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Application of an *in Vitro* Assay to Identify Chemicals That Increase Estradiol and Progesterone Synthesis and Are Potential Breast Cancer Risk Factors

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BACKGROUND: Established breast cancer risk factors, such as hormone replacement therapy and reproductive history, are thought to act by increasing estrogen and progesterone (P4) activity.

OBJECTIVE: We aimed to use *in vitro* screening data to identify chemicals that increase the synthesis of estradiol (E2) or P4 and evaluate potential risks.

METHOD: Using data from a high-throughput (HT) *in vitro* steroidogenesis assay developed for the U.S. Environmental Protection Agency (EPA) ToxCast program, we identified chemicals that increased estradiol (E2-up) or progesterone (P4-up) in human H295R adrenocortical carcinoma cells. We prioritized chemicals by their activity. We compiled *in vivo* studies and assessments about carcinogenicity and reproductive/developmental (repro/dev) toxicity. We identified exposure sources and predicted intakes from the U.S. EPA's ExpoCast.

RESULTS: We found 296 chemicals increased E2 (182) or P4 (185), with 71 chemicals increasing both. *In vivo* data often showed effects consistent with this mechanism. Of the E2- and P4-up chemicals, about 30% were likely repro/dev toxicants or carcinogens, whereas only 5–13% were classified as unlikely. However, most of the chemicals had insufficient *in vivo* data to evaluate their effects. Of 45 chemicals associated with mammary gland effects, and also tested in the H294R assay, 29 increased E2 or P4, including the well-known mammary carcinogen 7,12-dimethylbenz(a)anthracene. E2- and P4-up chemicals include pesticides, consumer product ingredients, food additives, and drinking water contaminants.

DISCUSSION: The U.S. EPA's *in vitro* screening data identified several hundred chemicals that should be considered as potential risk factors for breast cancer because they increased E2 or P4 synthesis. *In vitro* data is a helpful addition to current toxicity assessments, which are not sensitive to mammary gland effects. Relevant effects on the mammary gland are often not noticed or are dismissed, including for 2,4-dichlorophenol and cyfluthrin. Fifty-three active E2-up and 59 active P4-up chemicals that are in consumer products, food, pesticides, or drugs have not been evaluated for carcinogenic potential and are priorities for study and exposure reduction. <https://doi.org/10.1289/EHP8608>

Introduction

People are exposed to chemicals that affect hormonal regulation through diet (e.g., food and water), consumer products, pesticides, pharmaceuticals, and industrial processes. Although there is concern about adverse health effects of exposure to endocrine-disrupting chemicals (EDCs) and pollutants (Birks et al. 2016; Gore et al. 2015; Karwacka et al. 2019; La Merrill et al. 2020), most chemicals' endocrine-disrupting capabilities remain largely untested.

One concern is whether exposure to EDCs, which interfere with the body's hormonal systems, can increase the risk and progression of breast cancer, the most common invasive cancer in women worldwide and the second-leading cause of cancer-related deaths in American women (Siegel et al. 2019). The majority of breast cancers are hormonally responsive—that is, they are classified as estrogen (ER)- and progesterone (P4)-receptor (PR)-positive (Colditz et al. 2004)—and many established risk factors relate to changes in hormone levels, including hormone replacement therapy (Chlebowski et al. 2020), an increased number of menstrual cycles due to early menarche and/or late menopause, a late first birth, and nulliparity (Albrektsen et al. 2005; Colditz et al. 2004; Pathak and Whittemore 1992; Pike et al.

1983; Rosner et al. 1994). Because breast tumors can be dependent on hormones for their continued growth and progression, their treatment often relies on drugs that block estrogen action, either by antagonizing the ER or by inhibiting the synthesis of estradiol (E2) by the aromatase enzyme (Thorat and Balasubramanian 2020; Williams and Harris 2014). Some women with a genetic variant that causes elevated aromatase expression have had poor survival following ER-positive breast cancer (Friesenhengst et al. 2018). Preventative measures, such as the removal of the ovaries in women with high inherited risk of breast cancer (Thorat and Balasubramanian 2020), will often reduce the levels of endogenous hormones, and aromatase inhibitors have been shown to reduce breast cancer incidence by 49% in a study with almost 4,000 high-risk postmenopausal women (Cuzick et al. 2020).

The relationship between hormone exposure—especially of E2 and P4—and breast cancer is well documented in experimental animals (Cogliano et al. 2011; Rudel et al. 2014). In both mice and rats treated with chemical carcinogens, hormone withdrawal (e.g., through ovariectomy) inhibited mammary tumor development, whereas hormone supplementation increased its incidence (Medina et al. 2001; Planas-Silva et al. 2008; Russo and Russo 1996; Shull et al. 2018; Thordarson et al. 2001; Welsch 1985). Hormone exposure during periods of mammary gland development including embryonic, puberty, and pregnancy can also interfere with mammary gland growth and may alter susceptibility to tumors or inhibit lactation, sometimes evidenced by decreased pup weights during lactation (Makris 2011; Rudel et al. 2011). Both E2 and P4 are reported to be rodent mammary gland carcinogens and to increase the risk of breast cancer following ionizing radiation (Helm and Rudel 2020; Rudel et al. 2007). E2 may increase mammary tumors by increasing epithelial cell proliferation (Fernandez and Russo 2010; Shull et al. 2018; Yager and Davidson 2006), whereas P4 is hypothesized to be tumor promoting owing to its roles in cell proliferation and stem cell activation, which can increase mutations and DNA damage (Briskin et al. 2015). E2 and P4 in combination may also increase risk, whereby E2 induces PR expression allowing P4 to agonize the receptor and induce proliferation of mammary stem

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and progenitor cells, thus promoting tumor progression (Briskin et al. 2015). Notably, the interaction between E2 and P4 (and ER/PR) in mammary tumor etiology is complex and still being elucidated (Sathyamoorthy and Lange 2020). Although E2 and P4 can be tumor promoting, their administration to nulliparous rodents at concentrations high enough to differentiate the mammary gland, which mimics the effects of pregnancy, has been shown to reduce mammary tumor incidence (Sivaraman et al. 1998). However, this hypothesized mechanism for the protective effect of pregnancy has been questioned (Hilakivi-Clarke et al. 2006).

Despite the potential effects of E2 and P4 on breast cancer risk and progression, little attention has been paid to chemicals that may affect steroidogenesis by increasing synthesis of these hormones, systemically or in the breast, or altering the activity of enzymes involved in steroidogenic pathways, including specific or nonspecific effects on P450 enzymes, which can alter endogenous hormone levels (Gore et al. 2015). The enzyme aromatase is one example of an enzyme whose activity can be regulated by chemical exposures by, for example, triazines, neonicotinoid pesticides, dichlorodiphenyltrichloroethane (DDT), and common phenolic chemicals such as bisphenol A and the cosmetic preservatives methyl and butyl paraben (Caron-Beaudoin et al. 2017; Sanderson et al. 2000; Williams and Darbre 2019). Instead, most research about EDCs as risk factors has focused on chemicals that bind to and activate hormone nuclear receptors, such as the ERs and androgen receptors, or which interfere with receptor-relevant signaling pathways (Judson et al. 2015), even though effects on steroidogenesis can occur independently of activity at hormone receptors (Mansouri et al. 2016; Sanderson 2006; Whitehead and Rice 2006). Because E2 and P4 are important risk factors for breast cancer, chemicals that increase their synthesis may also increase the risk for breast cancer and must be prioritized for further research and exposure reduction.

The Organisation for Economic Co-operation and Development's (OECD) H295R *in vitro* steroidogenesis assay, an internationally validated assay for regulatory contexts, has been used to study a chemical's impact on the steroidogenic pathway by measuring hormone concentrations following exposure to human H295R adrenocortical carcinoma cells, and similar approaches have been used in research settings (Caron-Beaudoin et al. 2016, 2017; Fan et al. 2007; Hecker et al. 2011; Pinto et al. 2018; Strajhar et al. 2017). Although initially only validated to measure the effects on E2 and testosterone, the H295R assay was subsequently modified to run in high-throughput (HT) format as part of the U.S. Environmental Protection Agency (EPA) ToxCast chemical screening program and to measure effects on 13 hormones involved in the steroidogenic pathway, including progestagens, corticosteroids, androgens, and estrogens (Haggard et al. 2018; Karmaus et al. 2016).

We used publicly available data from the HT-H295R assay to identify chemicals that increased E2 or P4 synthesis. To understand whether chemicals that increased E2 or P4 levels in H295R cells also show evidence of carcinogenicity or reproductive or developmental toxicity, we compiled *in vivo* evidence for these effects using data from the U.S. EPA, California EPA, and California Office of Environmental Health Hazard Assessment databases and review articles. We also used the U.S. EPA Exposure Forecasting (ExpoCast) research program data and the U.S. EPA Chemical and Products Database (CPDat) (Dionisio et al. 2018) to identify potential exposure sources (including pesticides, consumer products, industrial, diet, and pharmaceutical products) and predicted intake rates for active chemicals (Ring et al. 2019). We used these data to prioritize chemicals that increased the synthesis of E2 or P4 (i.e., E2- and P4-up chemicals) for exposure reduction and further study.

Methods

Description of the ToxCast HT-H295R Experiments

We identified E2- and P4-up chemicals using publicly available data of two HT-H295R steroidogenesis assay experiments conducted by the U.S. EPA and first reported by Karmaus et al. (2016) and Haggard et al. (2018). This HT-H295R assay was developed and conducted as part of the U.S. EPA's ToxCast screening program. In these experiments, 2,012 chemicals were selected from ToxCast Phase I, II, and III and the endocrine 1000 (E1K) libraries, which include potential EDCs and other chemicals of regulatory interest, such as pesticides. The methodology for these experiments has been described in detail by Karmaus et al. (2016) and Haggard et al. (2018) and is briefly summarized below.

Human H295R adrenocortical carcinoma cells were prestimulated with forskolin for 48 h, followed by a 48-h chemical exposure at a single maximum tolerated concentration (MTC), usually 100 μ M. The sample was diluted if the cell viability did not exceed 70% as tested using a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium (MTT) cytotoxicity assay. Concentrations of 13 steroid hormones (Figure 1) were measured in the culture media using high performance liquid chromatography with tandem mass spectroscopy. The hormones dehydroepiandrosterone (DHEA) and pregnenolone were frequently detected below the lower limit of quantification (LOQ) and were excluded from further analyses. A treatment was considered to have a significant effect on a hormone if it produced a fold change ≥ 1.5 that of the dimethyl sulfoxide (DMSO) solvent control. Treatments that significantly affected four or more hormones ($n = 571$) were then tested again using a six-point concentration–response (CR) format with the MTC as 100 μ M or, if that dose was cytotoxic, the maximum concentration with $>70\%$ viability in an MTT cytotoxicity assay, and using half-log serial dilutions below that. An additional 85 chemicals that were not tested at a single MTC were also tested in the CR format (Haggard et al. 2018), for a total of 656 chemicals tested in the CR format. Across all the chemicals, administered concentration ranged from 0.041 nM to 100 μ M.

Karmaus et al. (2016) and Haggard et al. (2018) then used different approaches to determine the chemical–hormone pair significance (i.e., the hit-call) for each chemical run in the CR format. Karmaus et al. (2016) determined significance using the ToxCast automatic data processing pipeline (tcpl) (Filer et al. 2017), which is usually applied to all ToxCast data to standardize it with other HT data and to provide a preliminary look at the data, with the expectation that subsequent analyses would improve interpretation (Haggard et al. 2018). To be assigned a positive hit-call, the chemical data had to fit a hill or gain–loss model, and the top of the curve (the efficacy) had to exceed a cut off of six times the baseline (which was taken by normalizing the response values of the two lowest tested concentrations) (Filer et al. 2017; Karmaus et al. 2016). In contrast, Haggard et al. (2018) first determined significance for each chemical concentration–hormone pair using an analysis of variance (ANOVA) followed by post hoc Dunnett's tests ($p \leq 0.05$) on fold-change data using the DMSO solvent as the control (Haggard et al. 2018). A chemical was defined as hormonally active on the basis of logic applied in the OECD Test Guideline (TG) No. 456 (OECD 2011)—that is, either two consecutive concentrations had a significant effect or a significant result was observed at the maximum tested noncytotoxic concentration (Haggard et al. 2018).

Identification of E2- and P4-Up Chemicals

We defined E2- and P4-up chemicals as those that significantly increased the concentrations of E2 or P4 in the CR experiments

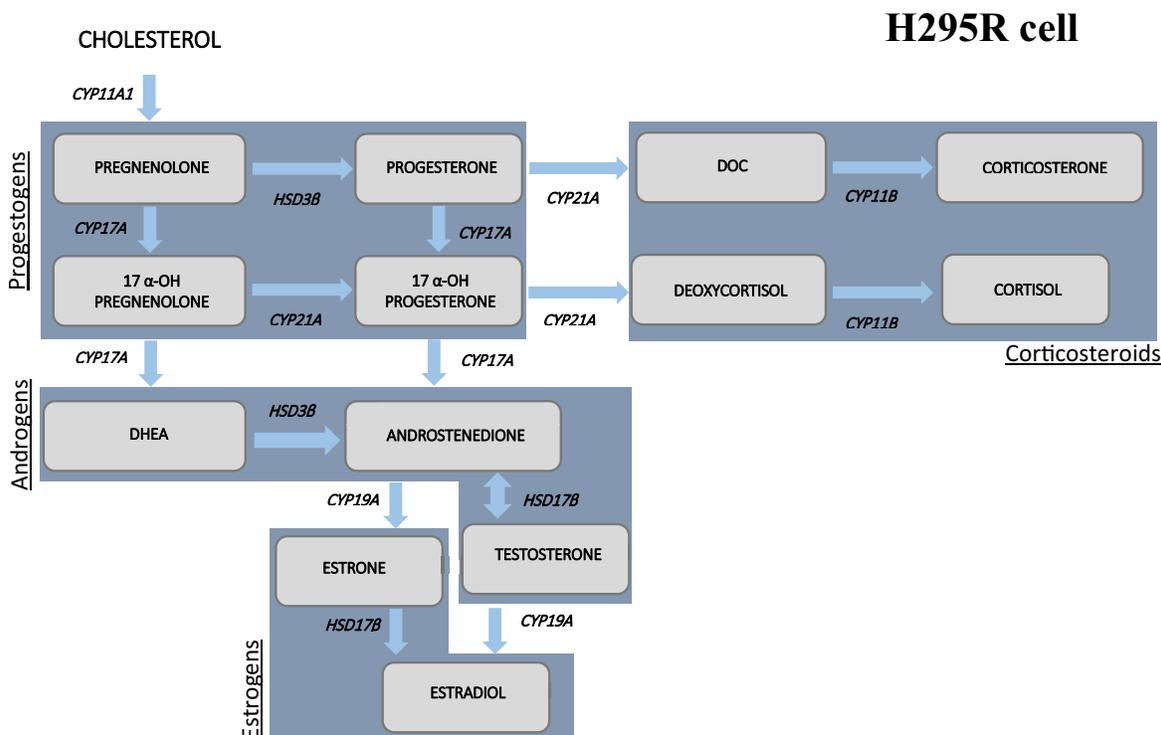


Figure 1. The steroidogenic pathway in H295R cells. The H295R assay is a high-throughput *in vitro* assay in H295R human adrenocortical carcinoma cells. The levels of 13 steroid hormones (shown in gray boxes) were measured to evaluate perturbations throughout the steroidogenic pathway. Enzymes are italicized. Arrows indicate direction of hormone synthesis. Steroid hormones are further grouped together by hormone type. Note: DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone.

