide ions showing significant change in intensity due to interactions fall mostly into the area of the SUS curve known to be unique for exposure to a TCDD containing mixture as expected.

After further univariate analysis of the results, using GraphPad Prism (version 5.0.1), 46 variables showed significant differences between treatment groups in a 2 way ANOVA using Bonferroni's post-hoc test; of these, a total of 39 showed evidence of significant interaction between coadministered compounds. The data suggest that each of the compounds tested (TCDD, PCB-52, or B*a*P) has a significant effect on the abundance of far fewer peptide ions than seen when either PCB-52 or B*a*P is administered to the cells in the presence of TCDD. Table 3 lists those variables which show significant interaction with the intensity values shown as a percentage of the intensity in the untreated cells. An additive effect is defined for these purposes as the effect of co-exposure being less than 10% different (relative to the control) from the predicted value of exposure to the individual compounds. It can be seen that the majority of these key peptides show evidence of significant non-additive effects. Twenty-three peptides varied significantly as a result of interactions between TCDD and PCB-52, and 22 showed evidence of interaction between TCDD and B*a*P. Only 6 of these variables were shared. In some cases, a contrary effect was noted upon coadministration on the abundance of certain peptides. For example, the peptide with an m/z value of 4934.61 when exposed to TCDD and PCB-52 together resulted in a greater than 4-fold difference from the value predicted by the sum of the effects of TCDD and PCB-52 when exposed individually. Figure 4 shows the changes in the 6 peptide variables, which are significantly influenced by interactions between TCDD and both cotoxicants.

4. DISCUSSION

Marker based detection tools appear to be valuable in identifying dioxins in the food chain and can assist in the removal of these toxicants. As dioxin is known to interact strongly with other chemicals,[13,25] an important first step to the development of such methods is the investigation of interactions of dioxins with potential co-contaminating chemicals and to examine the impact on marker discovery techniques. This investigation therefore studied the effect of mixtures of dioxin and other chemicals on H4IIE cells with the aim of illustrating this phenomenon *in vitro*. H4IIE cells were incubated with a range of concentrations of TCDD in the presence of B*a*P and PCB-52 and the effect on biological responses in cells evaluated through the use of EROD induction assays, AlamarBlue reduction measurements, and SELDI-TOF peptidomic profiling. The AlamarBlue reduction assay produced important information regarding the complex concentration dependency of these interactions. AlamarBlue is used as a measure of cell viability with a lower rate of reduction of the resazurin dye via NADP/NADPH dependent enzymes in the mitochondria associated with impaired cell viability. In this study, H4IIE cells were treated with mixtures of B*a*P and TCDD giving rise to changes in the rate of resazurin reduction relative to rates in cells exposed to TCDD only. This observed effect was concentration dependent but complex. The rate of reduction of resazurin in cells exposed to 2 μM B*a*P and above was elevated relative to control cells when co-exposed to TCDD concentrations of 0.01 μM to 0.2 μM. This effect diminished sharply at 1 μM TCDD. In contrast, levels of B*a*P below 2 μM produced on a nonsignificant response, as shown in the "scooped" profile of graphs in Figures 1 and S2 (Supporting Information). The precise mechanism by which resazurin reduction occurs is not

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Table 3. SELDI-TOF Variables Exhibiting Significant Interactions between TCDD and Either PCB-52 (A) or B*a*P (B)[a]

