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Effects and Interactions in an Environmentally Relevant Mixture of Pharmaceuticals

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With the goal of assessing the environmental risk of pharmaceuticals, we have previously observed that a mixture of 13 different drugs at environmentally relevant concentrations had adverse consequences on human and zebra fish cells *in vitro*. Here we aimed to identify both main and interaction effects within the same environmentally relevant mixture of pharmaceuticals. We studied *in vitro* cytotoxicity in *Escherichia coli*, human embryonic HEK293, and estrogen-responsive OVCAR3 tumor cells using fractional-factorial experimental design. Our approach identified a subset of compounds of primary environmental concern, namely atenolol, bezafibrate, ciprofloxacin, and lincomycin, that had statistically significant effects on prokaryotic and eukaryotic cells at environmentally relevant exposure levels (ng/l). Drugs could interact and behave as chemosensitizers, with joint effects representing a statistically significant element of mixture toxicity. Effects and interactions were concentration dependent, confirming the difficulty of dose extrapolation in mixture toxicity data. This study suggests that a thorough investigation of mixture effects can direct environmental concerns toward a handful of pharmaceuticals, which may represent an actual risk at environmental concentrations. We indicate that risk identification may strongly depend on the use of environmentally relevant exposure scenarios. Antagonistic-synergistic interactions and dose dependency of effects may hamper the modeling and prediction of mixture toxicity with pharmaceuticals. Hazard identification for micropollutants depends heavily on appropriate study designs, and we indicate the use of *in vitro* cytotoxicity threshold and statistical design of experiments (DOEs) as a valid approach.

***Key Words:* *E. coli*; HEK293; interaction; mixtures; OVCAR3; pharmaceuticals.**

Pharmaceuticals are a class of emerging environmental micropollutants that are extensively and increasingly being used in human and veterinary medicine. A number of active pharmaceutical ingredients (APIs) are commonly excreted unmetabolized or as active metabolites by humans and animals after medication, escaping conventional biological wastewater treatment and reaching surface, ground, and drinking water. Recent studies have documented the presence of a wide variety of pharmaceuticals in the environment worldwide, including antibiotics, anesthetics, anti-inflammatories, antitumor compounds, estrogens, lipid-reducing agents, diuretics, antidepressants, as well as illicit drugs (Castiglioni *et al*., 2006; Khetan and Collins, 2007).

This ecotoxicology issue is particularly challenging with respect to three factors: pharmaceuticals are present in the environment in mixtures; these mixtures are constituted by diverse chemicals with diverse modes of action and unknown subtherapeutic side effects, especially on nontarget organisms; each API occurs in concentrations in the range of nanogram to microgram per liter. Exploring the toxicological risks associated with micropollutants of mixed pharmaceutical origin is a prime concern for the health of aquatic organisms and humans.

We addressed the problem of very low concentrations by using sensitive *in vitro* tests and a panel of model cell lines, which offer a range of advantages (Pomati *et al*., 2006, 2007; Schirmer, 2006). To deal with complex mixtures of diverse chemicals, we drew inspiration from the approach proposed by Feron and Groten (2002; Groten *et al*., 2001). This is based on the identification of the top chemicals composing a mixture, on the study of those top chemicals as a simplified mixture followed by an assessment of the combined action of components, to ultimately identify the specific effects responsible for mixture toxicity.

Previously, we assembled a mixture that mimics the concentration and combination of drugs as detected in a typical aquatic environment contaminated by pharmaceuticals (Pomati *et al*., 2006). This simplified ''model'' mixture of APIs comprised 13 drugs: atenolol, bezafibrate, carbamazepine, cyclophosphamide, ciprofloxacin, furosemide, hydrochlorothiazide, ibuprofen, lincomycin, ofloxacin, ranitidine, salbutamol, and sulfamethoxazole. We observed that the model mixture had similar effects in human embryonic cells (HEK293) and zebra fish liver cells *in vitro*, with regard to inhibition of cell proliferation and molecular mechanisms of action (Pomati

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et al., 2006, 2007). We hypothesized effects that were specifically caused by individual drugs and the occurrence of potential interactions.

Here, we performed a component interaction analysis (Groten *et al*., 2001) aimed at the identification of the APIs in the "model" mixture that had a direct effect on cell proliferation or were able to interact *in vitro* with other compounds with this respect. We chose three standard lines: HEK293, human ovarian carcinoma (OVCAR3), and *Escherichia coli* (strain JM109). While *E. coli* represented our prokaryotic model, human cell lines were selected based on available data regarding their genetic profile. In particular, HEK293 cells lack the expression of hormone receptors, an important characteristic for studying basal toxicity associated with our drug mixture. On the contrary, OVCAR3 cells express androgen and estrogen receptors (Hamilton *et al*., 1983), representing, therefore, a good model to assess possible endocrine-disrupting effects of pharmaceuticals. We identified DOE (Box *et al*., 1978; Feron and Groten, 2002; Steel and Torrie, 1960) as the optimal statistical approach to investigate main effects and drug-drug interactions (DDIs) occurring at environmentally relevant exposure levels among the 13 APIs composing the mixture. Our aim was to reduce this mixture to a subset of priority pharmaceuticals to further characterize, monitor, and control in the environment.

MATERIALS AND METHODS

Factorial DOEs. Experiments were designed to address *in vitro* cell proliferation in standard conditions, based on a trial series of factorial experiments in which both variation in experimental replicates and reproducibility were assessed (Pomati *et al*., unpublished). To detect main and interactive (nonadditive) effects in a mixture of more than two chemicals we used factorial DOE, in which n compounds are studied at x dose levels (x^n experiments), with 2^n designs being the most common (two-level factorial) (Box *et al*., 1978; Steel and Torrie, 1960). In the case of 13 pharmaceuticals, a full two-level factorial DOE would include 2^{13} test solutions, each represented by a unique mixture of the 13 drugs, assembled by selectively combining APIs at the defined levels of exposure. This type of DOE generally includes a test solution named centerpoint (0) in which all factors are studied at the linear average value between their maximum (1) and minimum (− 1) levels of exposure and allows for an accurate estimation of pure error, lack-of-fit (LOF) error, and curvature (Box *et al*., 1978; Snedecor and Cochran, 1989).