conducted by Karmaus et al. (2016) and Haggard et al. (2018), per the “Methods” section, as described above. These were identified from the subset of 656 chemicals tested in the CR format, of which 654 had adequate CR data for analysis (Haggard et al. 2018). We used the Haggard et al. (2018) hit-call data, which employed an ANOVA-based approach, to identify the E2- and P4-up chemicals because this approach was developed and validated for this assay by the OECD in TG 456 (OECD 2011) and the interlaboratory validation report for the OECD TG 456 (Hecker et al. 2011). The U.S. EPA’s ToxCast data, which uses the tcpl hit-call data from Karmaus et al. (2016), can also be used to identify E2- and P4-up chemicals, and we included the chemical concentration resulting in 50% of maximal hormone increase (AC50) and the chemical concentration resulting in 10% of maximal hormone increase (AC10) values from the tcpl data in Excel Tables S1 and S2. The ANOVA-based approach may be more sensitive to effects at the lower doses because tcpl uses the lower doses to establish a baseline or control response, whereas the ANOVA compares responses at each dose to the control and so is able to detect increases at the two lower doses. The hit-call data we used can be found in the Supplemental Data File 2 of Haggard et al. (2019), an extension of the Haggard et al. (2018) experiments and data, which reports the direction of steroidogenesis for each hormone measured after chemical treatment. If a chemical was run multiple times, we used only the data from the earliest block (as indicated by the lowest numbered plate). All data processing and analysis that followed was conducted using R (version 3.6.3; R Development Core Team; <https://github.com/SilentSpringInstitute/Cardona-and-Rudel-2021/>).

Prioritization Based on Potency and Efficacy Measures

We used three measures to represent the potency and/or efficacy of each E2- and P4-up chemical: *a*) the maximal fold change (MFC)

in hormone concentration; *b*) the lowest effective concentration (LEC) at which the tested chemical produced a significant hormone increase; and *c*) the adjusted maximal mean Mahalanobis distance (adj.maxmMd). The adj.maxmMd, described by Haggard et al. (2018), is a unitless statistical value reflecting a chemical’s magnitude of steroidogenic pathway disruption for 11 hormones across the tested chemical concentration range; it controls for correlation and covariance among the hormone analytes (Haggard et al. 2018).

To find the MFC, a proxy for efficacy, we used the raw hormone data found in the Haggard et al. (2018) Supplemental Data File 3. Using the raw hormone data, we first calculated the fold change relative to the average of the DMSO solvent control duplicates from the same plate at each of the tested concentrations. We then used Haggard et al. (2018) Supplemental Data Files 4 and 7 to identify the chemical concentrations with significant hormone increases. Following OECD hit-call logic, we identified the chemical concentrations with consecutive significant hormone increases or a significant hormone increase at the highest tested noncytotoxic chemical concentration. Of these chemical concentrations, the highest fold change was chosen as the MFC.

We also identified the LEC, a proxy for potency, using the raw hormone data from the Haggard et al. (2018) Supplemental Data File 3. As with the MFC, we used the Haggard et al. 2018 Supplemental Data Files 4 and 7 to identify the chemical concentrations with consecutive significant hormone increases, or significance at the highest tested noncytotoxic chemical concentration, and then selected the lowest chemical concentration from these as the LEC. Furthermore, we compared the LEC values with the potency values calculated using the tcpl: the AC50 and the AC10. We compiled the AC50 and AC10 values for each hormone-chemical pair using the ToxCast and Tox21 Summary Files for invitroDBv3.2 (U.S. EPA 2019a). Tcpl calculated AC50s only for chemicals it identified as active, and AC10s for

chemicals that were able to fit a model even if their hit-call was not active. We conducted a Pearson's correlation analysis to assess the relationship between the LEC and the AC50 and AC10 values.

Last, the adj.maxmMd calculated by Haggard et al. (2018) was used to take both potency and efficacy into account and identify chemicals with a clear response above assay noise considering data for 11 hormones (excluding DHEA and pregnenolone owing to their frequent detection below the lower LOQ). The adj.maxmMd for each chemical tested can be found in the Haggard et al. (2018) Supplemental Data File 11.

We defined E2- and P4-up chemicals with higher potency and efficacy as active on the basis of the following criteria: $MFC \geq 1.5$, $LEC \leq 33 \mu M$, and $adj.maxmMd > 0$. These active chemicals were the focus of subsequent analysis because they are less likely to be false-positives (based on an $MFC \geq 1.5$, as suggested by the OECD interlaboratory analysis) and more likely to be active at environmentally relevant concentrations (by limiting to more potent chemicals). An $adj.maxmMd > 0$ is also less likely to result in a false-positive for steroidogenic pathway disruption, per Haggard et al. (2018). We defined the excluded chemicals as borderline active. We also removed the following synthetic or endogenous steroid hormones because they can interfere with steroidogenesis by acting as substrates: 17-methyltestosterone, 17 α -estradiol, 17 α -ethinylestradiol, 17 α -hydroxyprogesterone, 17 β -estradiol, 4-androstene-3,17-dione, 5 α -dihydrotestosterone, androsterone, dehydroepiandrosterone, equilin, estrone, P4, and testosterone propionate.

We classified the active chemicals into three groups corresponding to their potency and efficacy by ranking them on the basis of the average of their MFC percentile rank and LEC percentile rank. We considered those chemicals in the top 25% of the rank as having higher (compared with the other chemicals in the ranking) efficacy and potency, chemicals in the middle 25–75% as having intermediate potency and efficacy, and chemicals in the bottom 25% as having lower potency and efficacy.

Activity at the ER

Although chemicals that increase E2 or P4 will not necessarily also activate the ER directly, we wanted to identify the E2- and P4-up chemicals that may also act on the ER to provide insight on whether the two activities appear to be independent vs. dependent. We gathered the ER area under the curve (AUC), as calculated by Judson et al. (2015), which is a score between 0 and 1 describing the probability of a chemical to be active at the ER on the basis of its potency and efficacy across 18 of ToxCast's *in vitro* assays that measure ER-associated pathways such as binding, dimerization, and ER-dependent cell proliferation (Judson et al. 2015). We considered chemicals positive for ER interaction if they had an AUC score ≥ 0.1 , positive per Judson et al. (2015). Chemicals with an $AUC \geq 0.01$ but < 0.1 were considered as having ambiguous activity, and chemicals with an $AUC < 0.01$ were considered likely *in vivo* inactive (Judson et al. 2015). The ER AUC for agonism and antagonism, describing the probability of a chemical to be either an ER agonist or an ER antagonist, was also calculated by Judson et al. (2015), and we considered both probabilities, identifying the activity with the higher probability as being the more likely.

In Vivo End Points and Chemical Cancer Risk Assessment

We conducted three analyses to evaluate whether *in vivo* effects consistent with increased E2 or P4 are observed for chemicals that were active *in vitro*. First, we looked for any evidence of carcinogenicity or reproductive or developmental toxicity in authoritative databases and mammary gland-focused review articles.

Second, we evaluated how many of the chemicals that have been listed in review articles as causing mammary gland tumors or other mammary gland effects also show *in vitro* E2- and P4-up activity. Finally, because the authoritative carcinogenicity or reproduction and developmental toxicity assessments are general and may not specifically reflect effects due to increased E2 or P4, we conducted a more focused, but preliminary, primary literature review for a subset of the most potent and efficacious E2- and P4-up chemicals to identify *in vivo* effects that may be consistent with increased E2 and P4, focusing on mammary gland effects. Of course, the end points we looked for in rodent studies are imperfect proxies for *in vivo* effects in humans, but relevant data on humans is generally not available. The details of these analysis follow.

To collect information on *in vivo* reproductive toxicity, developmental toxicity and carcinogenicity, we used the U.S. EPA's Toxicity Value Database (ToxValDb) (Judson 2019; Williams et al. 2017). ToxValDb summarizes *in vivo* studies by various parameters, such as type of study, species tested, and toxicity values [e.g., no observed effect level (NOEL) or lowest observed effect level (LOEL)].

Reproductive and Developmental Toxicity. We used the ToxValDb study summary file (Judson 2019) to identify mammalian reproductive and developmental toxicity (repro/dev) toxicity studies for the E2- and P4-up chemicals. Toxicity values had to be reported in units of milligrams per kilogram per day so that studies were comparable. We classified developmental toxicity studies as those with a risk assessment class labeled as developmental, and reproductive toxicity studies as those with a risk assessment class labeled as reproductive, reproduction:chronic, or reproduction:acute. Studies labeled as reproductive developmental were considered both reproductive and developmental toxicity studies. The studies were then identified as reporting either an effect level (e.g., a benchmark dose or LOEL) or an NOEL. We assigned each of the E2- and P4-up chemicals to one of three categories; *a*) likely repro/dev toxicant (chemicals with an effect level < 100 mg/kg per day in either a reproductive or developmental toxicity study); *b*) unlikely repro/dev toxicant (chemicals with both a reproductive and developmental study showing an NOEL ≥ 100 mg/kg per day), or *c*) as not having enough information to determine repro/dev toxicity (chemicals that did not meet the criteria to be labeled as likely or unlikely). We also classified chemicals as likely repro/dev toxicants if they were listed as such by the California EPA under the Proposition 65 program (Prop65) (OEHHA 2021) or if they were included in a review of chemicals that alter mammary gland development (Rudel et al. 2011). Because study results may contradict each other, we prioritized the labels in the following order: likely repro/dev label, followed by unlikely, and then inadequate evidence.

Carcinogenicity. We used the ToxValDb cancer summary file (Judson 2019), which compiles cancer classifications by authoritative organizations including the U.S. EPA's Office of Pesticide Programs, the U.S. EPA's Integrated Risk Information System, and the National Toxicology Program's Report on Carcinogens. Based on the classification by these organizations, chemicals were assigned into one of three carcinogenicity categories: *a*) likely carcinogen (e.g., classified as a known, probable, or possible carcinogen); *b*) unlikely carcinogen (e.g., classified as having evidence of noncarcinogenicity or unlikely to be carcinogenic); or *c*) inadequate evidence to assess carcinogenicity (e.g., not classifiable as to human carcinogenicity or inadequate data for evaluation). If two organizations' cancer classifications for a given chemical contradicted each other, we used the likely carcinogen label, followed by the unlikely carcinogen, and then the inadequate evidence labels to assign our simplified cancer category to the chemical. See Excel Table S3 for a list of the cancer classifications assigned by organizations and Excel

Table S4 for subsequent categorization of the classifications into one of our three categories. Chemicals listed as carcinogens by the California EPA under Prop65 (OEHHA 2021) or as causing rodent mammary gland tumors by Rudel et al. (2007) were also classified as likely carcinogens. Chemicals not included in any of these sources were classified as having inadequate evidence to classify. We also noted whether any of the chemicals had been listed as having observed mammary tumors in pesticide U.S. EPA Reregistration Eligibility Documents (Cardona and Rudel 2020).

We also used existing reviews of chemicals with mammary gland effects to determine the frequency of effects on E2 and P4 synthesis among these chemicals. We reviewed how many of the ~250 mammary carcinogens or mammary gland developmental disruptors listed in three review articles published by this team (Cardona and Rudel 2020; Rudel et al. 2007, 2011) were also tested in the HT-H295R assay, and if so, whether they increased E2 or P4.

As an initial assessment of whether chemicals that increased E2 or P4 synthesis *in vitro* may also have *in vivo* effects suggestive of E2 or P4 increases, we conducted a preliminary literature review for the five most potent (i.e., the lowest LEC) and the five most efficacious (i.e., the highest MFC) E2- and P4-up chemicals in the higher efficacy/potency classification. We did not review data for the failed drug candidates. For pesticides, we reviewed the U.S. EPA's pesticide registration documents using the U.S. EPA's Pesticide Chemical Search website and registration review dockets (<https://iaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:1:0::NO:1>), focusing on reviewing human health risk assessments, carcinogenicity evaluations, and reproductive/developmental studies. For nonpesticidal chemicals and pesticides with no information in the Pesticide Chemical Search website, we used the PubMed search engine (or Google if studies were limited) and searched the chemical name or CASN followed by "mammary," "breast," "reproductive," or the name of the hormone that it increased *in vitro* (e.g., "estradiol" or "progesterone") (see Excel Table S5 for search terms). In PubMed, we used filters to limit to studies focusing on "human" or "other animal", with "full text", published after the year 2000 and in English. For each chemical, we limited to reviewing the first 20 results ordered by best match. As evidence of increased E2 or P4 synthesis *in vivo*, we looked for studies conducted in rodents or human epidemiological studies reporting; a) evidence of effects on hormone secretion or levels; b) effects on the mammary gland or lactation—including decreased pup body weight (BW); c) effects on reproductive or developmental end points consistent with increased E2 or P4—including altered litter size and implantation sites. For E2-up chemicals we specifically looked for increased E2 or aromatase expression, for uterotrophic responses in immature females (but not considering studies with ovariectomized females) and for accelerated female sexual maturation. For P4-up chemicals we looked for increases in P4 or in gestational length, as previous experimental studies in rodents have proposed that increased gestational length may be due to increased P4 levels (Chwalisz 1994; Vinggaard et al. 2005). We are not aware of any end point commonly reported for mammary gland that would reflect local P4 levels or activity.

We noted effects on other less commonly reported end points if we thought they might reflect E2 or P4 pathways, for example, vaginal cornification. If a study appeared to be designed to measure a relevant end point but did not see any effect of the chemical, then we have noted it. If a chemical is known to have activity at the ER or PR, we noted that in addition. However, our review was limited to identifying *in vivo* effects that are consistent with the mechanism of enhanced E2 and P4 synthesis. We did not investigate the entire literature on each of the high potency or efficacious chemicals or conduct a weight of evidence review. As a

result, if studies did not report relevant effects, we did not review them to determine whether the methods were sufficiently sensitive and the study appropriately designed to have been able to detect such effects.