peptide *m/z*	CTRL intensity	TCDD	PCB-52	SUM of TCDD and PCB-52 effect	TCDD + PCB52 treatment
			intensity as % of control		
Additive Interactions					
2167.22	2.54	136*	148***	184	186***
2261.04	1.49	125	138	163	161*
2352.62	2.34	117	142**	159	149***
2367.99	2.10	124	130	154	145**
2390.42	2.22	119	129	148	142*
2408.93	2.42	116	123	139	143**
Non-additive Interaction-Lower than Sum					
2180.97	1.43	151	159*	210	193***
2305.23	1.85	128	139	167	155**
3012.17	4.74	72.9***	84.4	57.3	93.5
3048.69	2.39	106	151***	157	121
3555.87	1.51	67.4	40.6*	8.00	56.1
3559.09	3.10	94.4	95.0	89.4	128*
4047.77	3.51	73.7	60.6**	34.4	63.3*
4139.08	2.33	56.1	40.1**	−3.80	45.4*
4145.39	8.23	82.0**	83.0**	65.0	89.5
4934.61	2.62	59.1	41.3**	0.40	50.2*
5382.89	7.06	112	123***	135	111
5699.03	1.68	127*	115	142	107
5879.45	0.93	75.2	41.1***	16.3	57.0
5890.91	2.31	92.6	79.2**	71.8	85.9
6692.54	2.08	119	111	130	122*
8465.32	7.85	109***	134***	143	80.2***
Non-additive Interaction-Greater than Sum					
3495.26	1.16	146	161	208	244***

peptide *m/z*	CTRL intensity	TCDD	B*a*P	SUM of TCDD and B*a*P effect	TCDD + B*a*P treatment
			intensity as % of control		
Additive Interactions					
4745.04	6.14	101	111	112	119.8*
5382.89	7.06	112	102	114	122.1***
5551.88	1.48	110	122	132	131.2*
5699.03	1.68	127*	110	136	133.5***
9933.04	1.96	112	112	124	129.9**
2872.6	2.55	86.7	85.0	71.7	64.5*
Non-additive Interactions-Lower than Sum					
2167.22	2.54	136*	119	156	109.6
2749.83	2.07	64.7	65.9	30.6	50.2**
3012.17	4.74	72.9***	81.1	54.0	69.3***
3944.01	2.27	87.0	84.1	71.1	237.6***
4934.61	2.62	59.1	69.9	29.0	185.1***
4943.74	11.3	96.0	95.9	91.8	132.1***
4987.45	1.72	125	137	162	107.1*
5152.08	0.72	93.9	98.2	92.1	268.5*
8465.32	7.85	109***	141***	151	134.3***
12123.9	0.76	90.5	85.9	76.4	200.0***
Non-additive Interactions-Greater than Sum					
5434.59	2.37	102	104	107	118.6*
5546.82	1.15	107	115	123	165.6***
5549.82	1.39	110	120	132	143.7***
6120.96	0.85	115	104	120	153.6*
6122.37	0.88	117	105	122	153.8*
6124.18	0.92	116	105	121	150.0*

[a]Non-additive interactions are those which differ from the value predicted by the sum of differences of each variable in the individual treatment groups by more than ±10%. Significant difference by ANOVA compared to the control (*, p = <0.05; **, p = <0.01; ***, p = <0.001). Variables appearing in both tables are marked in bold.

known, but the link to energy metabolism would indicate that this combination of chemicals has an effect on the mitochondrial cytochrome based electron transport chain leading to an increase in the rate of reduction.[26] These findings provide novel evidence of interactions between TCDD and other tested compounds on the metabolic system of H4IIE cells and complement findings from performed SELDI analysis in that H4IIE cells co-exposed to dioxin and other toxicants respond in complex ways.