Considering replication needs, costs, and logistics, a full two-level factorial design with 13 chemicals is experimentally intractable. We therefore chose a fractional-factorial DOE of resolution IV, reducing the 2^{13} tests of a full design to 32 test solutions plus the centerpoint. With this design, all main effects associated with single chemicals were readily determinable; however, two-factor interactions were confounded with other two-factor interactions (aliasing structure of the DOE). The test solutions required (32 + centerpoint) were randomized across four experimental blocks. Each block was a completely independent experiment, carried out sequentially by using the same batch of reagents/media/disposables, and composed of eight test solutions (replicated four times) and the centerpoint (replicated 12 times). This design gave an accurate and independent estimate of all sources of variance in our tests, including pure error and repetition variability (blocks).

Concentrations used for the 13 pharmaceuticals investigated here are reported in Table 1. Given the previously employed APIs and their environmentally relevant experimental concentrations named here X_i, i = 1, … 13 (see Table 1 in Pomati *et al*., 2006), we subsequently defined level 1 and − 1 for each drug as the corresponding concentration $X_i \times 10$ and $X_i \times 10^{-1}$, respectively. To test the dose dependency of mixture effects, we raised by a factor of 10 the corresponding X_i values. The design matrix, test units, blocking system, and run order for these two-level fractional-factorial experiments are available as Supplementary Material online (Suppl_DOE.xls).

Table 1
Tested Pharmaceuticals and Corresponding DOE Exposure Levels

	Concentration level (ng/l)		
Pharmaceutical	− 1	0	1
Atenolol*	100	5000[a]	10000
Bezafibrate**	10	500[a]	1000
Carbamazepine	100	5000	10000
Ciprofloxacin*	10	500[a]	1000
Cyclophosphamide	1	50	100
Furosemide	100	5000	10000
Hydrochlorothiazide	100	5000	10000
Ibuprofen	10	500	1000
Lincomycin**	10	500[a]	1000
Ofloxacin	10	500	1000
Ranitidine	10	500	1000
Salbutamol	1	50	100
Sulfamethoxazole	10	500	1000

[a]This level of exposure represents the TTC proposed in this study for statistically significant factors.
* $p \leq 0.1$, ** $p \leq 0.05$.

Test chemicals. Reagents and chemicals, including pharmaceuticals, were obtained from Sigma-Aldrich Co. (Dorset, UK) apart from ciprofloxacin and ranitidine that were purchased from ICN Biochemicals (Meckenheim, Germany) and GlaxoSmithKline (Philadelphia, PA), respectively. Stock solutions of single APIs were prepared in methanol as reported previously (Pomati *et al*., 2006) and stored at − 20°C. Drug stocks were mixed in methanol at the appropriate levels according to DOE combinations, diluted in methanol to a final concentration 100 times higher than the corresponding test level, and stored at − 20°C in darkness. Appropriate aliquots of these concentrated test solutions were evaporated to dryness in a speed-vac and suspended in culture media immediately prior to testing on cell lines.

Eukaryotic cell titer proliferation assays. HEK293 and OVCAR3 cells (ATCC number CRL-1573 and HTB-161, respectively) were maintained in a humidified incubator at 37°C with 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM glutamine (Euroclone, West Yorks, UK) containing 10% fetal bovine serum (FBS) (Euroclone). Grown in T-75 flasks, a batch of mid-exponential cultures was harvested by the standard trypsin (Euroclone) method, aliquoted, resuspended in DMEM supplemented with 40% FBS and 10% dimethyl sulfoxide, and stored in liquid nitrogen until needed for testing. Cell lines were verified as being free from *Mycoplasma* infection using the PCR-based method by Tang *et al*. (2000).

Aliquots of cells were thawed, grown to mid-exponential phase, harvested, and resuspended in DMEM 10% FBS. After enumeration and dilution, 4×10^3 HEK293 or 3×10^3 OVCAR3 cells per well were seeded in random order in 96-well flat-bottom microtiter plates using a volume of 50 µl of DMEM 10% FBS. Plates were incubated for 4 h at 37°C. Experimental wells were then added with 50 µl of DMEM 10% FBS supplemented with two times concentrated test solutions by following the run order as specified by the DOE. Two wells in each plate were added with 100 µl of media and no cells, for

contamination inspection and for media background absorbance subtraction (no-cells controls). Plates were incubated at 37°C with 5% CO_2 for 48 h.

Four hours prior to the end of the assay, experimental wells were added with 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) dye solution from the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI), and plates were incubated for 4 h at 37°C. Absorbance (optical density [OD]) was then recorded at 492 nm (OD_{492}; dye) and 620 nm (OD_{620}; reference for background) using a microplate reader Biotrak II (Amersham Biosciences, Little Chalfont, UK) set to shake before measuring. Absorbance was taken in two repeated measures for every plate. For each experimental well, the final OD values used for statistical analyses were calculated as follows:

$$OD_{final} = \left\{OD_{492\_sample} - OD_{492\_no\text{-}cells}\right\} - \left\{OD_{620\_sample} - OD_{620\_no\text{-}cells}\right\},$$

where every OD represents the average of the two technical repeats.

***Escherichia coli* proliferation assay.** *Escherichia coli* JM109 was maintained on a shaker at 37°C in Luria-Bertani (LB) broth, with cultures kept in mid-exponential growth by subculturing when OD_{620} reached circa 0.6. Test cultures were mixed thoroughly and diluted 27 times in LB medium at room temperature, to obtain an early exponential cell density. In random order, 270 µl of early exponential cell suspension was seeded in 96-well flat-bottom microtiter plates. Experimental wells were added with 30 µl of LB supplemented with 10 times concentrated test solutions by following the run order as specified by the DOE. Plates were incubated in a 37°C chamber for 3 h. Wells were mixed by pipetting, and OD_{620} was recorded with a microplate reader as reported above. Absorbance was taken in two repeated measures for every plate, and final OD_{620} values were calculated by averaging the two technical repeats.

Statistical analyses. Experimental results were used to estimate parameter values in the polynomial function of the general linear model (GLM) specified by the DOE utilizing multiple regression analysis and the least square method. Effect values for each factor were estimated as two times the regression coefficients as calculated by multiple linear regression. Prior to model fitting, Bartlett and Levene tests were employed to assess the assumption of homogeneity of variances in experimental units (Snedecor and Cochran, 1989). Analysis of residuals revealed two outliers in the OVCAR3 data set that were transformed by Winsorizing (Dixon, 1960). When polynomial models were fit to the data sets, significant levels of parameter values were determined by ANOVA using *t*-test, while the overall utility and adequacy of each model was obtained studying curvature and LOF by *F*-test.