Exposure Potential and Chemical Use

We gathered measures of exposure potential (i.e., the likelihood of general population exposure) and exposure sources for the active E2- and P4-up chemicals. We used data from the U.S. EPA's HT exposure prediction meta-model, which estimates the population intake rate of chemicals ($n = 479,926$) by using structural and physiochemical properties, production information, and predicted exposure pathway(s) leading from source to consumer (e.g., pesticides, industrial processes, consumer products and diet) (Ring et al. 2019). Using these predictors, the model estimates a median intake rate for the general U.S. population (units of milligrams per kilogram of BW per day) with a 95% credible interval (CI; quantiles for 0.025 and 0.975) for each chemical. This CI holds that the true median has a 95% probability of falling within the interval. Because of the tremendous uncertainty in these exposure estimates and the lack of any estimate of high-end population exposures, we used the upper quantile of the CI; if an upper 95% CI was not calculated, we used the predicted median instead.

To identify potential sources of exposure, we used the U.S. EPA CPDat (Dionisio et al. 2018), which compiles data on chemical composition of products and product/chemical usage as reported in material data safety sheets, ingredient lists or online retail sites. Each chemical is assigned a cassette or a product use category composed of terms describing the product/chemical usage. We used these categories to assign the E2- and P4-up chemicals into one or more of the following exposure sources: consumer (e.g., in furniture, toys or apparel), industrial (e.g., product manufacturing or processing), pesticide, diet (e.g., food flavoring/preservatives/colorants or food/water contaminants), and pharmaceutical; Excel Table S6 provides a descriptor for each. We also used data source information from CPDat (Dionisio et al. 2018) to assign chemicals into the exposure source categories; for example, every chemical/cassette pair originating from the Retail Product Categories was categorized as consumer use. Some sources were not exposure-source specific so we manually categorized these cassettes and product use categories based on their terms (Excel Table S7). Some chemical/cassette pairs were classified into multiple exposure sources, as is the case for pesticides whose data originated from an industry-specific source and a consumer use-specific source, and were thus categorized under the pesticide, consumer use, and industrial categories.

To provide additional detail, we identified several exposure source categories that describe the functional uses of a chemical or the type of product it may be found in. These included antimicrobials, cigarettes, drinking water contaminants, flame retardants, food additives, food contact, food residue, fragrance, hair-dyes, human metabolites, personal care products, plastics, or textiles—descriptions of these can be found in Excel Table S8. Some of these categories were chosen because they have previously been reported as associated with breast cancer or as containing endocrine disruptors (e.g., personal care products, textiles, fragrance, antimicrobials), or because they have wide exposure potential (e.g., water and food-related contamination). To assign chemicals into these exposure sources, we used text search to match the terms in each chemical's corresponding cassette or product use category to our categories of interest. A list of cassettes and/or product use categories and the associated categories can be found in Excel Table S9.

If a chemical was not assigned to an exposure source using CPDat (Dionisio et al. 2018) data, we used the exposure pathway assumed by Ring et al. (2019) to classify the chemical. Ring et al.

(2019) used machine learning to assign a likely exposure pathway on the basis of a chemical's structure and physicochemical properties.

We also identified the chemicals that are found in currently used pesticide products, pharmaceuticals, or consumer products; or if they are biomonitored in the Centers for Disease Control and Prevention (CDC) National Health and Nutrition Examination Survey (NHANES) because there is suggested exposure in the U.S. population. To identify current U.S. EPA-registered pesticides, we used the Pesticide Product Information System (PPIS) (U.S. EPA 2021), which lists pesticide products registered for use in the United States and their active ingredients. To identify pharmaceuticals in current U.S. Food and Drug Administration (FDA)-approved products, we used the *Orange Book: Approved Drug Products with Therapeutic Equivalence Evaluations* publication (FDA 2020), which lists drugs approved for use by the FDA and their active ingredients. Although there is no comprehensive list of chemicals in consumer goods, we noted if our chemicals were listed in CPDat (Dionisio et al. 2018) as consumer use products, which is compiled from material data safety sheets, ingredient lists and online retail sites. Finally, we used NHANES list of biomonitored chemicals, to identify if any of the E2-up or P4-up chemicals are biomonitored; this was downloaded from the CompTox Chemical Dashboard's downloadable list of NHANES chemicals (Williams et al. 2017).

Results

E2- and P4-up Activity

Based on an HT CR screening of 654 chemicals (of an initial 2,012) in the H295R steroidogenesis assay (Haggard et al. 2018, 2019), 418 chemicals significantly increased E2 (274) or P4 (283), with 139 increasing both (Excel Tables S1 and S2). To be included in the 418 chemicals, the chemical also had to show a significant effect at two consecutive concentrations or at the highest noncytotoxic concentration tested (i.e., the MTC), consistent with Haggard et al. (2018) criteria.

Of these 418 E2- and/or P4-up chemicals, we classified 296 as active (based on our previously defined criteria for potency and efficacy and excluding hormones) and these were the focus of subsequent analyses receiving an efficacy/potency score of higher, intermediate, or lower (Figure 2; Excel Tables S1 and S2) based on the average of their respective efficacy (i.e., the MFC) and potency (i.e., the LEC) percentile ranks (Excel Tables S1 and S2) as described in the "Methods" section. This set includes 182 E2-up and 185 P4-up chemicals, with 71 increasing synthesis of both E2 and P4. We classified the 122 chemicals that did not meet the criteria for active as borderline actives (indicating exclusion based on our criteria for potency and efficacy) or hormone substrates (indicating a hormone that may act on the steroidogenesis pathway as a substrate); these are included in Excel Tables S1 and S2 and can be identified in the efficacy/potency column. Figure 2 shows the 296 active chemicals that increased E2 and P4 arranged by their potency (i.e., the LEC) and efficacy (i.e., the MFC) (Excel Tables S1 and S2).

For the 182 active E2-up chemicals, the LECs ranged from 0.0041 to 33 μ M (first and third quartile: 1.2, 20), and the MFCs ranged from 1.5 to 82.1 (first and third quartile: 1.8, 2.6). The E2-up chemicals with the highest efficacy/potency on the basis of the average of the LEC and MFC percentile ranks (indicating highest potency and efficacy) were three pesticides (hexythiazox, oxyfluorfen, pirimiphos-methyl) and two failed drug candidates (CP-61237 and PharmaGSID47263) (Excel Table S1).

For the 185 active P4-up chemicals, the LECs ranged from 0.037 to 33 μ M (first and third quartile: 1.2, 11), and the MFCs

ranged from 1.5 to 39.3 (first and third quartile: 1.9, 4.7). The P4-up chemicals with the highest efficacy/potency on the basis of the average of the LEC and MFC percentile ranks (indicating highest potency and efficacy) were imazalil, prochloraz, and triflumizole (three pesticides), mifepristone (a drug), and 3,3'-dimethylbenzidine (used in producing dyes and pigments) (Excel Table S2).

We compared the LEC measurements that were calculated using data of Haggard et al. (2018) with the AC50 and AC10 potency estimates calculated using the tcpl (Filer et al. 2017) (Figure S1). Only 25% (46) of the 182 E2-up chemicals were considered active using tcpl compared with 60% (111) of the 185 P4-up chemicals; these tcpl actives also had an AC50 available. An additional 86 E2-up and 42 P4-up chemicals had an AC10 calculated by tcpl but were not considered active in the ToxCast hit-calls. In general, for E2-up chemicals the AC50 approximated the LEC most closely [E2-up: $r(44) = 0.92$, $p < 20.2^{-16}$; P4-up: $r(109) = 0.74$, $p < 2.2^{-16}$] and these values were more highly correlated compared with the AC10s (Figure S1). For both E2- and P4-up chemicals, the correlations between LEC and AC10s limited to the tcpl actives were generally lower than with the AC50s but still highly correlated [E2-up: $r(44) = 0.81$, $p = 1.3^{-11}$; P4-up: $r(109) = 0.69$, $p < 2.2^{-16}$], and for the E2-up chemicals, correlations were further reduced when combining the chemicals that the tcpl considered to be active or inactive [E2-up: $r(130) = 0.66$, $p < 2.2^{-16}$; P4-up: $r(151) = 0.68$, $p < 2.2^{-16}$].

ER Agonism or Antagonism

Some of the chemicals that increased E2 or P4 synthesis also showed ER agonism, but most did not (Excel Tables S1 and S2). Ten of the 182 E2-up chemicals and 15 of the 185 P4-up chemicals had an AUC ≥ 0.1 and were thus considered active at the ER; all 10 of the E2-up ER-active chemicals were agonists whereas 14/15 P4-up chemicals were. An additional 34 E2-up chemicals and 33 P4-up chemicals had an AUC ≥ 0.01 but < 0.1 and so were considered to have ambiguous ER activity. Twenty-four of the E2-up chemicals and 32 of the P4-up chemicals had not been tested for ER activity. A majority of the chemicals were not active at the ER (114 E2-up, 105 P4-up).

Carcinogenicity, Reproductive, and Developmental Toxicity Findings

To investigate whether chemicals that increased E2 or P4 *in vitro* may be reproductive toxicants, developmental toxicants, or carcinogens *in vivo*, we compiled data from the U.S. EPA's ToxValDb, from California Prop65 listings, and from review articles of chemicals observed to produce rodent mammary gland tumors or alter mammary gland development. We gathered data for the 182 E2-up chemicals and 185 P4-up chemicals that had been prioritized by potency and efficacy. As described in the "Methods" section, we conducted three analyses to evaluate whether *in vivo* effects consistent with increased E2 or P4 are observed for the chemicals that are active *in vitro*.

We found that 33% (60) of the 182 E2-up chemicals were likely repro/dev toxicants and 30% (54) were likely carcinogenic; 12% (21) were positive for both end points (Figure 3A). Meanwhile, 6% (11) of chemicals were unlikely repro/dev, and 13% (24) were designated unlikely carcinogenic (Figure 3A). Of the 185 P4-up chemicals, 33% (61) were likely repro/dev toxicants and 28% (50) were likely carcinogens; 9% (16) were positive for both of these end points (Figure 3B). In contrast, 5% (8) of chemicals were classified as unlikely repro/dev and 11% (20) as unlikely carcinogenic (Figure 3B). The majority of the chemicals did not have enough information to determine repro/dev toxicity [61% (111) of E2-up, 63% (116) of P4-up] or

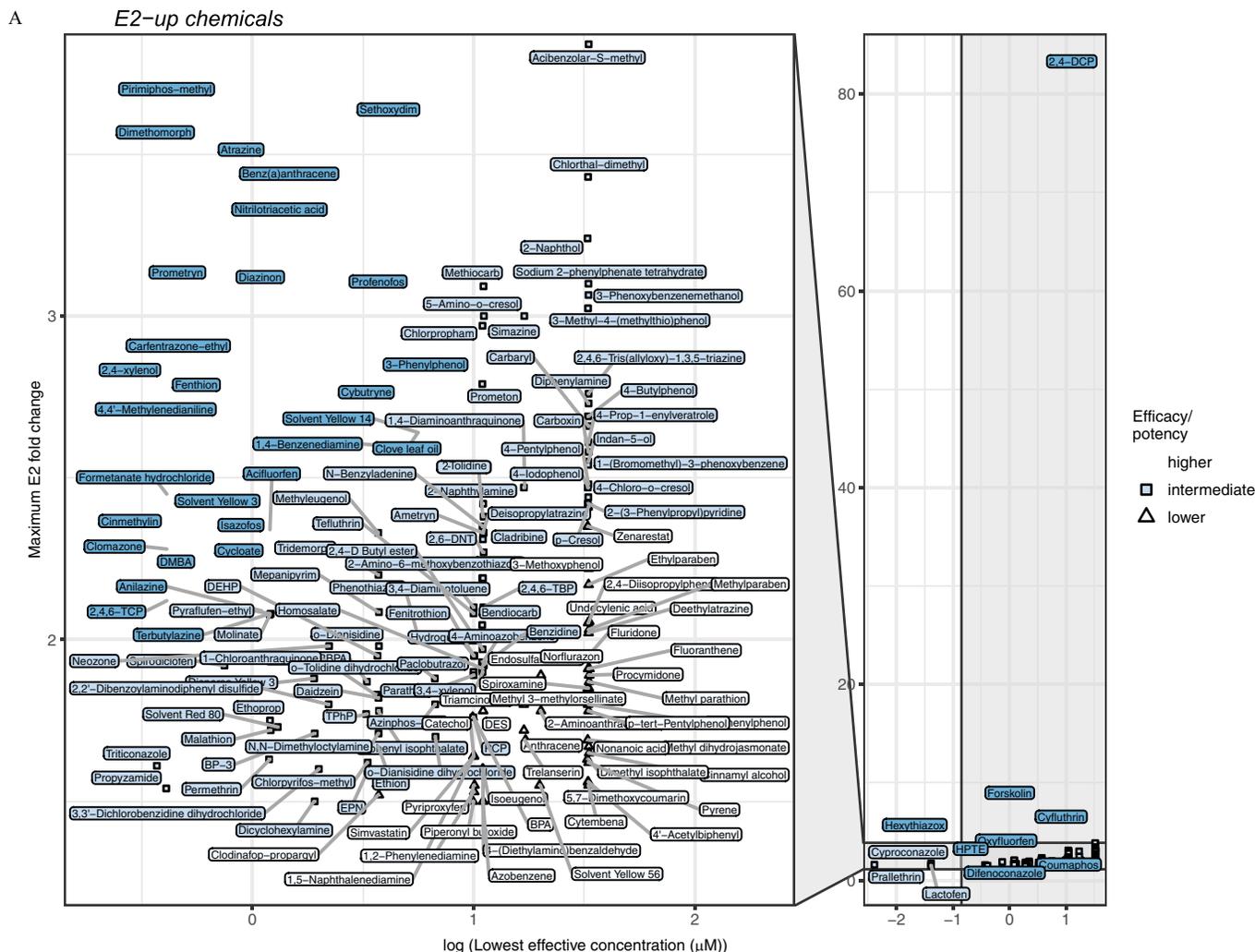


Figure 2. Chemicals that increased estradiol (A) or progesterone (B) with their corresponding log(LEC) and MFC values. The LEC and MFC were obtained from data by Haggard et al. (2018) and are plotted along the *x*- and *y*-axes, the LEC is logged. For both (A) and (B), the plot on the right shows the entire range of values for log(LEC) and MFC, whereas the insert on the left shows a subsection of the plot for added clarity to the chemical names. A chemical's combined efficacy/potency classification can be identified by shape and/or color: chemicals labeled as higher are the top 25% of chemicals with highest potency and efficacy, chemicals labeled as intermediate are the middle 50%, and chemicals labeled as lower are the bottom 25%. Failed drug candidates were removed from the plots. Values used to generate the figure can be found in Excel Tables S1 and S2. Note: 2-HEA, 2-hydroxyethyl acrylate; 2,3-DNT, 2,3-dinitrotoluene; 2,4-DCP, 2,4-dichlorophenol; 2,4,6-tribromophenol; 2,4,6-trichlorophenol; 2,5-DCP, 2,5-dichlorophenol; 3,3',5,5'-TBBPA, 3,3',5,5'-tetrabromobisphenol A; 4-COT, 4-chloro-2-methylaniline; BBPA, di(5-nonyl) adipate; BP-3, benzophenone-3; BPA, bisphenol A; DBNPA, 2,2-dibromo-3-nitropropionamide; DCDPA, 4-(2-phenylpropan-2-yl)-*N*-[4-(2-phenylpropan-2-yl)phenyl]aniline; DEHP, di(2-ethylhexyl) phthalate; DES, diethylstilbestrol; DMBA, 7,12-dimethylbenz(a)anthracene; EPN, epinephrine; HPTE, 2,2-bis(4-hydroxyphenyl)-1,1,1-trichloroethane; LEC, lowest effective concentration; MCI, 5-chloro-2-methyl-3(2H)-isothiazolone; MFC, maximal fold change (compared with dimethyl sulfoxide control); NDGA, nordihydroguaiaretic acid; PCP, pentachlorophenol; TGSA, 4,4'-sulfonylbis[2-(prop-2-en-1-yl)phenol] (a *p,p'*-bisphenolic compound); TOCP, tri-*o*-cresyl phosphate.

carcinogenicity [57% (104) of E2-up, 62% (115) of P4-up]; 43% (78) of E2-up chemicals and 44% (82) of P4-up chemicals had insufficient data to evaluate either end point (Figure 3).