The induction of EROD activity in H4IIE cells or activation of the CYP1A1 pathway in the CALUX assay by dioxin-like chemicals and the interference on these systems by non-dioxin-like compounds have been investigated previously.[24,25,27] The 4-parameter fitting data produced from the matrix of concentrations of compounds that cells were exposed to in this study (shown in Table 1) demonstrated patterns of interference by B*a*P, PCB52, and PCB-153 with the induction of EROD through increasing TCDD concentrations. It is difficult to associate a clear pattern to these interactions because effects are multiphasic within the concentration ranges examined, and antagonistic and synergistic effects are evident depending on chemical concentrations used in binary mixtures. The matrix of concentrations of each compound tested makes it difficult to discern patterns that do not follow monophasic dose−response relationships. Figure 2 shows the level of EROD induction in cells exposed to TCDD was reduced by co-exposure to 0.02 nM to 10 nM B*a*P, yet co-exposure to 1000 nM and 10000 nM B*a*P resulted in a level of EROD induction above the maximum by 1×10^{-10} M TCDD alone. Interestingly, this multiphasic response to B*a*P in the presence of TCDD replicates the characteristic "scooped" pattern observed during monitoring of AlamarBlue responses, with suppression relative to control at lower concentrations but synergistic induction at higher levels. Findings from co-exposure of the cells to PCB-52 or PCB-153 suggest that the mechanism of action of these compounds in suppressing the level of EROD induction by TCDD is similar and evident within the same defined concentration range. At an EROD induction of around 50%, increasing concentrations of both PCBs reduced EROD activity significantly, although the effects of co-exposure of these chemicals at TCDD concentrations which induce maximal EROD activity were different. Observations such as the raised curve profile at 10,000 nM PCB-52 (Table 1) are interesting because of their transient nature. The EROD induction data shown in Figure 2 did not indicate a synergistic relationship between PCB-52 and TCDD but rather an antagonistic effect, indicating further complexity in this system. These findings also indicate that the pattern of antagonism of PCB-52 on TCDD induced EROD activity is variable and dependent upon the concentration of both compounds. Although findings were reproducible on a day-to-day basis, at some treatment conditions the level of variability was high relative to other measurements in the same experiment; this is observable in the error bars shown in Figure 2. This elevated variability has been remarked upon in other published work,[28] and nonchemical agents including stress and temperature have all been shown to have a significant impact on

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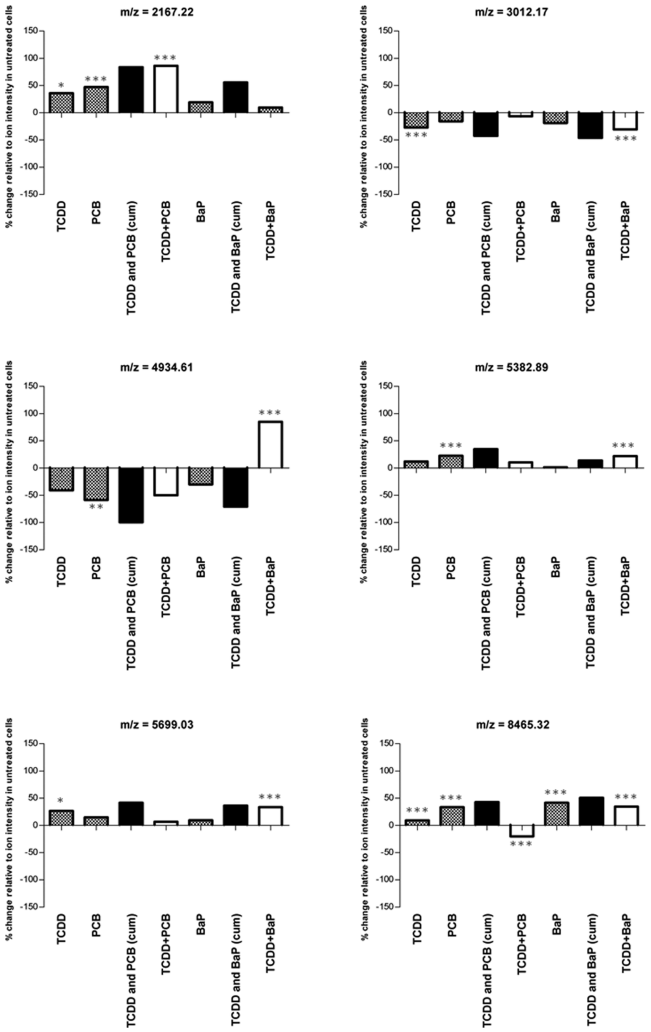

Figure 4. Relative intensity of peptide ions as determined by SELDI-TOF analysis of extracts of H4IIE cells exposed to combinations of TCDD, PCB-52, and B*a*P for 24 h showing evidence of non-additive interactions upon co-exposure. Significant difference by ANOVA (with Bonferroni's post-hoc test from untreated cells shown by asterisks (p values: * = <0.05, ** = <0.01, and *** = <0.0001). Note that the values presented are normalized relative intensity values, although significance testing was performed on raw intensity values; thus error bars are not presented.

cellular and organism responses to dioxin.[13,28,29] These results emphasize the complexity of interactions of dioxin-like toxic effects with other chemicals.