In the fractional DOE, two-factor interaction terms were confounded between each other. To obtain meaningful interaction data, we performed statistical analyses in two phases. Initially, all factors (13 pharmaceuticals) were studied to identify which ones contribute more significantly to the observed responses. Interaction terms were not included in this first screening. Based on the statistical significance of main effects, factors with a p value ≤ 0.5 were selected for a second round of multiple linear fit and ANOVA, this time including two-factor interaction terms in the polynomial model. This procedure of reducing the number of factor terms allowed the testing of main effects and DDIs with a less confounded aliasing structure. Also, phase 2 analysis always provided the best model and the best fit as assessed by R^2 values and ratios of R^2-adj/R^2 closer to 1 (see Supplementary Material online).

We increased the power of our analysis by considering data as statistically significant with a confidence limit of $p \leq 0.1$, correcting for multiple hypothesis testing. This strategy increases the potential for discovery while raising the chance of type I errors and false-positive detection. To quantify the expected proportion of false positives as a consequence of our unconventional confidence limit, we performed false discovery rate (FDR) tests and introduced a q value, defined to be the FDR analogue of the p value (Supplementary Table S10). The q value of an individual hypothesis test is the expected proportion of false positives in that data set among all factors called significant (Storey and Tibshirani, 2003). The expected proportion and the identity of false positives in our study were subsequently assessed by means of an independently designed set of confirmation experiments (see Supplementary Material online, Suppl_DOE.xls). This approach allowed us to detect reproducible effects that would have been missed using a more conventional and conservative confidence limit of $p \leq 0.05$.

Statistical DOEs, analyses, and interaction plots were performed using R (http://www.r-project.org) and MINITAB 14.1 for PC (Minitab Inc., State College, PA). Results were visualized in color-coded image maps (CIMs) using CIMminer (Weinstein *et al.*, 1997). Some DOE interaction terms were confounded with effects from blocks and, therefore, could not be studied. These include cyclophosphamide × hydrochlorothiazide and lincomycin × sulfamethoxazole for *E. coli*, and bezafibrate × furosemide, ciprofloxacin × lincomycin, bezafibrate × ciprofloxacin, furosemide × lincomycin, bezafibrate × lincomycin, and ciprofloxacin × furosemide for OVCAR3. Raw data are available as Supplementary Material online together with the DOE matrices (Suppl_DOE.xls).

RESULTS AND DISCUSSION

Component Analysis

In the first stage of our study, we tested the effects associated with single drugs composing our mixture. Resulting p values and main effects for *E. coli*, HEK293, and OVCAR3 cells are reported in Figure 1 as clustered and CIMs. Considering effects and statistical significance, *E. coli* and HEK293 appeared, by cluster analysis, to respond in a similar way compared to OVCAR3 (Figs. 1A–B). No API had statistically significant effects on cell proliferation simultaneously for all models (Fig. 1A). This could be the result of differences in the expression of specific drug target sites. Comprehensively, only four pharmaceuticals showed statistically significant effects for $p \leq 0.1$: atenolol, bezafibrate, ciprofloxacin, and lincomycin.

Atenolol had slight stimulatory consequences on *E. coli* ($p = 0.12$, $q = 0.36$) and HEK293 ($p = 0.093$, $q = 0.63$) cell proliferation. This drug binds to β-adrenergic receptors in human cells, enhancing several metabolic functions including carbohydrate oxidation and glycogenolysis (Mora-Rodriguez *et al.*, 2001). These receptors are commonly found in the kidneys, which explains the specific response observed here on HEK293. The recently discovered bacterial adrenergic receptor QseC may instead account for the impact of atenolol on the proliferation of *E. coli* (Clarke *et al.*, 2006). This may signify that β-blockers have subtle effects on both target and nontarget organisms at environmentally relevant concentrations.

Bezafibrate clustered alone in the effects of CIM, having marked stimulatory effects on both our model eukaryotic cells, HEK293 ($p = 0.04$, $q = 0.63$) and OVCAR3 ($p = 0.042$, $q = 0.29$), and no statistically significant effect on *E. coli*. This lipid-regulating agent decreases plasma triglicerides and cholesterol levels by increasing fatty acids catabolism via induction of peroxisomes activity. Bezafibrate is an agonist of peroxisome proliferator–activated receptor alpha (PPAR-α), a member of the nuclear hormone receptor superfamily, which is intimately connected to cellular metabolism (carbohydrate, lipid, and protein) and cell differentiation (Takeuchi *et al.*, 2006). The PPAR-α isoform is present both in tumorigenic cells and in kidney cells, and its activation generally results in