In addition, this research team previously compiled ~250 chemicals with reported mammary gland tumors or other mammary gland effects, such as altered mammary gland development (Cardona and Rudel 2020; Rudel et al. 2007, 2011). For some of these chemicals, although mammary effects were reported, they were also dismissed as not treatment related (Cardona and Rudel 2020). Forty-five chemicals from the lists of chemicals with mammary gland effects (excluding endogenous or synthetic steroidal hormones) were tested in the H295R assay, and 29 of the 45 increased E2 ($n=21$) or P4 ($n=23$). Six of those had been removed from our detailed analysis because of their lower potency (LEC > 33 μM) or efficacy (MFC < 1.5); these six are 2,4-

diaminotoluene, etridiazole, captafol, biochanin A, 1,2-diphenylhydrazine, and propazine (Table 1). It is notable that these six compounds that we classified as borderline active were associated with *in vivo* mammary gland effects including tumors. The remaining twenty-three chemicals with mammary effects that we had classified as active for E2-up or P4-up included 19 that induced mammary tumors (Table 1). Notable in our list of E2/P4-up chemicals is 7,12-dimethylbenz(a)anthracene (DMBA), a research chemical that is commonly used to induce mammary gland tumors in experimental animal models (Abba et al. 2016; Currier et al. 2005; Liu et al. 2015; Welsch 1985). Also on the list are chemicals, or chemical classes, that consistently produce mammary tumors (Rudel et al. 2007, 2014) including aromatic amines (such as benzidine compounds, anilines, and diaminotoluenes), nitro polycyclic aromatic hydrocarbons (PAHs), and triazines (Table 1).

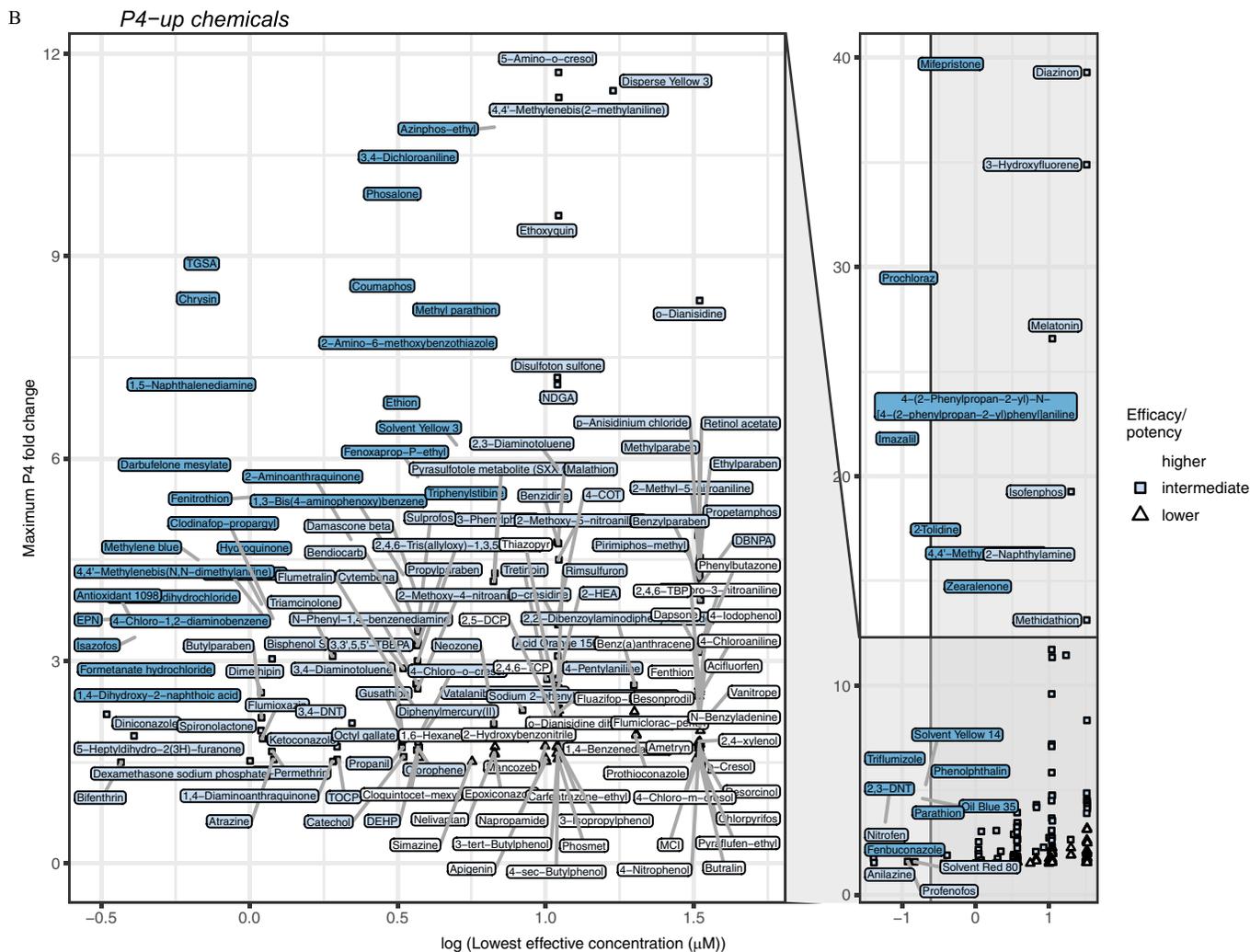


Figure 2. (Continued.)

Literature Review for the Most Active Chemicals

We conducted a preliminary literature review for the five most potent and five most efficacious chemicals that increased E2 or P4 *in vitro* to identify reports of *in vivo* effects that are consistent with increased E2 and/or P4 levels, as described in the “Methods” section. We limited to chemicals in the higher efficacy/potency classification. These findings are described in Table 2.

Of the chemicals that increased E2, the most efficacious chemicals (i.e., the highest MFC) were the herbicide precursor 2,4-dichlorophenol (2,4-DCP), three pesticides (oxyfluorfen, cyfluthrin, and coumaphos), and the supplement forskolin (Figure 2). The most potent chemicals (i.e., the lowest LEC) were four pesticides (hexythiazox, difenoconazole, pirimiphos-methyl, and dimethomorph) and the methoxychlor metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Figure 2). These represent 10 unique chemicals with LECs ranging from 0.04 to 11 μM and MFCs ranging from 1.9 to 82.1 (Table 2). Of these 10, 3 were classified likely and 3 unlikely to be carcinogenic, 4 had inadequate evidence; 5 were classified likely repro/dev toxicants, and 5 inadequate evidence (Table 2).

We found reports of mammary tumors or other mammary gland effects for four of these 10 E2-up chemicals: 2,4-DCP, cyfluthrin, HPTE (as methoxychlor), and hexythiazox (Table 2). In addition, possible effects on lactation are suggested for four chemicals where lower pup weight during lactation was reported: hexythiazox, difenoconazole, cyfluthrin, and oxyfluorfen. For

most (7) of the top 10 E2-up chemicals, we found reports of some effect potentially related to increased E2 synthesis. In some cases, potentially relevant effects were dismissed as not treatment related—owing to a lack of statistical significance (i.e., cyfluthrin) or not following a monotonic increasing dose-response pattern (oxyfluorfen)—or as unimportant (i.e., 2,4-DCP) (Table 2). For two chemicals (coumaphos and pirimiphos-methyl) the U.S. EPA reported no E2-relevant effects in summary documents, but the underlying studies or data evaluations records were not available for review (Table 2). Where we did not find mammary gland effects, it may reflect limited assessment and reporting.

Of the chemicals that increased P4, the ones with the highest MFCs included a pharmaceutical (mifepristone), two pesticides (prochloraz and imazalil), a dye intermediate (3,3'-dimethylbenzidine), and the industrial chemical 4-(2-phenylpropan-2-yl)-*N*-[4-(2-phenylpropan-2-yl)phenyl]aniline (Figure 2). The chemicals with the lowest LECs were four pesticides (imazalil, triflumizole, prochloraz, and fenbuconazole) and the industrial chemical 2,3-dinitrotoluene (2,3-DNT) (Table 2). These represent eight unique chemicals with LECs ranging from 0.037 to 3.7 μM and MFCs ranging from 2.7 to 39 (Table 2). Of these eight, four were classified as likely and one as unlikely to be carcinogenic, three had inadequate evidence; three were classified as likely repro/dev toxicants and five had inadequate evidence (Table 2).

E2-up Chemicals (n=182)

Carcinogenicity	Developmental or Reproductive Toxicity		
	Likely (33% of total)	Unlikely (6% of total)	Inadequate evidence (61% of total)
Likely (30% of total)	21 (12%)	3 (2%)	30 (16%)
# with expected current use	18	3	23
Unlikely (13% of total)	17 (9%)	4 (2%)	3 (2%)
# with expected current use	15	4	3
Inadequate evidence (57% of total)	22 (12%)	4 (2%)	78 (43%)
# with expected current use	13	4	36

P4-up Chemicals (n=185)

Carcinogenicity	Developmental or Reproductive Toxicity		
	Likely (33% of total)	Unlikely (5% of total)	Inadequate evidence (63% of total)
Likely (28% of total)	16 (9%)	1 (1%)	33 (18%)
# with expected current use	15	1	24
Unlikely (11% of total)	17 (9%)	2 (1%)	1 (1%)
# with expected current use	14	2	0
Inadequate evidence (62% of total)	28 (15%)	5 (3%)	82 (44%)
# with expected current use	13	4	42

Figure 3. Number (and percentage) of chemicals ($n = 296$) that increased estradiol or progesterone synthesis according to level of evidence for reproductive or developmental (repro/dev) toxicity or carcinogenicity; the number of chemicals with expected current uses is shown below each cell. Chemicals were classified as likely repro/dev toxicants if they had an effect level below 100 mg/kg per day in a reproductive or developmental toxicity study in ToxValDb, were listed in the California Prop65 as developmental toxicants, or were identified by Rudel 2011 as mammary developmental toxicants. Chemicals were classified as unlikely repro/dev toxicants if they had a no effect level ≥ 100 mg/kg per day in both a reproductive and developmental toxicity study in ToxValDb. Information for carcinogenicity was gathered from a variety of sources including ToxValDb, California Prop65 chemical listings, and the rodent mammary carcinogens list of Rudel et al. 2007. A classification of “likely” includes chemicals that are known, probable, or possible carcinogens or for which mammary tumors were reported; “unlikely” includes chemicals classified as having evidence of noncarcinogenicity in humans or unlikely to be carcinogenic; “inadequate evidence” includes chemicals not classifiable as to human carcinogenicity or inadequate data for evaluation, including no data available. A chemical was categorized as currently used if found in pesticide products approved for use by the U.S. EPA, pharmaceuticals approved for use by the FDA, or consumer products as found in CPDat, or if biomonitoring in the CDC’s NHANES. Additional detail can be found in the “Methods” section and the specific chemicals in each cell can be identified using Excel Tables S1 and S2. Note: CDC, Centers for Disease Prevention and Control; CPDat, Chemical and Products Database; EPA, Environmental Protection Agency; FDA, U.S. Food and Drug Administration; NHANES, National Health and Nutrition Examination Survey; Prop65, Proposition 65 program; ToxValDb, Toxicity Value Database.

Of the eight P4-up chemicals, mammary tumors are reported for two (3,3'-dimethylbenzidine and a mixture that contains 2,3-DNT), whereas possible lactation-related effects are reported for three (mifepristone, triflumizole, and imazalil) (Table 2). Effects on hormone levels or other reproductive effects are reported for five chemicals (triflumizole, fenbuconazole, mifepristone, prochloraz, and imazalil); prochloraz increased serum concentrations of P4 *in vivo*, and prochloraz, imazalil, and triflumizole increased gestational length.

Exposure

We used data from the U.S. EPA’s ExpoCast HT exposure modeling (Ring et al. 2019) and a chemical uses database (Dionisio et al. 2018) to estimate general population exposure levels and classify the chemicals by type of use, for example, diet, consumer

products, pesticide, industrial, pharmaceutical. Of the 182 E2-up and 185 P4-up chemicals, 169 and 165 chemicals, respectively, had an identified exposure source or exposure prediction by the U.S. EPA (271 unique chemicals). Of the 169 E2-up chemicals, 93 are in consumer products, 121 in dietary sources, 103 in industrial sources, 127 in pesticide products, and 36 in pharmaceuticals (Figure 4A). Of the 165 P4-up chemicals, 84 are in consumer products, 110 in dietary sources, 110 in industrial sources, 108 in pesticide products, and 39 in pharmaceuticals (Figure 4A). Many of the chemicals that were identified in pesticide products have dietary exposures: 102 E2-up, and 87 P4-up (Excel Tables S1 and S2). Based on more specific data about exposure sources, 119/271 E2 and/or P4-up chemicals are used as food additives, 110 are found as food residue, 50 have food contact, and 77 are considered drinking water contaminants (Figure 4B). Fifty-eight are found in personal care products, with 17 of these found in

Table 1. Chemicals that increase estradiol (E2-up) and/or progesterone (P4-up) with reported mammary gland effects.