The kinetic response of induced EROD enzyme activity in cells to the immediate presence of B*a*P, PCB-52, and PCB-153 were another area where interference was observed. The nonclassical inhibition profiles of B*a*P and PCB-153 are unusual and may relate to findings indicating the participation of the chemicals themselves, either as electron donors or receptors, in the redox chain of the cytochrome system, and it is known that B*a*P is a substrate for EROD. Schlezinger et al.[36] suggested that this uncoupling, due to PCB-77 in particular, may have further downstream consequences by increasing reactive oxygen species production within exposed cells. The level and nature of inhibition of the EROD enzyme by PCB-52 in this study are similar to the findings of Edwards et al.[37] who calculated a mean K_i value for PCB-52 of 6.6 μM at 7500 and 15000 nM inhibitor levels in hepatic microsomes extracted from exposed rats. K_m values for uninhibited EROD activity reported in this study (0.59 μM) were also comparable to previously reported EROD activity of 0.49 μM by the same authors. The profile of plots of the rate of EROD activity by substrate concentration shown in Figure S3 (Supporting Information) and the deviation from first order Michaelis–Menten kinetics suggests competitive interaction with PCB-52 is responsible for the changes in rate of substrate conversion observed. In the inhibited curves, the R^2 value of the fit is less than 0.95, suggesting that the precise mechanism of inhibition may be more complex. As with the other interactions reported in this study, there was no clear dose-dependent relationship with 1000 nM PCB-52 showing a stronger inhibitory effect than 10000 nM PCB-52. These inhibitory effects may contribute to the overall activity measurements observed in the TCDD induced EROD activity experiments shown in Table 1 and Figure 2. However, in this study the toxicants were removed prior to measurement of activity, so any inhibition reflected in these findings would have to function through an irreversible or long-term, noncompetitive mechanism; uncoupling of the cytochrome redox system[29] could result in such an effect.

OPLS-DA treatment of SELDI-TOF acquired spectral data, produced from lysates of exposed cells, enabled the responses to the exposure of cells to chemicals of interest to be visualized and evaluated. These results demonstrated spectra produced from cells exposed to TCDD in the presence of PCB-52 or B*a*P to be clearly separated from each other and from the control group with no overlap. This gives an indication that both PCB-52 and B*a*P interact with TCDD to significantly influence the levels of a number of peptides in exposed H4IIE cells. This is confirmed by looking at the changes in abundance of the peptides in more detail. Table 3 shows there was very little commonality in the response of individual peptides to interactions between TCDD and PCB-52 and TCDD and B*a*P suggesting that a separate pathway may be acted upon by the interacting compounds. This is clearly observable in the peptides with m/z of 4934.61 and 12123.9 showing contrary patterns of response with these treatments. The loadings plot in Figure 3, bottom panel, indicates how these peptides contribute to the separation of the observations in the scores plot in Figure 3, top panel.

The non-additive effects detailed in Table 3, and observable in Figure 4, show an interesting pattern in that many of those variables which are affected by the combined action of TCDD and PCB-52 appear to exhibit a contrary response to treatment with TCDD and B*a*P. This could indicate 2 distinct modes of action being encapsulated by the binary mixture of these toxic groups. The identity of the peptides responsible for these peaks or the pathways they represent is not known at this time; however, the presence of groups of proteins which interact in this way strongly indicates that the interactions between dioxin and non-dioxin-like toxicants is of key importance in evaluating the risk from real exposure scenarios to animal and human health, and thus they must be considered when utilizing marker based methods to detect contaminant exposure. SELDI-TOF itself, however, appears to be of more limited use as a peptidomic tool, largely as a result of poor precision within sample replicates, which may mask effects on specific markers. A further drawback is that the technique is a *cul de sac* in terms of identification of the proteins and peptides in question. Any identification of peptides must occur by going back to the original sample and isolating potential targets before confirming their response within the SELDI system. More modern variants of the SELDI system