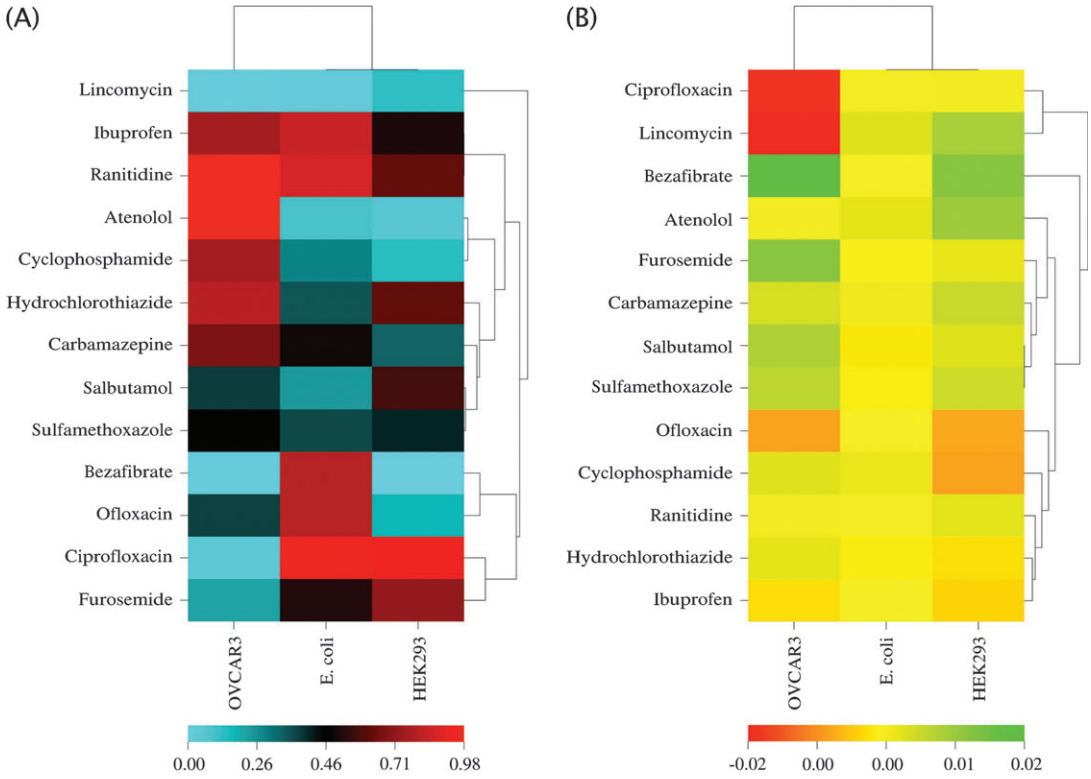

FIG. 1. Colored representations (CIMs) of result tables for component analysis, with 13 rows (drugs) and 3 columns (cell lines) ordered and clustered by average linkage using Euclidean distances. The color in each image reflects the estimated p values (A) and main effects (2 × linear regression coefficient) on cell proliferation (B) for single variables in the factorial DOE. Color scales are shown with aqua = high significance and red = no significance in (A) and red = inhibition and green = stimulation in (B), respectively.

antiproliferative stimuli (Chinetti *et al.*, 2000). In our experimental conditions, instead, bezafibrate had a positive consequence on the growth of both our eukaryotic lines at very low levels of exposure (Table 1). Fent *et al.* (2006a) have reported no detectable estrogenic effects of bezafibrate at exposure levels comparable to ours using a yeast system expressing human estrogen receptor alpha (YES) assay. Another fibrate, gemfibrozil, has been shown, however, to affect PPAR-β expression and to have a potential for endocrine disruption in goldfish (Mimeault *et al.*, 2005, 2006). We suggest, as a precautionary principle, that bezafibrate should be further investigated as a possible "Trojan horse" endocrine disruptor (Sharpe and Irvine, 2004).

Ciprofloxacin and lincomycin clustered together outside the main clade comprising the other drugs, having distinct negative effects on OVCAR3 proliferation ($p = 0.069$, $q = 0.32$ and $p = 0.047$, $q = 0.29$, respectively) and no statistically significant effect on HEK293 (Fig. 1B). Antibiotics may stimulate bacterial growth at low concentrations (Pomati *et al.*, 2004), and lincomycin appeared to promote *E. coli* proliferation ($p = 0.048$, $q = 0.36$) at environmentally relevant levels. Fluoroquinolones such as ciprofloxacin inhibit bacterial DNA gyrase sub-A and slightly inhibit the eukaryotic topoisomerase II (Cortázar *et al.*, 2007). Lincosamides such as lincomycin interfere with the transpeptidase reaction in the 50S subunit of bacterial ribosomes with a mechanism similar to macrolide antibiotics (Champney and Tober, 2000), some of which can affect the physiology of eukaryotic cells (Tirado *et al.*, 2005). Both fluoroquinolones and lincosamides have been described to affect the growth of human cell lines, although at concentrations much higher than those utilized here (Duewelhenke *et al.*, 2007). We can only speculate on the reasons why these drugs affected OVCAR3 and not HEK293 cells. The seemingly specific inhibitory effect of lincomycin and ciprofloxacin on OVCAR3 can be caused either by differences in gene expression profiles in the two cell lines or simply by the higher rate of generation that tumor cells have compared to HEK293. A fast growing line such as OVCAR3 would be more sensitive than slow growing cells to chemicals affecting cell replication.

Bezafibrate, ciprofloxacin, and lincomycin have been repeatedly found in surface and ground waters (Khetan and Collins, 2007). Given their ability to interfere with proliferation in eukaryotic cells, studies on these drugs should be advanced by long-term effects on aquatic species. In addition, monitoring of drinking water supplies for these pharmaceuticals should be encouraged to manage exposure risks in particularly sensitive human conditions such as pregnancy.