Chemical name	CASN	Effects on mammary gland	E2-up	P4-up	Carcinogenicity ^d
1,2-Diphenylhydrazine	122-66-7	Tumor ^b	✓ ^c	—	Likely
2-Amino-5-azotoluene	97-56-3	Tumor ^b	✓	✓	Likely
2-Methoxy-5-nitroaniline	99-59-2	Tumor ^b	—	✓	Likely
2,4-Diaminotoluene	95-80-7	Tumor ^b	—	✓ ^c	Likely
3,3'-Dimethoxybenzidine	119-90-4	Tumor ^b	✓	✓	Likely
3,3'-Dimethylbenzidine	119-93-7	Tumor ^b	✓	✓	Likely
4,4'-Methylenebis(2-methylaniline)	838-88-0	Tumor ^b	—	✓	Likely
7,12-Dimethylbenz(a)anthracene	57-97-6	Tumor ^b	✓	—	Likely
Ametryn	834-12-8	Tumor ^{d,e}	✓	✓	Inadequate evidence
Atrazine	1912-24-9	Tumor ^b and alters mammary gland development ^f	✓	✓	Likely
Benzidine	92-87-5	Tumor ^b	✓	✓	Likely
Biochanin A	491-80-5	Alters mammary gland development ^f	—	✓ ^c	Inadequate evidence
Bisphenol A	80-05-7	Alters mammary gland development ^f	✓	—	Inadequate evidence
Captafol	2425-06-1	Tumor ^b	—	✓ ^c	Likely
Catechol	120-80-9	Tumor ^b	✓	✓	Likely
Chlorpyrifos	2921-88-2	Other effect ^{d,e}	—	✓	Unlikely
Cytembena	21739-91-3	Tumor ^b	✓	✓	Likely
Diethylstilbestrol	56-53-1	Tumor ^b and alters mammary gland development ^f	✓	—	Likely
Diphenylamine	122-39-4	Other effect ^e	✓	✓ ^c	Unlikely
Etridiazole	2593-15-9	Tumor ^e	✓ ^c	✓ ^c	Likely
Hydroquinone	123-31-9	Tumor ^b	✓	✓	Likely
Malathion	121-75-5	Tumor and other effect ^{d,e}	✓	✓	Likely
Methyleugenol	93-15-2	Tumor ^b	✓	—	Likely
Parathion	56-38-2	Tumor and other effect ^{d,e}	✓	✓	Likely
Phosmet	732-11-6	Tumor ^e	—	✓	Likely
Propazine	139-40-2	Tumor ^{d,e}	✓ ^c	—	Unlikely
Simazine	122-34-9	Tumor ^b	✓	✓	Likely
Terbutylazine	5915-41-3	Tumor ^{d,e}	✓	✓ ^c	Inadequate evidence
Zearalenone	17924-92-4	Alters mammary gland development ^f	—	✓	Inadequate evidence

Note: Reported mammary gland effects gathered from the data of Rudel et al. (2007, 2011) and Cardona and Rudel (2020). —, effects on relevant end points were not assessed or reported in the studies we reviewed; ✓, chemicals that increase estradiol or progesterone, respectively, in the HT-H295R assay using the ANOVA hit-call based method presented by Haggard et al. (2018) and described in the “Methods” section of this article; ANOVA, analysis of variance; CASN, Chemical Abstracts Service Registry Number; Prop65, Proposition 65 program; ToxValDb, Toxicity Value Database.

^aInformation from variety of sources including ToxValDb, California Prop65 chemical listings and the rodent mammary carcinogens list of Rudel et al. 2007. A classification of “likely” includes chemicals that are known, probable, or possible carcinogens or for which mammary tumors were reported; “unlikely” includes chemicals classified as having evidence of noncarcinogenicity in humans or unlikely to be carcinogenic; “inadequate evidence” includes chemicals not classifiable as to human carcinogenicity or inadequate data for evaluation, including no data available. See the “Methods” section of this article for additional detail.

^bReported by Rudel et al. 2007.

^cChemical was classified as borderline active in increasing estradiol or progesterone using potency and efficacy criteria defined in the “Methods” section of this article.

^dU.S. EPA dismissed the observed mammary gland effect (reported by Cardona and Rudel 2020).

^eReported by Cardona and Rudel 2020.

^fReported by Rudel et al. 2011.

hair dye (Figure 4B). Twenty-two are reported in textile-related products, 36 in plastic-related products, and 14 in flame retardants (Figure 4B).

The U.S. EPA’s exposure modeling (Ring et al. 2019) generated median population exposure estimates for 181/182 E2-up chemicals and 183/185 P4-up chemicals. As discussed in the “Methods” section, because of uncertainties in these estimates, we used upper CI estimates of the predicted medians (UCI median). For the E2-up chemicals, UCI median exposure ranged from <0.0001 to 8.74 mg/kg BW/day, and 33 chemicals had a UCI median >0.01 mg/kg BW/day (Figure 5). For the P4-up chemicals, UCI medians ranged from <0.0001 to 52 mg/kg BW/day, and 28 chemicals had a predicted exposure >0.01 mg/kg BW/day (Figure 5). Some of the highest exposure E2-up chemicals were nitrilotriacetic acid (a consumer-use product used for chelating), atratic acid (a natural compound often used as a drug), 10-undecenoic acid (used in drugs and consumer products), dimethyl isophthalate (found in food additives and adhesives), and *p*-cresol (used as a disinfectant and antiseptic); for P4-up, they included 3-isopropylphenol, *p*-cresidine (used in dyes and pigments), 2,4,6-tribromophenol (a fungicide and intermediate in flame retardant production), 2,5-dichlorophenol (an intermediate in pesticides), and 3-*tert*-butylphenol (Figure 5).

We identified 119/182 E2-up chemicals and 115/185 P4-up chemicals that have a higher likelihood of exposure because they

are found in current U.S. EPA-registered pesticide products (55 E2-up and 37 P4-up), are active ingredients in current FDA-approved drug products (5 E2-up and 8 P4-up), are reported in consumer use products (76 E2-up and 77 P4-up), or are currently biomonitored in NHANES (30 E2-up and 28 P4-up) (Excel Tables S1 and S2). These include many common pesticides (e.g., diazinon, atrazine, cyfluthrin, malathion, permethrin, imazalil) and commercial chemicals [e.g., di(2-ethylhexyl) phthalate, 2,4-DCP, tetrabromobisphenol A, methyl paraben, nitriloacetic acid].

Interestingly, 53 of the 104 active E2-up chemicals that have not been evaluated for carcinogenicity are among the 119 chemicals currently used in consumer goods, pesticides, or drugs (Figure 3A; Excel Tables S1 and S2). The two chemicals in this group with a higher potency/efficacy and the highest predicted exposure rates are 2,4-DCP (a precursor and metabolite of the herbicide 2,4-D) and 2,4-dimethylphenol (used as a fungicide and disinfectant, also known as 2,4-xyleneol). Sixty-two of the 111 E2-up chemicals with inadequate evidence to assess reproduction and development are also currently used (Figure 3A; Excel Tables S1 and S2); the two chemicals with a higher potency/efficacy as well as highest predicted exposure are nitrilotriacetic acid (used in washing and cleaning products) and 2,4-dimethylphenol. For P4-up chemicals, 59 of 115 chemicals with inadequate evidence to assess carcinogenicity have current

Table 2. *In vivo* findings for chemicals that increased the synthesis of estradiol (E2-up) or progesterone (P4-up) with the highest potency and/or efficacy.

Chemical name (hormone increased)	LEC (μM)	MFC	Mammary gland effects	Other E2- or P4-up-relevant <i>in vivo</i> effects ^d	Carcinogenicity ^b and reproductive/developmental toxicity ^c assessments
2,4-Dichlorophenol (E2-up)	11	82.1 ^d	A multigeneration reproductive study in rats found significant mammary gland swelling after weaning in all treated groups of both generations; and mammary gland whitening and stiffening at the highest dose in the parental group and all doses in the offspring (Aoyama et al. 2005). These effects were not considered in setting the NOAEL.	Significantly higher uterine weight in weanlings and lower number of implantation sites and live births in F1 females were reported in a multigeneration reproductive study (Aoyama et al. 2005). Study authors proposed steroidogenesis as possible mechanism in light of low ER activity; higher serum E2 measurements were also reported at all doses although these had high variability and were not considered statistically significant (Aoyama et al. 2005). Human: Prenatal exposure was significantly associated with an earlier menarche in girls (Harley et al. 2019). A study using NHANES data determined association with earlier menarche in girls using the sum of 2,4-DCP and 2,5-DCP urinary concentrations, but not 2,4-DCP as a single compound (Buttke et al. 2012). Studies have reported no association with serum estradiol levels (Aker et al. 2016, 2019; Pollack et al. 2018).	Carc: inadequate evidence; repro/dev: likely
Coumaphos (E2-up)	3.3	4.1 ^d	—	The U.S. EPA did not report E2-up-relevant effects in publicly available summaries (U.S. EPA 2006, 2016a, 2020a). Underlying studies and Data Evaluation Records were not readily available for review.	Carc: unlikely; repro/dev: likely
Cyfluthrin (E2-up)	3.7	5.3 ^d	A combined chronic toxicity/carcinogenicity study in rats reported mammary gland adenocarcinomas at the highest dose in females (dismissed by the U.S. EPA owing to a lack of statistical significance). There were also two mammary adenocarcinomas reported in dosed male rats, although these were not discussed. Both the male and female adenocarcinomas were above historical control rates (Haseman et al. 1998; U.S. EPA 2001c). Mammary hyperplasia, inflammation, and fibroadenomas were also reported in some low-dose males and females (U.S. EPA 2001c). A carcinogenesis study in Wistar rats was cited by the U.S. EPA as not having mammary tumors (U.S. EPA 2001c), but the mammary gland was not evaluated in this study (U.S. EPA 2001a).	A multigeneration study in rats reported course tremors in pups during lactation, decreases in pup mean body weights and decreases in mean litter weights (U.S. EPA 2002a). A supplemental to the multigeneration rat study reported a lower number of estrous cycles at both doses tested in parent generation (effect considered incidental as it was not observed in offspring nor in previous reproductive study, even though previous study was conducted at higher doses) (U.S. EPA 2001a). A 3-generation reproduction study reported lower viability through lactation period, lower lactation index and decreased pup body weight gains (U.S. EPA 2001d). At high dose in the female pubertal rat assay, vaginal opening was significantly delayed and mean age at first vaginal estrus was delayed (not significant) (U.S. EPA 2017).	Carc: unlikely; repro/dev: likely
Difenoconazole (E2-up)	0.1 ^e	1.9	—	Reduced pup bodyweight at mid and high doses in a multigeneration study (U.S. EPA 1994). In a rat developmental toxicity study, treatment resulted in a nonstatistically significant reduction in the mean number of fetuses per dam, and a non-significant increase in the mean number of reabsorptions per dam and post implantation loss in the highest dose group (U.S. EPA 1994). Increased reabsorptions in rats (U.S. EPA 1998). In summary documents, the U.S. EPA reported that there were no toxic effects observed in offspring at doses lower than in the parents, and no significant effects on the development of neoplasms (U.S. EPA 1998, 2012b). Underlying studies and Data Evaluation Records were not readily available for review.	Carc: likely; repro/dev: likely
Dimethomorph (E2-up)	0.41 ^e	3.61	—	Increased reabsorptions in rats (U.S. EPA 1998). In summary documents, the U.S. EPA reported that there were no toxic effects observed in offspring at doses lower than in the parents, and no significant effects on the development of neoplasms (U.S. EPA 1998, 2012b). Underlying studies and Data Evaluation Records were not readily available for review.	Carc: unlikely; repro/dev: inadequate evidence
Forskolin (E2-up)	1.2	7.7 ^d	—	The majority of studies reviewed were conducted <i>in vitro</i> (Excel Table S5), and because of forskolin's rapid metabolism <i>in vivo</i> , effects <i>in vivo</i> may be limited (Hecker et al. 2011).	Carc: inadequate evidence; repro/dev: inadequate evidence
Hexythiazox (E2-up)	0.04 ^e	4	Produced benign mammary gland tumors in male rats at the highest tested dose; the U.S. EPA classified as likely human carcinogen on the basis of the mammary tumors and liver tumors (U.S. EPA 2011, 2012a)	Decreased pup weight during lactation (U.S. EPA 2011, 2012a)	Carc: likely; repro/dev: inadequate evidence

Table 2. (Continued.)

Chemical name (hormone increased)	LEC (μM)	MFC	Mammary gland effects	Other E2- or P4-up-relevant <i>in vivo</i> effects ^d	Carcinogenicity ^b and reproductive/developmental toxicity ^c assessments
HPTE (methoxychlor metabolite) (E2-up)	0.08 ^e	2	Presumed altered mammary gland development based on experiments with methoxychlor (Rudel et al. 2011)	Uterine weights of sexually immature female mice were increased 2.6-fold after treatment with HPTE, 3.8-fold after treatment with E2, and 8.9-fold after treatment with both (Waters et al. 2001). Hypothesized to be a more potent endocrine disruptor than its parent compound, methoxychlor (Aoyama and Chapin 2014; Cummings 1997), whose <i>in vivo</i> effects include uterine hypertrophy, hormonal imbalances, altered mammary gland development, and formation of cystic ovaries (Aoyama and Chapin 2014; Rudel et al. 2011). The pattern of gene expression produced in uterine and ovarian tissues after treatment of sexually immature mice was linked to the reproductive and developmental effects observed in rodents after treatment with methoxychlor, regulating many of the same genes as E2 (Waters et al. 2001). HPTE is also considered to be a strong ER agonist and increased uterine weight in ovariectomized mice in an ER-dependent manner (Hewitt and Korach 2011).	Carc: inadequate evidence; repro/dev: inadequate evidence
Oxyfluorfen (E2-up)	1.1	5.3 ^d	—	Decreased pup body weight during lactation and decreased litter size at birth in the highest dose in both generations of a two-generation reproductive rat study (U.S. EPA 1993b); the U.S. EPA attributed lower pup weight to systemic toxicity because the most pronounced effects were seen after pups started eating the test diet (U.S. EPA 2019b).	Carc: likely; repro/dev: likely
Pirimiphos-methyl (E2-up)	0.4 ^e	3.7	—	U.S. EPA did not report E2-up-relevant effects in publicly available summaries (U.S. EPA 2009, 2016b). Underlying studies were not readily available for review.	Carc: inadequate evidence; repro/dev: inadequate evidence
2,3-Dinitrotoluene (P4-up)	0.06 ^e	3.5	Technical grade dinitrotoluene (containing <5% 2,3-DNT) induced mammary fibroadenomas in rats and nonfunctioning ovaries in mice (NIOSH 1985). These effects may be due to the 2,4-DNT isomer, which is linked to mammary fibroadenomas (IARC 1996; NIOSH 1985).	—	Carc: inadequate evidence; repro/dev: inadequate evidence
4-(2-Phenylpropan-2-yl)-N-[4-(2-phenylpropan-2-yl)phenyl]aniline (P4-up) (DCDPA)	1.2	24.3 ^d	—	Health Canada did not report P4-up-relevant or repro/dev toxicity in a publicly available summary report (Health Canada 2017). Underlying study was not available for review.	Carc: inadequate evidence; repro/dev: inadequate evidence
Fenbuconazole (P4-up)	0.1 ^e	2.7	—	A two-generation reproductive study in rats reported a higher number of dams not delivering viable offspring and decreased live litters at same dose maternal toxicity was observed (U.S. EPA 1993a, 2020b). A rat developmental toxicity study reported a lower number of live fetuses per dam at the mid and high doses and an increase in resorption sites and postimplantation loss at the high dose (U.S. EPA 1993a, 2020b).	Carc: likely; repro/dev: likely
Imazalil (P4-up)	0.04 ^e	21.8 ^d	—	Longer gestational length, smaller litter size at birth, and decreased implantation sites (nominal trend; statistical significance not calculated) in rats (U.S. EPA 2000). Reduced litter size and number of live pups in all treated groups along with increased number of resorptions at the high dose in a mouse developmental toxicity study (Pesticide residues in food 2018). In a multigeneration study in rats (U.S. EPA 2000), survival during lactation was significantly lower at all doses in the F1 pups; the effect was dismissed owing to lack of dose response in F2 pups (Pesticide residues in food 2018). A statistical reexamination controlling for litter size showed significance only at the higher dose in the F1 pups (U.S. EPA 2000). Imazalil sulfate increased the number of resorbed fetuses at the mid and high doses and significantly decreased litter size and number of live fetuses in a prenatal developmental toxicity study in rats (U.S. EPA 2018b).	Carc: likely; repro/dev: likely

Table 2. (Continued.)