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use a modular chip interface which can be coupled to more sensitive and powerful TOF mass spectrometers to allow in-line identification by trypsin fragment analysis.[30]

Studies of the interaction of PCBs with TCDD in a number of systems tend to concentrate on dioxin-like PCBs to investigate their contribution to toxicity. Investigations into the interactions of dioxin and non-dioxin like compounds are a much less developed area. A defining characteristic of findings reported here is, however, the absence of simple interaction patterns. Commercial PCB oil mixtures such as Aroclor 1254 contain a mixture of dioxin-like and non-dioxin-like PCB compounds. These mixtures have long been known to have antagonistic effects on EROD induction in H4IIE cells.[27] Toxicological studies have used various toxic end points in the study of these mixtures and are a good source of information on the interaction of dioxinergic chemicals with other PCBs.[19] Importantly, studies addressing the interactions of dioxin with other chemicals show similar findings in cell culture and live animal work on a wide range of biochemical and clinical end points.[31,32] Dioxin is a toxicant which is known to affect almost every tissue type in the body. In particular, nontargeted proteomic methods have revealed the complexity and range of responses in cells similar to that shown here.[33] It can be inferred that the interactive effects seen in single tissue type cell culture would be further complicated in terms of proteomic or metabolomic responses seen in plasma or urine of an exposed animal. This work further reinforces the purported role of dioxin as a potentiator of other biological challenges. Some authors suggest that the way dioxin interacts with other toxicants may be a more important aspect of dioxin toxicity than the direct toxicity of dioxin itself.[14,34] This study has illustrated significant interactions between TCDD and B*a*P or *ortho*-PCBs on the metabolic activity of H4IIE cells. The fact that these interactions are evident in each of the targeted and untargeted techniques discussed herein indicates that such effects occur within many distinct biochemical pathways in a pleiotropic manner, even within a single cell line. The results from this study further emphasize the complexity of the interactions and highlight the need for continued and more integrated study in this area. The detection of dioxin exposure using markers within biological systems would be a useful tool in safeguarding public health and preventing the financial and reputational damage caused by dioxin contamination in the human food chain. In particular, the findings of this paper were considered in the decision to utilize real-world sources of dioxin in the conduct of an in vivo study investigating markers of dioxin exposure in rats.[35] The findings presented here emphasize the importance of both considering the source of the dioxin-like contamination and investigating the potential interactions of co-contaminating compounds in developing biomarker based detection approaches.

■ ASSOCIATED CONTENT

Supporting Information

OPLS-DA misclassification table of SELDI-TOF data; interaction effects of TCDD and PCB-52 on AlamarBlue reduction; timecourse of ethoxyresorufin conversion; inhibition of EROD activity in the presence of toxicants; and example of SELDI-TOF spectra obtained from analysis of H4IIE cell lysates. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

PCB, polychlorinated biphenyl; PAH, polynuclear aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; B*a*P, benzo-*a*-pyrene; SELDI-TOF, surface enhanced laser desorption/ionization-time-of-flight; MS, mass spectrometry; CYP1A1, cytochrome P450 1A1; CALUX, chemical activated luciferase gene expression; EROD, ethoxyresorufin-*O*-deethylase

■ REFERENCES

(1) Casey, D. K., Lawless, J. S., and Wall, P. G. (2010) A tale of two crises: the Belgian and Irish dioxin contamination incidents. *Br. Food J. 112*, 1077−1091.

(2) Hoogenboom, R., Bovee, T., Portier, L., Bor, G., van der Weg, G., Onstenk, C., and Traag, W. (2004) The German bakery waste incident, use of a combined approach of screening and confirmation for dioxins in feed and food. *Talanta 63*, 1249−1253.

(3) Llerena, J. J., Abad, E., Caixach, J., and Rivera, J. (2003) An episode of dioxin contamination in feedingstuff: the choline chloride case. *Chemosphere 53*, 679−683.

(4) Hoogenboom, L. A. P., Kan, C. A., Bovee, T. F. H., van der Weg, G., Onstenk, C., and Traag, W. A. (2004) Residues of dioxins and PCBs in fat of growing pigs and broilers fed contaminated feed. *Chemosphere 57*, 35−42.