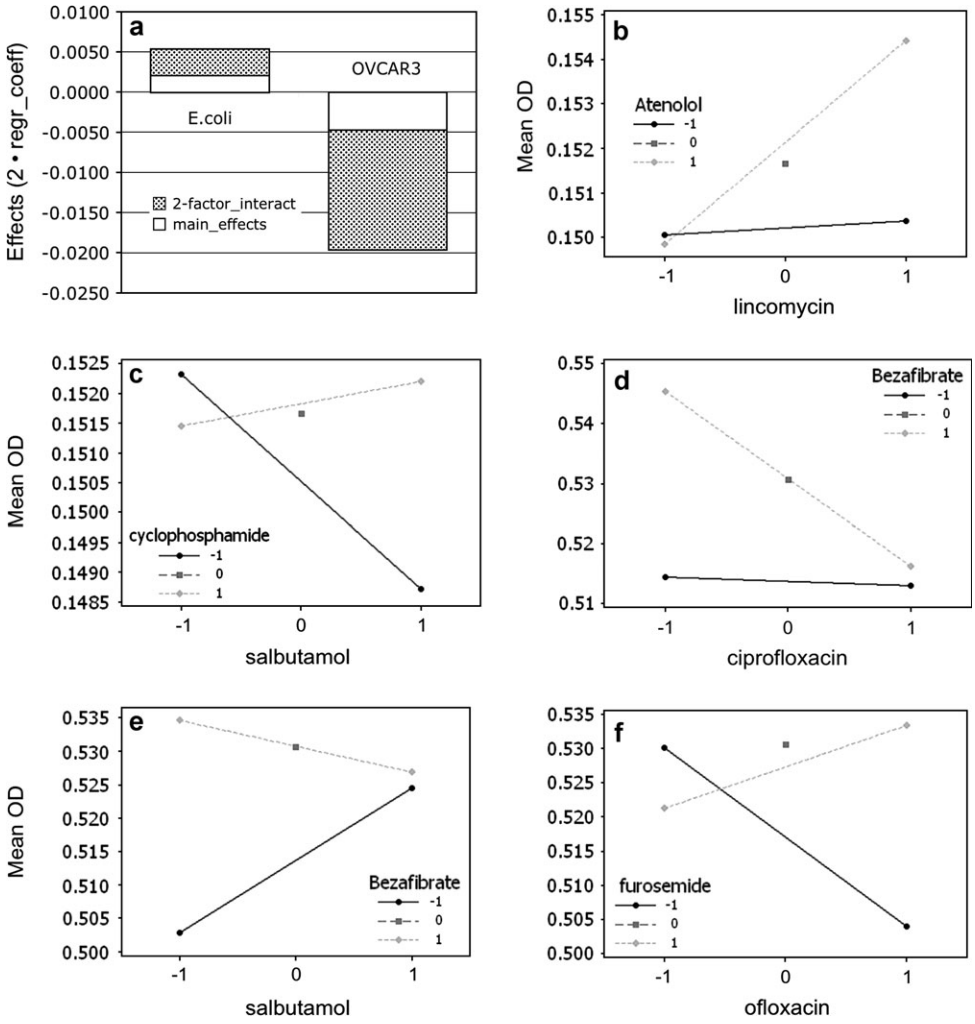

FIG. 2. Role and identity of interaction terms in mixture toxicity: sum of main effects and DDIs composing total mixture toxicity (A), interaction plots for significant DDIs in *E. coli* (B, C), and OVCAR3 (D–F). Exposure levels (− 1, 0, 1) as reported in Table 1.

Interaction Analysis

In a second phase of our study, we tested the DOE for interactions using a reduced set of terms. We focused on two-factor interactions since joint effects between three drugs are generally infrequent. No statistically significant DDIs were detected testing HEK293 cell proliferation. This could be due to limited variety of specific drug target sites in this cell line, with which drugs may influence each other's effects. Statistically significant DDIs were instead identified in *E. coli* and OVCAR3, where two-factor interactions appeared to account for a large part of total mixture effects (Fig. 2A). This may indicate that, when cells are responsive, DDIs are a fundamental component of mixture toxicity.

Statistically significant DDIs (Figs. 2B–F) in *E. coli* (atenolol × lincomycin $p = 0.084$, $q = 0.36$; cyclophosphamide × salbutamol $p = 0.077$, $q = 0.36$) and in OVCAR3 cells (bezafibrate × ciprofloxacin $p = 0.099$, $q = 0.37$; bezafibrate × salbutamol $p = 0.081$, $q = 0.34$; furosemide × ofloxacin $p = 0.023$, $q = 0.29$) were mainly antagonistic. It appears from our data that antagonistic actions are more probable than synergistic effects (e.g., atenolol × lincomycin, Fig. 2B), when combining chemicals that have diverse modes of action. This is consistent with the consideration that increasing the number of chemicals in a complex mixture, the chance of antagonism in the combined action raises as compared to synergism (Brack, 2007).

We observed how negligible negative effects on cell proliferation could interfere with each other and become statistically significant and stimulatory (Figs. 2C and 2F) or combined positive effects can have a negative influence on growth (Fig. 2E). In particular, the positive effect of bezafibrate on OVCAR3 proliferation appeared to be opposed by ciprofloxacin and salbutamol (Figs. 2D–E). Alternatively, bezafibrate may sensitize OVCAR3 cells at the highest dose tested here toward negative effects by other drugs (Figs. 2D–E). The importance of similar interactions and chemosensitizers

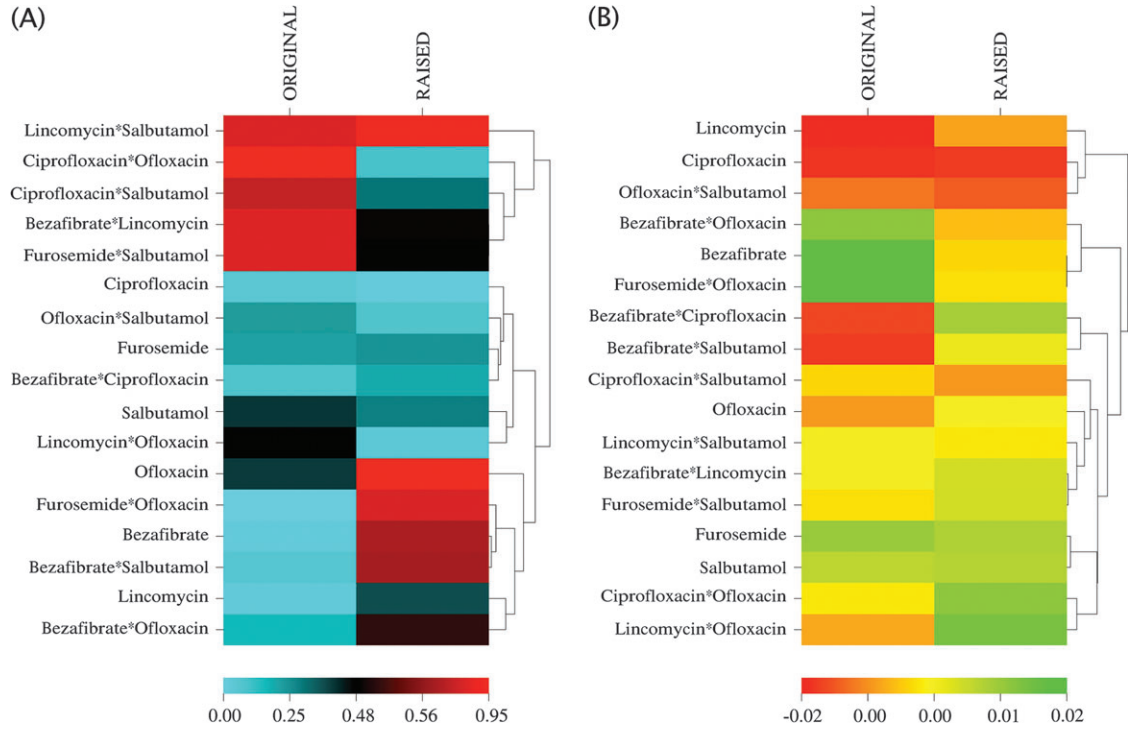

FIG. 3. CIMs of result tables for OVCAR3 factorial experiments, with cluster-ordered rows depicting selected terms (drugs and DDIs) and columns comparing original exposure and raised level data sets. The color in each image reflects the estimated p values (A) and effects (2 × linear regression coefficient) on cell proliferation (B) for main variables and two-factor interactions in the DOE. Clustering and color scales are as in Fig. 1.

has been recently highlighted (Luckenbach and Epel, 2005). Similarly, the statistically most significant DDI detected in this study was the combined and stimulatory action of furosemide and ofloxacin in OVCAR3 (Fig. 2F). Although we could not discern statistically significant main effects for neither of these drugs, furosemide has been previously demonstrated to exhibit weak estrogenic activity by YES assay (Fent *et al.*, 2006a). Combinatorial exposure to furosemide and ofloxacin may therefore potentially be endocrine disrupting.