Chemical name (hormone increased)	LEC (µM)	MFC	Mammary gland effects	Other E2- or P4-up-relevant <i>in vivo</i> effects ^d	Carcinogenicity ^b and reproductive/developmental toxicity ^c assessments
Mifepristone (P4-up)	0.4	39 ^d	—	Administration to rats during early pregnancy induced mammary gland dysplasia resulting in adverse effects on lactation, including a lower expression of the milk protein β-casein, milk yields, and litter growth rates (Zhu et al. 2020). Effects on serum P4 levels were conflicting (Barnhart et al. 2004; Niinimäki et al. 2009; Zhu et al. 2020). Mifepristone is a well-established PR antagonist (Ho et al. 2002; Kim et al. 2020).	Carc: inadequate evidence; repro/dev: inadequate evidence
Prochloraz (P4-up)	0.1 ^e	28.9 ^d	—	Induced higher testicular P4 concentrations in rat fetuses after perinatal exposure (Vinggaard et al. 2005) and higher serum P4 levels after pubertal exposure (Blystone et al. 2007). Higher testicular and plasma P4 levels were also observed in rat fetuses exposed perinatally although effects were reversed in pups (Laier et al. 2006). Pregnant dams administered prochloraz had a longer gestational length (Vinggaard et al. 2005).	Carc: likely; repro/dev: inadequate evidence
Triflumizole (P4-up)	0.04 ^d	5.9	—	In a multi-generation rat study terminated early, administration increased gestational length at all doses and increased estrous cycle lengths and incomplete vaginal cornification at the highest dose (U.S. EPA 2002b, 2012c). Lower bodyweight in pups during lactation, lower survival indices, and smaller litter sizes have also been reported (U.S. EPA 2002b, 2012c).	Carc: unlikely; repro/dev: inadequate evidence
3,3'-Dimethylbenzidine (P4-up)	0.4	16.6 ^{d,e}	Significantly increased incidence of mammary lesions (including carcinoma, fibroadenoma, and hyperplasia) in rats administered by gavage in sesame oil over a 30-d period (U.S. EPA 2008). Increased mammary tumors in female rats exposed to 3,3'-dimethylbenzidine dithydrochloride in drinking water (Morgan et al. 1990; NTP 1991; Rudel et al. 2007; U.S. EPA 2008). Induced mammary tumors in rats administered by subcutaneous injections or subcutaneous implantation, and in the offspring of mice injected subcutaneously during gestation (U.S. EPA 2008).	—	Carc: likely; repro/dev: inadequate evidence

Note: Chemicals were selected for literature review from those classified as having a "higher" efficacy/potency. The top five efficacious (i.e., the highest MFC) and top five most potent (i.e., the lowest LEC) chemicals of the E2-up chemicals and of the P4-up chemicals were then selected; failed drug candidates were excluded. For two P4-up chemicals, the most efficacious chemicals were also the most potent. Excel Table S5 lists the studies we reviewed for this table; additional details of the literature review are in the "Methods" section of this article.

^aEffects on relevant end points were not assessed or reported in the studies we reviewed; 2,3-DNT, 2,3-dinitrotoluene; 2,4-DCP, 2,4-dichlorophenol; 2,5-DCP, 2,5-dichlorophenol; care, carcinogenicity; E2, estradiol; EPA, Environmental Protection Agency; ER, estrogen receptor; F1, first filial generation; F2, second filial generation; HPTe, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane; LEC, lowest effective concentration; MFC, maximal fold change; NHA/NEs, National Health and Nutrition Examination Survey; NOAEL, no observed adverse effect level; P4, progesterone; PR, progesterone receptor; Prop65, Proposition 65 program; repro/dev, reproductive/developmental; ToxValDb, Toxicity Values database.

^bE2- and/or P4-up-relevant *in vivo* effects were searched for include evidence of increased hormone secretion or levels, lower pup weight or lower pup survival during lactation period, altered litter size and implantation sites, increased aromatase expression, uterotropic responses in immature females, accelerated female sexual maturation or changes in estrous cyclicity, and increased gestational length. See the "Methods" section of this article for additional detail.

^cCancer classifications are based on the U.S. EPA's ToxValDb, California's Prop65 chemical listings and the rodent mammary carcinogens list of Rudel et al. 2007. A classification of "likely" includes chemicals that are known, probable, or possible carcinogens or for which mammary tumors were reported; "unlikely" includes chemicals classified as having evidence of noncarcinogenicity in humans or unlikely to be carcinogenic; "inadequate evidence" includes chemicals not classifiable as to human carcinogenicity or inadequate data for evaluation, including no data available. See the "Methods" section of this article for additional detail.

^dChemicals were classified as "likely" reproductive/developmental (repro/dev) toxicants if they had an effect level <100 mg/kg per day in reproductive or developmental studies in ToxValDb, were listed in the California Prop65 as developmental toxicants, or were identified by Rudek 2011 as mammary developmental toxicants. Chemicals were classified "unlikely" repro/dev toxicants if they had a repro/dev effect level, or a no effect level, ≥ 100 mg/kg per day in both reproductive and developmental toxicity studies in ToxValDb. See the "Methods" section of this article for additional detail.

^eChemical considered due to high efficacy (i.e., high MFC).

^fChemical considered due to high potency (i.e., low LEC).

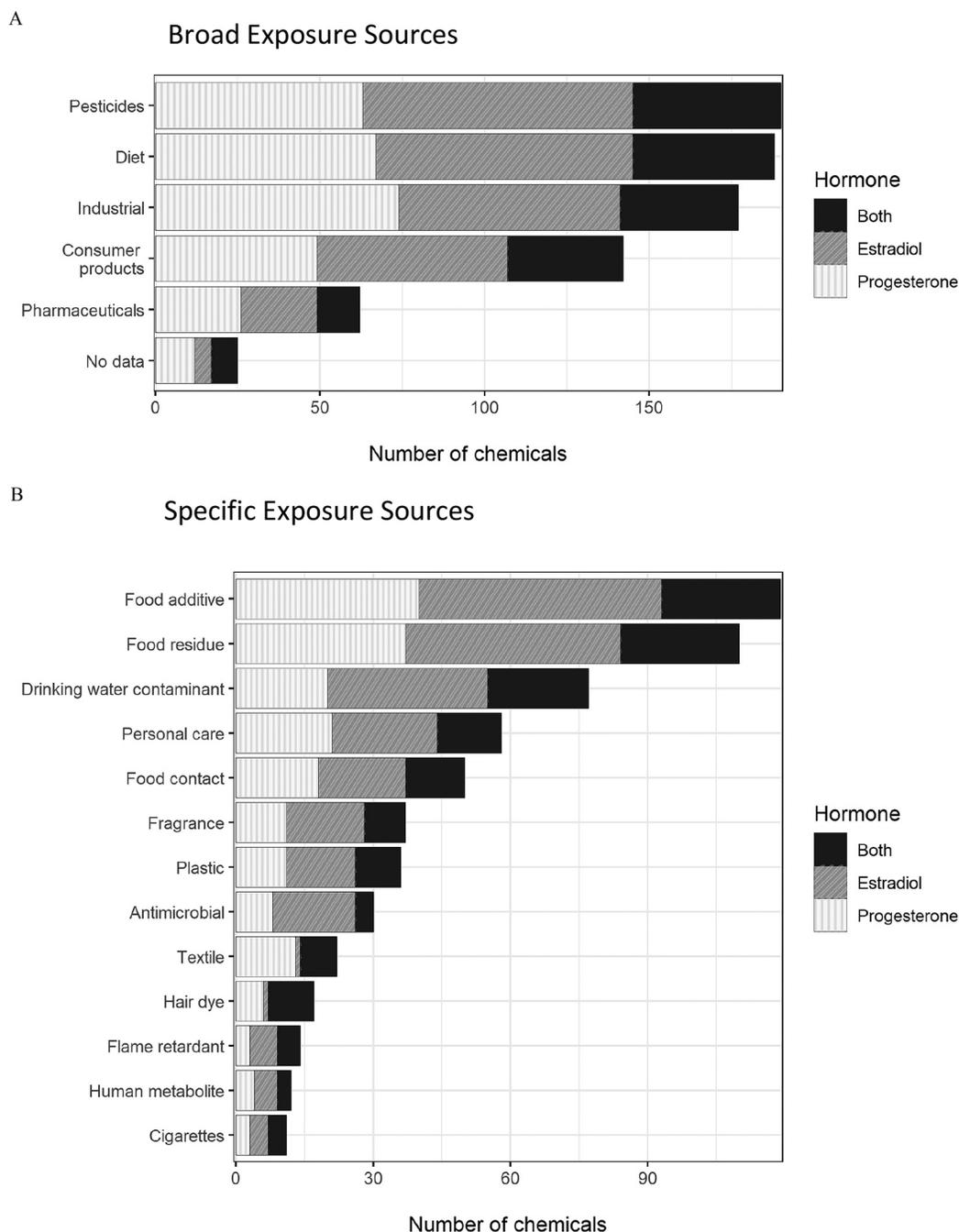


Figure 4. (A) Broad and (B) specific exposure sources for chemicals ($N = 296$) that increased estradiol or progesterone synthesis. Exposure sources were gathered from CPDat or Ring et al. (2019) and assigned into five broad categories: consumer products, diet, industrial use, pesticide products, or pharmaceuticals (A). Within these five broad categories, we also identified exposure sources that are more specific and which may be of regulatory interest (B). See the “Methods” section for additional detail. Individual chemicals could be assigned into more than one of the broad or specific sources of exposure. Within each source we use texture to indicate the number of chemicals that increased estradiol, progesterone, or both. Information on which chemicals comprise the exposure sources is in Excel Tables S1 and S2. Note: CPDat, Chemical and Products Database.

known uses (Figure 3B; Excel Tables S1 and S2). Among these chemicals, the higher potency/efficacy P4-up chemicals with the highest predicted exposure rates are 3,4-dichloroaniline (a precursor of the commonly used herbicide propanil and an azo dye for polyester fabrics), 2,3-DNT (an isomer of dinitrotoluene that is used in explosives), and 4,4'-sulfonylbis[2-(prop-2-en-1-yl)phenol] (a *p,p'*-bisphenolic compound also known as TGSA). Of the P4-up chemicals with the inadequate evidence to assess reproduction and development, 66 of 116 chemicals have current known uses (Figure 3B; Excel Tables S1 and S2).

Discussion

In this article, we used publicly available data to introduce a new set of breast cancer-relevant chemicals based on their ability to increase the synthesis of E2 and/or P4 in a CR screening of 654 chemicals (from an initial 2,012) in the HT-H295R steroidogenesis assay (Haggard et al. 2018, 2019; Karmaus et al. 2016). We identified 182 chemicals that increased E2 and 185 that increased P4, for a total of 296 unique chemicals (with 71 that increased both) of concern for breast cancer and other endocrine-related

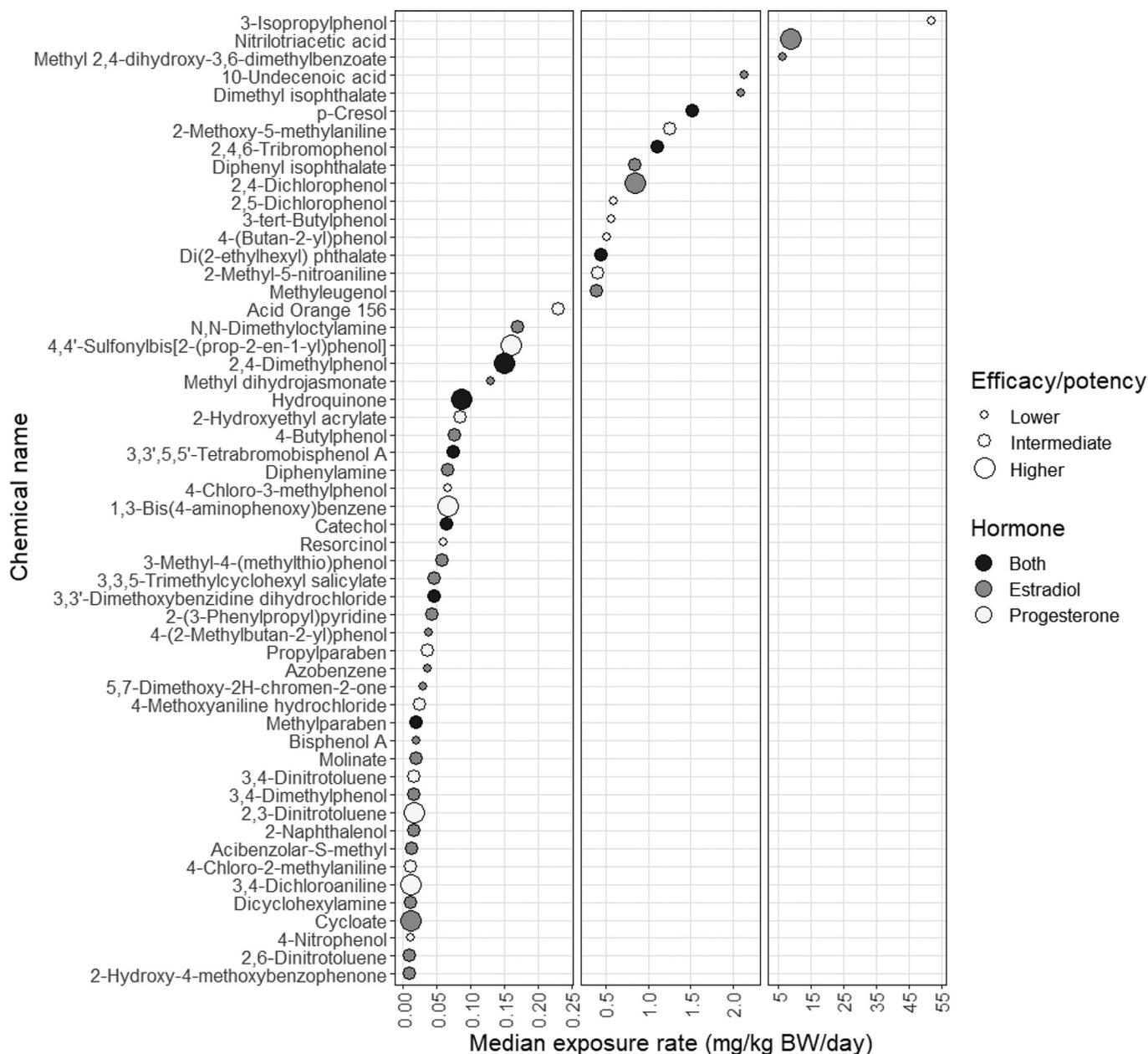


Figure 5. Chemicals that increased estradiol and/or progesterone synthesis with median exposure rates (upper credible interval estimate) >0.01 mg/kg BW/day. For chemicals that increased both estradiol and progesterone synthesis, the efficacy/potency assignment is that of the hormone activity with the higher efficacy/potency. Median exposure rates gathered from Ring et al. (2019). See the “Methods” section for additional detail. Data used to generate the graph can be found in Excel Tables S1 and S2, in the column titled “Predicted median intake rate (mg/kg BW/day).” Note: BW, body weight.