(5) Schoeters, G., and Hoogenboom, R. (2006) Contamination of free-range chicken eggs with dioxins and dioxin-like polychlorinated biphenyls. *Mol. Nutr. Food Res. 50*, 908−914.

(6) Reiner, E. J. (2010) The analysis of dioxins and related compounds. *Mass Spectrom. Rev. 29*, 526−559.

(7) Benedict, W. F., Gielen, J. E., Owens, I. S., Niwa, A., and Nebert, D. W. (1973) Aryl hydrocarbon hydroxylase induction in mammalian liver-cell culture 0.4. stimulation of enzyme-activity in established cell lines derived from rat or mouse hepatoma and from normal rat-liver. *Biochem. Pharmacol. 22*, 2766−2769.

(8) Behnisch, P. A., Hosoe, K., and Sakai, S. (2001) Bioanalytical screening methods for dioxins and dioxin-like compounds - a review of bioassay/biomarker technology. *Environ. Int. 27*, 413−439.

(9) Hoogenboom, L., Goeyens, L., Carbonnelle, S., van Loco, J., Beernaert, H., Baeyens, W., Traag, W., Bovee, T., Jacobs, G., and Schoeters, G. (2006) The CALUX bioassay: current status of its application to screening food and feed. *Trends Anal. Chem 25*, 410−420.

(10) Murk, A. J., Legler, J., Denison, M. S., Giesy, J. P., vandeGuchte, C., and Brouwer, A. (1996) Chemical-activated luciferase gene expression (CALUX): A novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. *Fundam. Appl. Toxicol. 33*, 149−160.

(11) Birnbaum, L. S. (2005) Risk characterization of dioxins. *Toxicol. Lett. 158*, S10−S10.

(12) Van den Berg, M., et al. (2006) The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol. Sci. 93*, 223−241.