Dose Dependency of Mixture Effects

With the aim of verifying whether observed effects for drugs were consistent across a wider range of concentrations, we raised all exposure levels (− 1, 0, 1; Table 1) by a factor of 10. Given their responsiveness, we chose OVCAR3 cells as the model line and analyzed for main effects first and then for DDIs. Based on all the data regarding main effects and interactions observed here in OVCAR3, we selected the most statistically significant variables and compared their p values (Fig. 3A) and effects (Fig. 3B) between original and increased exposure levels.

Raising concentrations resulted in a change in statistical significance or effect for most of the terms analyzed (Fig. 3). Some drugs (including bezafibrate and lincomycin) and DDIs (e.g., furosemide × ofloxacin) appeared to be statistically significant only at relevant environmental concentrations while other terms gained significance with increasing exposure levels, such as the antagonistic action between lincomycin and ofloxacin ($p = 0.06$, $q = 0.32$, Fig. 3A). We also observed dose-dependent transitions in the effects of several statistically significant DDIs (Fig. 3B). Only few terms, and in particular ciprofloxacin, seemed to maintain consistence of effects or statistical significance with exposure levels departing from environmentally relevant scenarios. Changes in mixture effects with increasing concentration may not be surprising considering the evidence for complex interactions occurring among our pharmaceuticals (Fig. 2).

Advantages and Limitations of the Approach

Several articles have previously demonstrated the usefulness of factorial DOE to analyze components and interactions in mixtures (Feron and Groten, 2002; Groten *et al.*, 2001), including its application to cell lines *in vitro* (Audet *et al.*, 2002; Cortin *et al.*, 2005; Tajima *et al.*, 2002; Zupke *et al.*, 1998). Here, a panel of three cell lines was selected to reach a sufficient level of target site diversity to provide meaningful biological responses in a simplified scenario (Schirmer, 2006). In addition to being more sensitive with regard to subtle effects many cell lines, including OVCAR3, have been extensively characterized for their drug sensitivity (Scherf *et al.*, 2000).

In this study, the use of randomization and blocked designs markedly increased the precision of *in vitro* experiments. The effects of blocks (pure error associated with the repetition of

tests) were always the most statistically significant factor in our regression analysis (Supplementary Material online). This reveals the importance of blocking and randomizing experimental designs when dealing with the high intrinsic variability associated with the set up of cell line–based bioassays (Rey deCastro and Neuberg, 2007). Also, repetition in blocks assured that treatments were tested over a wider range of laboratory conditions, increasing the scope of our assays (Steel and Torrie, 1960). We further demonstrated the efficacy of using linear assumptions when studying drug mixtures at low exposure levels. Curvature and LOF tests clearly indicated the utility and adequacy of GLM analysis for all experimental sets in this investigation (Supplementary Material online).

Nevertheless, our study has certain limitations. We verified the robustness of our approach with a separate run of confirmation experiments that were redesigned to study only the most statistically significant six factors (bezafibrate, ciprofloxacin, furosemide, lincomycin, ofloxacin, and salbutamol) in OVCAR3 cells (see Supplementary Material online, Suppl_DOE.xls). For this purpose, we employed a different DOE (resolution VI, three blocks) and analyzed for main effects and DDIs (Supplementary Material and Table S9). Statistical significance and effect values were confirmed with two exceptions: lincomycin and ciprofloxacin were inconsistent with regard to their statistical significance and main effect, respectively (data not shown). This was expected by our calculated FDR. We employed designs that were carefully chosen to have the highest possible resolution optimizing the laboratory effort, considering the combinatorial analysis of 13 variables. To decrease this residual FDR, we suggest the employment of blocked and full-factorial designs with a reduction of factor terms in future studies.

One of the constraints of this study rests in the use of a single toxicological end point to determine effects and interactions. Although we are currently planning experiments in which multiple dependent variables are tested, such as gene expression, the end point chosen here has its own limits. MTS tetrazolium is reduced by mitochondrial activity into a colored formazan product and is therefore an indirect measure of cell proliferation. Mitochondria are, however, a secondary but still significant toxicological target for many drugs (Duewelhenke *et al.*, 2007; Scatena *et al.*, 2007a, 2007b). Various catabolic and anabolic processes, calcium fluxes, various oxygen-nitrogen reactive species, or other signal transduction pathways interact in mitochondria to maintain cell homeostasis and to mediate cellular responses such as apoptosis. Changes in mitochondrial activity, as detected by formazan-based cell bioassays, may therefore represent themselves an interesting end point for toxicity testing and inference.

Implications for Risk Assessment of Pharmaceuticals

Compared to other industrial pollutants that are used for a range of applications and only incidentally have toxic effects, pharmaceuticals have been expressly designed to exert biological responses. Pharmacologically, every water-borne drug could be considered as a potential environmental concern. Our work indicates that only a limited number of APIs in our set of 13, namely atenolol, bezafibrate, ciprofloxacin, and lincomycin, have a statistically significant effect on prokaryotic and eukaryotic cells at environmentally relevant exposure levels. Given the sensitivity of *in vitro* tests and the resolution of the experimental design employed here, our investigation suggests that after systematic screening only a handful of pharmaceuticals out of the repertoire of water-borne drugs may represent an actual risk at environmental concentrations.