outcomes. Many of these have not previously been identified as potentially related to breast cancer risk; for example, they are not included in previous reviews of chemicals that may affect breast cancer (Rodgers et al. 2018; Rudel et al. 2007, 2011, 2014). Thus, this work demonstrates the application of *in vitro* screening approaches to prioritize chemicals for exposure reduction and further study. The 296 chemicals were selected from a larger set of 418 that increased E2 or P4 and that we classified as active on the basis of having a higher potency (LEC ≤33 uM) and efficacy (≥1.5-fold change) that may indicate a lower likelihood of being false-positives and a higher likelihood of being active at environmentally relevant concentrations. The full list of 418 chemicals, which includes the borderline active chemicals that did not meet our potency and efficacy criteria, can be found in Excel Tables S1 and S2.

Our discussion first covers our findings related to *in vivo* effects for these chemicals, followed by our findings about likely exposures. Then we discuss strengths and limitations of our approach, including for the HT-H295R assay. Finally, we summarize conclusions and make recommendations.

In Vivo Findings

We found that chemicals that increased E2 and/or P4 were more likely to be carcinogens and/or repro/dev toxicants than to not cause those types of effects. Many of the chemicals were identified as likely repro/dev toxicants [33% (60) E2-up and 33% (61) P4-up] or potentially carcinogenic [30% (54) E2-up and 28% (50) P4-up] by authoritative sources or in comprehensive reviews (Figure 3). Generally, fewer than 6% of the chemicals appeared to be unlikely

reproductive or developmental toxicants (NOEL \geq 100 mg/kg-day) (Figure 3). Fewer than 13% were classified as unlikely to be carcinogenic (Figure 3); however, this number could be smaller if mammary tumors were observed but dismissed, as we have previously reported for pesticides (Cardona and Rudel 2020) and as we found to be the case for some of the E2- and P4-up chemicals we identified (Table 2). In addition, well over half of the chemicals with observed mammary gland effects and that were tested in the HT-H295R assay increased E2 or P4 (Table 1), so this mechanism appears to be common in chemicals that cause mammary gland tumors and other effects. Of course, the effects we identified are not necessarily a consequence of the E2-up activity of the chemical, and there are several examples of chemicals with multiple activities related to estrogen pathways, for example, HPTE (methoxychlor).

Through our preliminary literature review of the 18 most potent and efficacious E2- and P4-up chemicals, we found *in vivo* evidence of effects that may be due to increased hormone synthesis, including increased hormone concentrations, mammary gland effects including tumors, and other reproductive and developmental toxicity (Table 2). Of concern, we found cases where effects that are plausibly related to the mechanism of increasing E2 or P4 were dismissed in regulatory evaluations, and cited as not statistically significant or not biologically relevant. For example, a multigeneration reproduction and development study of 2,4-DCP (a substrate and degradant of the widely used pesticide 2,4-D)—which produced the highest increase in E2 production in the H295R assay—observed alterations in mammary gland histopathology at all doses in all generations (Aoyama et al. 2005). Despite these histopathological changes, a no observed adverse effect level (NOAEL) was set at the mid dose, ignoring these and other estrogenic effects. Surprisingly, these effects were not mentioned in the abstract or conclusions of the article or as a keyword, making it impossible to find this article by searching “mammary,” our primary search term for mammary effects, in PubMed. We also found that cyfluthrin, one of the more potent E2-up chemicals, produced mammary adenocarcinomas in male and female rats in the cancer bioassay, yet the tumors in females were dismissed by the U.S. EPA based on statistical significance and the tumors in males were not discussed (U.S. EPA 2001c). The adenocarcinomas in the males and females exceeded rates in historical controls (Haseman et al. 1998), and mammary hyperplasia, inflammation, and fibroadenomas were also reported in some low-dose males and females (Table 2) (U.S. EPA 2001c). It is possible that severe BW decreases observed at the mid and high doses (U.S. EPA 2001c) masked tumors in the mammary gland as it has been previously shown that weight decreases can lead to reduced mammary tumors in rodents (Haseman et al. 1998). The acaricide hexythiazox was also found to produce mammary tumors, although a lack of mutagenicity was cited as a reason to develop a reference dose over a cancer slope factor (U.S. EPA 2011, 2012a). Although not mutagenic, hexythiazox may act as an EDC and increase risk of mammary gland tumors and lactation effects through the E2 synthesis pathway, given that there was also an observed decrease in pup weight during lactation (U.S. EPA 2011, 2012a).

Because testing of pesticides is required before registration (including tests for developmental and reproductive toxicity and cancer), whereas there are no similar testing requirements for many other common chemicals, finding that many of the chemicals with *in vivo* evidence are pesticides is not surprising. However, as we have reported previously (Cardona and Rudel 2020), we have found many examples where mammary tumors were dismissed for pesticides, so pesticide registrations for chemicals that cause mammary tumors and also increase E2 or P4 or have other endocrine activity should be reevaluated. Pesticides

that increased E2 or P4 in the HT-H295R assay and have reported mammary gland tumors but were classified as unlikely carcinogenic by the U.S. EPA's pesticide office include atrazine (E2-/P4-up), simazine (E2-/P4-up) and propazine (E2-up). In addition, of the chemicals with inadequate evidence for carcinogenicity, two pesticides—ametryn (E2- and P4-up) and terbutylazine (E2-up)—have reported mammary tumors *in vivo*, but these were also dismissed by the U.S. EPA (Cardona and Rudel 2020). There are likely to be additional pesticides where mammary tumors were dismissed that were not identified in Cardona and Rudel 2020, given that that report only included pesticides where mammary tumors were reported in the Reregistration Eligibility Decisions, and the cancer bioassays or their summaries were not reviewed. For example, in this article we also identified cyfluthrin as one of the most effective/potent E2-up chemicals, and in searching for *in vivo* data, we discovered that mammary tumors had been reported and then dismissed in studies submitted to the U.S. EPA's Office of Pesticide Programs (U.S. EPA 2001c).

To investigate potentially relevant *in vivo* effects for the 20 most potent and efficacious E2- and P4-up chemicals, we read original studies (or data evaluation records for pesticides) and because mammary effects were not consistently reported, we had to review study findings extremely closely to identify relevant effects. A more systematic approach would be a useful next step but is challenging because mammary gland assessment methods are not standardized or routine (Makris 2011; Rudel et al. 2011), hormone measurements may not be made with sufficiently sensitive methods (OECD 2018a; Schwarzman et al. 2015), and underlying study data for many pesticides are not available and so cannot be reviewed (Cardona and Rudel 2020; <https://iaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:1:0::NO:1>). Taken together, these limitations in the conduct and reporting of studies suggest that *in vivo* effects that result from chemicals that increase E2 and P4 levels may have been missed in many cases.

Although we did not conduct a primary literature review to identify relevant *in vivo* findings for the remaining E2- and/or P4-up chemicals, a few chemicals and chemical groups stood out because of their well-established connections with breast cancer. These include DMBA [a chemical commonly used to induce mammary tumors as an experimental model of breast cancer (Welsch 1985)], aromatic amines (such as benzidine compounds, anilines, and diaminotoluenes), and nitro PAHs, all of which consistently induce mammary tumors in rodents (Rudel et al. 2014). Rodent cancer bioassays are the most common approach to predicting if a chemical may cause cancer in humans (IARC 2019) and they are typically used to identify carcinogens and establish exposure limits. Russo and Russo (1996) documented many aspects of mammary gland hormonal regulation, development, and carcinogenesis that are similar in rats and humans, although some important differences have also been highlighted (Thayer and Foster 2007). It is also interesting to note that chemically induced mammary tumors are more often seen in rats compared with mice (Dunnick et al. 1995; Thayer and Foster 2007), and the observation that aromatase promoters are not present in mammary adipose in mice (Zhao et al. 2012, 2016) might explain this difference in part.

Notably, eight triazine pesticides (terbutylazine, deisopropylatrazine, deethylatrazine, phenothiazine, anilazine, simazine, atrazine, and 2,4,6-tris(allyloxy)-1,3,5-triazine) increased E2, and the latter four also increased P4 levels, suggesting that effects on steroidogenesis may be a mechanism of action for the observed increases in mammary tumors in rats after administration of several triazines (Cardona and Rudel 2020; Rudel et al. 2007). Those tumors were dismissed as not being relevant to humans because they were attributed to a persistent estrous via

attenuation of luteinizing hormone surge (U.S. EPA 2018a). However, the observed increases in E2 and P4 by the triazines are consistent with previous *in vitro* reports of increased E2 production (Hecker et al. 2011; Higley et al. 2010; Tinfo et al. 2011), and P4 production (Pogrmic-Majkic et al. 2014; Tinfo et al. 2011), including in ovarian granulosa cells and H295R cells. Increased E2 has been reported *in vivo* in male rats (Stoker et al. 2000) and in ovariectomized female rats (Cooper et al. 2007). Triazines have also been shown to induce aromatase activity *in vitro* (Caron-Beaudoin et al. 2016; Heneweer et al. 2004; Holloway et al. 2008; Sanderson et al. 2000, 2001; Tinfo et al. 2011), suggesting aromatase induction as a possible mechanism for increasing E2 production. These effects represent an important mechanism of action that may be operative in humans by increasing local or systemic hormone levels and breast cancer risk. We propose that additional research to better characterize the significance of these effects should be a priority given that U.S. regulatory agencies have dismissed the mammary tumors but these herbicides are widely used and are common drinking water contaminants in agricultural areas. There is precedent for reviewing previously published toxicity studies and modifying the conclusions in response to new information about mechanism; such changes may be appropriate for the triazines and other chemicals discussed here, such as 2,4-DCP and cyfluthrin. For example, a multigeneration study of dibutyl phthalate was revised after low-incidence effects on the testis, epididymis, and penis of F1 male offspring, which had originally been dismissed as not treatment related, were reviewed in light of knowledge that phthalates inhibit fetal testosterone synthesis (Makris et al. 2013).

In addition, some chemicals have previously been shown to have potential E2/P4-related repro/dev effects in epidemiologic studies, including phenols and parabens. For instance, peripubertal exposure to methyl paraben (E2-up, P4-up, weak ER agonist) from personal care products was associated with an earlier age of breast development, pubic hair development and menarche in girls; peripubertal propyl paraben (P4-up, weak ER agonist) urinary concentrations were associated with earlier pubic hair development in girls and earlier genital development in boys; and peripubertal exposure to 2,5-DCP (E2-up) was associated with later-onset pubic hair development in girls (Harley et al. 2019). A possible explanation for some of these results is that children going through early puberty are more likely to use personal care products that contain these chemicals (Harley et al. 2019), so additional studies are required to establish a clear relationship. Harley et al. (2019) also reported that prenatal exposure to 2,4-DCP (E2-up) was associated with earlier age at menarche. A study in pregnant women reported that butyl and propyl paraben (both P4-up, weak ER agonists) concentrations were associated with increased odds of preterm birth, decreased gestational age at birth, decreased birth weight and decreased body length (Rattan et al. 2017). Butyl paraben levels in young adults were also associated with shortened menstrual cycles (reviewed by Rattan, Zhou et al. 2017). Another study of pregnant women reported decreased serum E2 and a decreased E2/P4 ratio associated with urinary butylparaben (P4-up, weak ER agonist) (reviewed by Rattan, Zhou et al. 2017). In addition, a case report of gynecomastia among male refugees exposed to lice treatments in a detention center investigated estrogenic (via the ER) and antiandrogenic effects of the lice treatments, which contained phenothrin, permethrin, and piperonyl butoxide. Although these investigators identified phenothrin as having some antiandrogen activity (Brody 2003), it is interesting to note that all three of those pesticides increased E2 in the HT-H295R assay, and permethrin also increased P4, so it is possible that effects on steroidogenesis

might be involved in this outbreak. Genetic polymorphisms in aromatase and other CYP enzymes may influence individual responses to these chemicals in humans. In addition, there is supporting evidence of paraben effects in rodents, given that early life oral exposure to methyl paraben (E2- and P4-up, weak ER agonist) led to histological abnormalities in mammary glands and increased pup mortality that may be due to effects on lactation (Manservigi et al. 2015). We suggest that it should be a priority to evaluate whether exposure to chemicals that increased E2 or P4 are associated with breast cancer incidence or progression, breast density, breast development, or effects on lactation. Some methodological challenges must be addressed to allow measuring human exposures in a meaningful way and for measuring some of these outcomes.

Taken together, our analysis provides preliminary and compelling evidence that chemicals that increased E2 and P4 in the HT-H295R assay are of toxicological concern because many of these chemicals affected the mammary gland (e.g., causing tumors) and/or induced other repro/dev effects *in vivo*. However, almost half of the chemicals have not been tested *in vivo* or do not have adequate *in vivo* data to determine repro/dev toxicity or carcinogenicity (Figure 3; Excel Tables S1 and S2), and so these should be priorities for further study. Prioritization of these for further evaluation could consider exposure potential as well as potency and efficacy. For example, we identified 53 active E2-up and 59 P4-up chemicals that have not been evaluated for carcinogenicity and are currently used in consumer goods, pesticides, drugs, or are biomonitored in NHANES (Figure 3; Excel Tables S1 and S2) and so these should be considered for carcinogenicity assessment, carefully considering hormonal mechanisms. Similarly, there are 62 active E2-up and 66 P4-up chemicals with current uses that have inadequate evidence to assess repro/dev toxicity (Figure 3), so they should be prioritized for *in vivo* study.