(13) Dhanabalan, S., and Mathur, P. P. (2009) Low dose of 2,3,7,8 tetrachlorodibenzo-p-dioxin induces testicular oxidative stress in adult rats under the influence of corticosterone. *Exp. Toxicol. Pathol. 61*, 415−423.
(14) Mocarelli, P., et al. (2008) Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. *Environ. Health Perspect. 116*, 70−77.
(15) Simanainen, U., Adamsson, A., Tuomisto, J. T., Miettinen, H. M., Toppari, J., Tuomisto, J., and Viluksela, M. (2004) Adult 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure and effects on male reproductive organs in three differentially TCDD-susceptible rat lines. *Toxicol. Sci. 81*, 401−407.
(16) Kan, C. A., and Meijer, G. A. L. (2007) The risk of contamination of food with toxic substances present in animal feed. *Anim. Feed Sci. Technol. 133*, 84−108.
(17) Gidarakos, E., Petrantonaki, M., Anastasiadou, K., and Schramm, K. (2009) Characterization and hazard evaluation of bottom ash produced from incinerated hospital waste. *J. Hazard. Mater. 172*, 935−942.
(18) Lavric, E. D., Konnov, A. A., and De Ruyck, J. (2004) Dioxin levels in wood combustion - a review. *Biomass Bioenergy 26*, 115−145.
(19) Kodavanti, P. R. S., Kannan, N., Yamashita, N., Derr-Yellin, E. C., Ward, T. R., Burgin, D. E., Tilson, H. A., and Birnbaum, L. S. (2001) Differential effects of two lots of Aroclor 1254: Congener-specific analysis and neurochemical end points. *Environ. Health Perspect. 109*, 1153−1161.
(20) US EPA (1993) Provisional Guidance for Quantitative Risk Assessment of Polycyclic Aromatic Hydrocarbons, EPA/600/R-93/089.
(21) Simon, T., Britt, J. K., and James, R. C. (2007) Development of a neurotoxic equivalence scheme of relative potency for assessing the risk of PCB mixtures. *Regul. Toxicol. Pharmacol. 48*, 148−170.
(22) Tan, Y. S., Li, D. M., Song, R. J., Lawrence, D., and Carpenter, D. O. (2003) Ortho-substituted PCBs kill thymocytes. *Toxicol. Sci. 76*, 328−337.
(23) World Health Organisation (1998) Environmental Health Criteria 202, Selected Non-heterocyclic Polycyclic Aromatic Hydrocarbons, 202.
(24) Peters, A. K., van Londen, K., Bergman, A., Bohonowych, J., Denison, M. S., van den Berg, M., and Sanderson, J. T. (2004) Effects of polybrominated diphenyl ethers on basal and TCDD-induced ethoxyresorufin activity and cytochrome P450−1A1 expression in MCF-7, HepG2, and H4IIE cells. *Toxicol. Sci. 82*, 488−496.
(25) Sanctorum, H., Elskens, M., and Baeyens, W. (2007) Bioassay (CALUX) measurements of 2,3,7,8-TCDD and PCB 126: interference effects. *Talanta 73*, 185−188.
(26) O'Brien, J., Wilson, I., Orton, T., and Pognan, F. (2000) Investigation of the AlamarBlue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem. 267*, 5421−5426.
(27) Bannister, R., Davis, D., Zacharewski, T., Tizard, I., and Safe, S. (1987) Aroclor-1254 as a 2,3,7,8-tetrachlorodibenzo-para-dioxin antagonist - effects on enzyme-induction and immunotoxicity. *Toxicology 46*, 29−42.
(28) vanBirgelen, A. P. J. M., Fase, K. M., vanderKolk, J., Poiger, H., Brouwer, A., Seinen, W., and vandenBerg, M. (1996) Synergistic effect of 2,2′,4,4′,5,5′-hexachlorobiphenyl and 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic porphyrin levels in the rat. *Environ. Health Perspect. 104*, 550−557.
(29) Schlezinger, J. J., and Stegeman, J. J. (2001) Induction and suppression of cytochrome P450 1A by 3,3′,4,4′,5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (Stenotomus chrysops). *Aquat. Toxicol. 52*, 101−115.
(30) Jourdain, S., Bulman, A., and Dalmasso, E. (2010) The Lucid Proteomics System for top-down biomarker research. *Arch. Physiol. Biochem. 116*, 158−162.
(31) Burgin, D. E., Diliberto, J. J., Derr-Yellin, E. C., Kannan, N., Kodavanti, P. R. S., and Birnbaum, L. S. (2001) Differential effects of two lots of Aroclor 1254 on enzyme induction, thyroid hormones, and oxidative stress. *Environ. Health Perspect. 109*, 1163−1168.
(32) Vanbirgelen, A. P. J. M., Vanderkolk, J., Fase, K. M., Bol, I., Poiger, H., Brouwer, A., and Vandenberg, M. (1995) Subchronic dose-response study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in female Sprague-Dawley rats. *Toxicol. Appl. Pharmacol. 132*, 1−13.
(33) Sala, G. L., Ronzitti, G., Sasaki, M., Fuwa, H., Yasumoto, T., Bigiani, A., and Rossini, G. P. (2009) Proteomic analysis reveals multiple patterns of response in cells exposed to a toxicant mixture. *Chem. Res. Toxicol. 22*, 1077−1085.
(34) Bock, K. W., and Kohle, C. (2006) Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem. Pharmacol. 72*, 393−404.
(35) O'Kane, A. A., Chevallier, O., Graham, S., Elliott, C. T., and Mooney, M. H. (2013) Metabolomic profiling of in vivo plasma responses to dioxin-associated dietary contaminant exposure in rats: implications for identification of sources of animal and human exposure. *Environ. Sci. Technol. 47*, 5409−5418.
(36) Schlezinger, J. J., Struntz, W. D. J., Goldstone, J. V., and Stegeman, J. J. (2006) Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners. *Aquat. Toxicol. 77*, 422−432.
(37) Edwards, P. R., Hrycay, E. G., and Bandiera, S. M. (2007) Differential inhibition of hepatic microsomal alkoxyresorufin O-dealkylation activities by tetrachlorobiphenyls. *Chem.-Biol. Interact. 169*, 42−52.