Statistically significant correlation has been previously found between fish cell–based *in vitro* assays and *in vivo* toxicity for several chemicals, including pharmaceuticals (Caminada *et al.*, 2006; Schirmer, 2006). Considering our centerpoint levels as a threshold of toxicological concern (TTC) for those drugs whose main effects were found to be statistically significant (Table 1), our *in vitro* assays were at least four orders of magnitude more sensitive then standard acute toxicity tests performed on aquatic animals and plants for APIs based on EC_{50}, LC_{50}, and other different end points (Crane *et al.*, 2006; Fent *et al.*, 2006b; Isidori *et al.*, 2007; Khetan and Collins, 2007). On the other hand, our TTC levels were comparable with lowest- or no-observed effect concentrations derived from standard chronic exposure tests using aquatic species (Crane *et al.*, 2006; Fent *et al.*, 2006b; Isidori *et al.*, 2007; Khetan and Collins, 2007). *In vitro* tests are systematically characterized by increased pharmacodynamic and reduced pharmacokinetic effects, representing a very sensitive model for toxicological investigation. We propose that *in vitro* cytotoxicity data, supported by a solid statistical design, could be useful in the initial identification of TTC levels for pharmaceuticals thereby contributing to the reduction of *in vivo* long-term exposure tests.

Environmental toxicology approaches to pharmaceuticals strongly rely on the study of single substances. Our study reinforces the view that such an approach underestimates the real environmental and health impact of APIs, given the ability of drugs to interact with each other (Khetan and Collins, 2007; Silva *et al.*, 2002). The unexpected consequences of the apparently innocuous musk fragrance galaxolide on cell physiology have also highlighted the need to determine whether cumulative exposure to common xenobiotics and APIs could lead to increased effects of normally mild toxicants (Luckenbach and Epel, 2005).

Our results confirm the paramount importance of identifying quantitative models to afford dose extrapolation in mixture toxicity data (Feron and Groten, 2002). We found that principal and joint actions at higher toxic effect levels of drugs seemed not to be able to predict individual or combinatorial effects of mixture components at more environmentally relevant doses. This consideration is highly relevant to the risk assessment of water-borne drugs, and it indicates that toxicity data obtained

with standard tests at doses of APIs that depart from real-world concentrations may not necessarily be representative for an environmentally relevant scenario.

CONCLUSIONS

This study represents a laboratory simplification of a very complex issue. We indicate standardization of *in vitro* toxicological assays as a research priority since assay conditions may strongly affect the identification of risk for micropollutants such as APIs. By using rigorously designed experiments and standard cell lines, we could detect specific, statistically significant and reproducible effects pointing at a handful of pharmaceuticals of toxicological concern at environmentally relevant exposure levels. We identified atenolol, bezafibrate, ciprofloxacin, and lincomycin as priority pharmaceuticals to be studied further for their long-term effects on aquatic species. They may also represent a potential hazard for particular human conditions, such as pregnancy or infancy, in case of chronic exposure via contaminated drinking water.

We found evidence that some drugs can interact at environmentally relevant exposure levels and that combined effects may represent a statistically significant component in mixture toxicity. Additionally, effects and interactions were dose dependent, suggesting that toxicity data for risk assessment should be obtained using mixed chemicals and exposure levels that are representative for the environment. *In vitro* tests are becoming an essential part of an integrated toxicological testing strategy, allowing reproducible events to be characterized at an appropriate level of complexity and under a high-throughput scenario. Our study provides a precautionary but not overprotective approach to the predictive hazard assessment of drug mixtures under realistic exposure levels, with the additional ethical and practical advantages of *in vitro* toxicology.

SUPPLEMENTARY DATA

Supplementary Material (Suppl_DOE.xls) and Tables S9 and S10 are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Audet, J., Miller, C. L., Eaves, C. J., and Piret, J. M. (2002). Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. *Biotechnol. Bioeng.* **80,** 393–404.

Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978). *Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building*. John Wiley & Sons, New York.

Brack, W. (2007). Integrated chemical and biological approaches for the analysis and confirmation of cause-effect relationships. *In Society for Environmental Toxicology and Chemistry (SETAC) 17th Annual Meeting 20–24. May 2007*, Porto, Portugal.

Caminada, D., Escher, C., and Fent, K. (2006). Cytotoxicity of pharmaceuticals found in aquatic systems: comparison of PLHC-1 and RTG-2 fish cell lines. *Aquat. Toxicol.* **79,** 114–123.

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., and Bagnati, R. (2006). Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* **78,** 8421–8429.

Champney, W. S., and Tober, C. L. (2000). Specific inhibition of 50S ribosomal subunit formation in *Staphylococcus aureus* cells by 16-membered macrolide, lincosamide, and streptogramin B antibiotics. *Curr. Microbiol.* **41,** 126–135.

Chinetti, G., Fruchart, J.-C., and Staels, B. (2000). Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation. *Inflamm. Res.* **49,** 497–505.

Clarke, M. B., Hughes, D. T., Zhu, C., Boedeker, E. C., and Sperandio, V. (2006). The QseC sensor kinase: a bacterial adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **103,** 10420–10425.

Cortázar, T. M., Coombs, G. H., and Walker, J. (2007). *Leishmania panamensis*: comparative inhibition of nuclear DNA topoisomerase II enzymes from promastigotes and human macrophages reveals anti-parasite selectivity of fluoroquinolones, flavonoids and pentamidine. *Exp. Parasitol.* **116,** 475–482.

Cortin, V., Garnier, A., Pineault, N., Lemieux, R., Boyer, L., and Proulx, C. (2005). Efficient in vitro megakaryocyte maturation using cytokine cocktails optimized by statistical experimental design. *Exp. Hematol.* **33,** 1182–1191.

Crane, M., Watts, C., and Boucard, T. (2006). Chronic aquatic environmental risks from exposure to human pharmaceuticals. *Sci. Total Environ.* **367,** 23–41.

Dixon, W. J. (1960). Simplified estimation from censored normal samples. *Ann. Math. Stat.* **31,** 385–391.

Duewelhenke, N., Krut, O., and Eysel, P. (2007). Influence on mitochondria and cytotoxicity of different antibiotics administered in high concentrations on primary human osteoblasts and cell lines. *Antimicrob. Agents Chemother.* **51,** 54–63.

Fent, K., Escher, C., and Caminada, D. (2006a). Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. *Reprod. Toxicol.* **22,** 175–185.

Fent, K., Weston, A. A., and Caminada, D. (2006b). Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* **76,** 122–159.

Feron, V. J., and Groten, J. P. (2002). Toxicological evaluation of chemical mixtures. *Food Chem. Toxicol.* **40,** 825–839.

Groten, J. P., Feron, V. J., and Sühnel, J. (2001). Toxicology of simple and complex mixtures. *Trends Pharmacol. Sci.* **22,** 316–322.

Hamilton, T. C., Young, R. C., McKoy, W. M., Grotzinger, K. R., Green, J. A., Chu, E. W., Whang-Peng, J., Rogan, A. M., Green, W. R., and Ozols, R. F. (1983). Characterization of a human ovarian carcinoma cell line (NIH:OVCAR-3) with androgen and estrogen receptors. *Cancer Res.* **43,** 5379–5389.

Isidori, M., Nardelli, A., Pascarella, L., Rubino, M., and Parrella, A. (2007). Toxic and genotoxic impact of fibrates and their photoproducts on non-target organisms. *Environ. Int.* **33,** 635–641.

Khetan, S. K., and Collins, T. J. (2007). Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. *Chem. Rev.* **107,** 2319–2364.

Luckenbach, T., and Epel, D. (2005). Nitromusk and polycyclic musk compounds as long-term inhibitors of cellular xenobiotic defense systems mediated by multidrug transporters. *Environ. Health Perspect.* **113,** 17–24.

Mimeault, C., Trudeau, V. L., and Moon, T. W. (2006). Waterborne gemfibrozil challenges the hepatic antioxidant defense system and down-regulates peroxisome proliferator-activated receptor beta (PPARbeta) mRNA levels in male goldfish (*Carassius auratus*). *Toxicology* **228,** 140–150.

Mimeault, C., Woodhouse, A. J., Miao, X. S., Metcalfe, C. D., Moon, T. W., and Trudeau, V. L. (2005). The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*. *Aquat. Toxicol.* **73,** 44–54.

Mora-Rodriguez, R., Hodgkinson, B. J., Byerley, L. O., and Coyle, E. F. (2001). Effects of beta-adrenergic receptor stimulation and blockade on substrate metabolism during submaximal exercise. *Am. J. Physiol. Endocrinol. Metab.* **280,** E752–E760.

Pomati, F., Castiglioni, S., Zuccato, E., Fanelli, R., Vigetti, D., Rossetti, C., and Calamari, D. (2006). Effects of a complex mixture of therapeutic drugs at environmental levels on human embryonic cells. *Environ. Sci. Technol* **40,** 2442–2447.

Pomati, F., Cotsapas, C., Castiglioni, S., Zuccato, E., and Calamari, D. (2007). Gene expression profiles in Zebrafish (*Danio rerio*) liver cells exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. *Chemosphere* **70,** 65–73.

Pomati, F., Netting, A. G., Calamari, D., and Neilan, B. A. (2004). Effects of erythromycin, tetracycline and ibuprofen on the growth of *Synechocystis* sp. and *Lemna minor*. *Aquat. Toxicol.* **67,** 387–396.

Rey deCastro, B., and Neuberg, D. (2007). The statistical performance of an MCF-7 cell culture assay evaluated using generalized linear mixed models and a score test. *Stat. Med.* **26,** 2501–2518.

Scatena, R., Bottoni, P., Botta, G., Martorana, G. E., and Giardina, B. (2007a). The role of mitochondria in pharmacotoxicology: a reevaluation of an old, newly emerging topic. *Am. J. Physiol. Cell Physiol.* **293,** C12–C21.

Scatena, R., Bottoni, P., Martorana, G. E., Vincenzoni, F., Botta, G., Pastore, P., and Giardina, B. (2007b). Mitochondria, ciglitazone and liver: a neglected interaction in biochemical pharmacology. *Eur. J. Pharmacol.* **567,** 50–58.

Scherf, U., Ross, D. T., Waltham, M., Smith, L. H., Lee, J. K., Tanabe, L., Kohn, K. W., Reinhold, W. C., Myers, T. G., Andrews, D. T., *et al.* (2000). A gene expression database for the molecular pharmacology of cancer. *Nat. Genet.* **24,** 236–244.

Schirmer, K. (2006). Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology* **224,** 163–183.

Sharpe, R. M., and Irvine, D. S. (2004). How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? *BMJ* **328,** 447–451.

Silva, E., Rajapakse, N., and Kortenkamp, A. (2002). Something from "nothing"-eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* **36,** 1751–1756.

Snedecor, G. W., and Cochran, W. G. (1989). *Statistical Methods* 8th ed., Iowa State University Press.

Steel, R. G. D., and Torrie, J. H. (1960). *Principles and Procedures of Statistics with Special Reference to the Biological Sciences*. McGraw-Hill Book Complany, Inc., New York.

Storey, J. D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* **100,** 9440–9445.

Tajima, O., Schoen, E. D., Feron, V. J., and Groten, J. P. (2002). Statistically designed experiments in a tiered approach to screen mixtures of *Fusarium* mycotoxins for possible interactions. *Food Chem. Toxicol.* **40,** 685–695.

Takeuchi, S., Matsuda, T., Kobayashi, S., Takahashi, T., and Kojima, H. (2006). *In vitro* screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)alpha and PPARgamma and quantitative analysis of *in vivo* induction pathway. *Toxicol. Appl. Pharmacol.* **217,** 235–244.

Tang, J., Hu, M., Lee, S., and Roblin, R. (2000). A polymerase chain reaction based method for detecting *Mycoplasma/Acholeplasma* contaminants in cell culture. *J. Microbiol. Methods* **39,** 121–126.

Tirado, O. M., Mateo-Lozano, S., and Notario, V. (2005). Rapamycin induces apoptosis of JN-DSRCT-1 cells by increasing the Bax: Bcl-xL ratio through concurrent mechanisms dependent and independent of its mTOR inhibitory activity. *Oncogene* **24,** 3348–3357.

Weinstein, J. N., Myers, T. G., O'Connor, P. M., Friend, S. H., Fornace, A. J., Kohn, K. W., Fojo, T., Bates, S. E., Rubinstein, L. V., Anderson, N. L., *et al.* (1997). An information-intensive approach to the molecular pharmacology of cancer. *Science* **275,** 343–349.

Zupke, C. A., Stefanovich, P., Berthiaume, F., and Yarmush, M. L. (1998). Metabolic effects of stress mediators on cultured hepatocytes. *Biotechnol. Bioeng.* **58,** 222–230.