Exposure Findings

Many of the E2- and P4-up chemicals represent common exposures. For example, 119/182 E2-up and 115/185 P4-up chemicals are reported to be currently used in pesticides, pharmaceuticals, and/or consumer products, or are found in NHANES biomonitoring samples (Figure 3; Excel Tables S1 and S2). Many of the chemicals in our E2- and P4-up chemical lists are pesticides (127 and 108, respectively), which is concerning, considering there are many ways that people can be exposed to pesticides, including aerial spraying, food residues, drinking water contamination, and use in the home. Indeed, many pesticides have been previously detected in environmental samples such as soil, water, indoor air and dust, food residues, and drinking water (Rudel et al. 2003; Zota et al. 2017). Consumer products are another major exposure source for these chemicals, including personal care products, such as hair dye, and materials used in buildings and furnishings, such as chemical flame retardants. This finding aligns with previous studies that have found various EDCs, such as phthalates, parabens and phenols, in commonly used products such as feminine hygiene products (Gao and Kannan 2020), hair products (Helm et al. 2018), and other personal care products (Dodson et al. 2012). Chemicals found in personal care products have also been found in in home air and dust (Rudel et al. 2003; Zota et al. 2017). Some of the consumer products also include pesticides that are used in or near homes and lawns, on children for lice control, or on pets, scenarios that present higher exposure potential. Imazalil, one of the most potent and effective P4-up chemicals, is a commonly used fungicide applied post-harvest to citrus fruits, which may result in elevated exposures through contact with and ingestion of citrus peels (Vass et al. 2015). A barrier to reducing exposure to these chemicals is that, with the exception of

pesticides, ingredient disclosure is not available for most chemicals in most categories of products (Egeghy et al. 2012). The exposure sources we gathered from the U.S. EPA's CPDat (Dionisio et al. 2018) are far from comprehensive, as there are no laws or regulations requiring full disclosure of consumer product ingredients.

The U.S. EPA's estimates of median population exposures identified 33 E2-up and 28 P4-up chemicals with exposure rates >0.01 mg/kg BW/day, and include butylphenols, parabens, BPA, benzophenone, nitrophenols, and fragrance chemicals (Figure 5; Excel Tables S1 and S2). Although there is substantial uncertainty in these estimates because information about chemical use is often not disclosed by manufacturers, the estimates can indicate which chemicals are expected to have ubiquitous population-level exposures. Moreover, it is well known that population distributions of environmental chemical exposure are lognormal and highly skewed, which means typically exposures among the higher exposed people can be several orders of magnitude above median exposures (Jia et al. 2008; Su et al. 2012). Thus, these median exposure estimates are of limited utility for identifying chemicals that pose a higher risk to subpopulations of frequent users, given that the median is low if most of the population does not use the chemical. For example, people using chemicals in the workplace, higher intensity users, children, and some racial/ethnic minorities are expected to have much higher exposures than those at the population median (Arcury et al. 2018; McKelvey et al. 2011; Nguyen et al. 2019, 2020; Trowbridge et al. 2020).

Strengths and Limitations of *in Vitro* Data from the H295R Steroidogenesis Assay

Based on our review of the HT-H295R assay data for E2 and P4 as well as analyses published by Haggard et al. (2018, 2019) and Karmaus et al. (2016), the HT-H295R assay appears to be a sensitive and robust HT assay that can measure chemical effects throughout the steroidogenic pathway using cells that are able to express the enzymes necessary to synthesize four major classes of hormones, including progestogens, corticosteroids, androgens, and estrogens. However, given that *in vitro* testing approaches can be vulnerable to interferences and other artifacts, it would be useful to supplement the HT-H295R assay with additional approaches for detecting effects on steroidogenesis.

Within the *in vitro* chemical screening programs Tox21 and ToxCast, two other assays—the Tox21 aromatase inhibition assay and the NovaScreen hCYP19A1 activation assay—relate to aromatase activity, which is one of the steroid biosynthesis mechanisms captured within the H295R assay and a mechanism that can influence E2 synthesis (Figure 1). However, neither of these assays provide insight into chemicals that increase aromatase activity, which can increase E2 synthesis. The Tox21 aromatase inhibition assay (Chen et al. 2015) only detects inhibition, so will not detect chemicals that increase E2. The cell-free NovaScreen hCYP19A1 activation assay only produced 3 positive results for aromatase activation, vs. 72 positives for aromatase inhibition, so it may not be sensitive to detecting activation (Williams et al. 2017).

We considered whether activity in steroidogenesis pathways might be accompanied by other endocrine activity, for example, activation at the ER. However, these activities appear to be fairly independent as only 10 of the 182 E2-up chemicals and 15 of the 185 P4-up chemicals were considered active at the ER (Judson et al. 2015).

Although the low-throughput OECD H295R assay also measures effects on steroidogenesis, it is not as efficient in screening many chemicals at once and only identifies chemicals that alter E2 and testosterone, ignoring effects on other hormones. However, a comparison of the HT-H295R assay results with the

OECD reference chemical interlaboratory results for the low-throughput H295R steroidogenesis assay, which is run without forskolin prestimulation, showed good reproducibility in testosterone and E2 responses, with the HT-H295R assay showing a sensitivity of 0.75, a specificity of 0.85 and an accuracy of 0.81 for increased E2 (Haggard et al. 2018). We also noted some reports of increased aromatase and increased E2 associated with neonicotinoid pesticides in another study that used the low-throughput H295R assay (Caron-Beaudoin et al. 2016), but this result was not clearly replicated in the HT-H295R assay.

Despite its strengths identifying chemicals that increased E2 and P4 *in vitro*, there are some important limitations in using the HT-H295R assay specifically and *in vitro* assays in general. For example, a requirement of the HT-H295R assay, and most of the ToxCast assays, is that chemicals are soluble in DMSO (Karmaus et al. 2016), so chemicals that do not meet this requirement cannot be tested. In addition, the assays are not set up to test volatile chemicals (Thomas et al. 2019). As a result, it is not known whether steroidogenesis is a pathway affected by common solvents that increase mammary gland tumor incidence but may have been considered too volatile to test with the HT-H295R assay, such as methylene chloride, dichloroethane, di-chloropropane, carbon tetrachloride, and benzene (Rudel et al. 2014). In addition, because of prestimulation of steroidogenesis with forskolin, the HT-H295R assay appears to be less sensitive to E2 increases, and this may result in false-negatives (Haggard et al. 2018). Consistent with this finding, we observed much more robust MFC responses for P4 compared with E2 (Figure 2). We also noted that compared with the ANOVA-based data analysis presented by Haggard et al. (2018), the tcpl returned a much smaller fraction of positive hit-calls for E2-up chemicals compared with P4-up chemicals: Only 25% of E2-up chemicals were also active in tcpl, compared with 60% of P4-up. This is possibly due to the low responsiveness for E2 increases, so data analysis choices are influential. In fact, the differences between hit-calls for the HT-H295R assay using tcpl (as in ToxCast) vs. ANOVA (as used by Haggard et al. 2018, 2019) raises concerns about insensitivity of the tcpl approach. For example, two of the most potent/effective E2-up chemicals based on the ANOVA in the reports by Haggard et al. (2018, 2019), cyfluthrin and hexythiazox, were not designated active for E2-up based on the tcpl analysis, yet we found that both of these have studies that reported mammary tumors (U.S. EPA 2011, 2012a). Of particular concern is the tcpl practice of using the two lowest doses, rather than the control, to establish a baseline and looking for a response above that at higher doses.

There are also limitations to using data from *in vitro* assays such as the HT-H295R assay to predict *in vivo* outcomes (e.g., an increase in E2 or P4 in a live animal or a downstream effect). For instance, although *in vitro* assays can identify activity of a parent chemical, *in vivo* this activity can be modified by the effects of absorption, distribution, metabolism, and excretion, which can substantially increase or decrease activity. Without metabolic capability, an important limitation is the potential for false-negatives because the H295R cells cannot produce an active metabolite (Hecker et al. 2011). The HT-H295R assay also cannot demonstrate how a chemical may affect steroidogenesis in other endocrine tissues such as the hypothalamus or the pituitary gland, including effects on the release of follicle stimulating hormone or luteinizing hormone (Hecker et al. 2011). Chemicals may also have multiple biological effects *in vivo* that may overshadow the effects of increased hormone levels or which make it hard to separate effects due to steroidogenesis vs. receptor agonism. For example, in our literature review of chemicals with high potency/efficacy, mifepristone both increased P4 and is considered a strong PR antagonist (Chwalisz 1994; Ho et al. 2002; Kim et al. 2020), thus the effects of increased P4 levels on the PR

may be blocked. HPTE increased E2, but is considered a strong ER agonist (Hewitt and Korach 2011), so it was difficult to tell which of the relevant outcomes observed were related to ER agonism vs. steroidogenesis.

Another uncertainty in generalizing from the H295R assay to steroidogenesis effects in other tissues or organisms is that cellular responses to chemicals will vary. For example, the aromatase promoter profile in H295R cells differs from that of breast adipose tissue or gonadal tissue (Bulun et al. 2004; Caron-Beaudoin et al. 2016), so it is not known whether mammary tissue responses to aromatase inducers will be the same as those observed in the H295R assay, which uses adrenocortical carcinoma cells. However, there is evidence to support the idea that some of the same promoters—PII and I.3—that activate aromatase transcript genes and activity in HR29R cells (Caron-Beaudoin et al. 2016; Watanabe and Nakajin 2004) are also active in adipose tissue (although used minimally) and are responsible for upregulating aromatase in breast adipose tissue of women with breast cancer (Agarwal et al. 1996; To et al. 2015; Zhao et al. 2016). Because aromatase is the enzyme that converts androgens to estrogens, higher levels of aromatase are predicted to lead to more E2 synthesis (Simpson 2003; Zhao et al. 2016).

Finally, *in vitro* assays are typically run in a cell line or primary cell culture derived from a single individual, so responses do not reflect genetic susceptibility or variation that exists in populations, which may make some individuals more prone or resistant to a given outcome. For example, women with a polymorphism that increased CYP19A1 gene expression (which encodes for aromatase) had a worse prognosis after breast cancer (Friesenhengst et al. 2018).

Despite the limitations of *in vitro* testing, these data can inform weight of evidence discussions about carcinogenicity or endocrine disruption by suggesting plausible mechanisms of action, and can identify new chemicals that alter steroidogenesis and so may pose a hazard. Although it would be useful to observe the effects of these chemicals on hormone synthesis in human breast tissue or gonads, this is difficult to do in practice. A crucial gap in screening programs for endocrine disruptors is that there are no adequate *in vivo* tests sensitive to local or systemic increases of E2 or P4. The rodent uterotrophic assay has in some cases mistakenly been assumed to be a useful screen for E2 activity *in vivo*, but it does not capture effects on estrogen synthesis if it is run in ovariectomized animals (OECD 2018a). For example, the U.S. EPA concluded that *o*-phenylphenol (an E2-up chemical) did not have sufficient evidence for an interaction with the estrogen pathway based on a negative result in a uterotrophic assay (U.S. EPA 2015).

Overall, the HT-H295R assay, with the ANOVA-based approach for assigning a hit-call, currently provides the best available *in vitro* prediction of effects on E2 and P4 synthesis in a HT format. New coculture models may provide even better breast cancer-relevant models for testing EDCs, for example, by increasing the sensitivity of E2-up responses, integrating measures of breast cell proliferation and aromatase activation, and by incorporating more relevant aromatase promoters (Yancu et al. 2020).

Conclusions and Recommendations

We identified 296 chemicals of particular concern for increasing breast cancer risk on the basis of their ability to increase the synthesis of E2 (182) and/or P4 (185). These chemicals were more likely to be carcinogens or repro/dev toxicants than to not cause those types of effects (Figure 3). *In vivo* effects of the 18 most potent and efficacious E2- and P4-up chemicals included increased hormone concentrations, mammary gland effects (including tumors), and other repro/dev toxicity. These examples of *in vivo* effects may be consistent with local or systemic increases in E2 and P4 levels and add confidence to the continued use of this assay as a screening

tool for identifying chemicals that may increase the risk of breast cancer. For example, of 45 chemicals previously reported to cause mammary tumors or other mammary effects and that were also tested in the HT-H295R steroidogenesis assay, 29 increased E2 or P4, including the well-known mammary gland carcinogen DMBA (Table 1). Among these are 6 chemicals that we classified as borderline active because of their relatively low potency or efficacy in the H295R assay, which suggests that even these may have important *in vivo* effects. E2- and P4-up chemicals included pesticides, consumer product ingredients, food additives, and drinking water contaminants.

Many of the E2- and P4-up chemicals have not been evaluated *in vivo* or have inadequate data to evaluate for carcinogenicity or repro/dev potential, so we submit that follow-up studies of these chemicals is a priority, especially because they can be found in common exposure sources. Fifty-three active E2-up chemicals and 59 active P4-up chemicals that are currently used in consumer products, food, pesticides, or drugs have inadequate information to assess carcinogenic potential, so these are important priorities for additional study and exposure reduction (Figure 3; Excel Tables S1 and S2). Future studies on steroidogenic effects should also prioritize the chemicals that increased E2 or P4 at the MTC but which were not tested in the CR format because they did not affect four or more hormones, as well as the chemicals that we considered to be borderline active because they did not meet our predefined potency and efficacy criteria (Excel Tables S1 and S2).

Where *in vivo* assessments exist already, we propose that another priority for future assessment is to carefully review existing toxicity studies and associated risk assessments for the E2- or P4-up chemicals to note whether there are related effects that are reported, reported and dismissed, or were not ascertained because the appropriate end points were not measured. Based on the *in vivo* studies we reviewed (Table 2), we found incomplete reporting of outcomes related to steroidogenesis, especially for mammary gland effects. For example, mammary gland histopathology was altered by 2,4-DCP at all doses in all generations in a multigenerational rodent reproduction and development study (Aoyama et al. 2005) but despite these changes, an NOAEL was set at the mid dose, ignoring these and other estrogenic effects. In other cases, *in vivo* effects plausibly related to the mechanism of increased E2 or P4 were also dismissed in regulatory evaluations and were regarded as not statistically significant or not biologically relevant (Table 2) (Pesticide residues in food 2018; U.S. EPA 2001b, 2001c, 2019a). Studies often do not include end points that would be sensitive to increases in E2 or P4 synthesis, including mammary gland assessments (Makris 2011; Rudel et al. 2011).

There are opportunities to increase the ability to detect mammary gland effects in toxicology studies, and previous research by ourselves and others provides direction (Davis and Fenton 2013; Makris 2011; Rudel et al. 2011). Specifically, classical transverse or cross-sectioning of the mammary gland that includes the skin provides insufficient mammary tissue to detect hyperplasia or inflammation, and so frontal or longitudinal sectioning is recommended instead to increase by 8–10 times the mammary epithelium present for detection of morphological changes, hyperplasia, and inflammation that might reflect locally increased hormone action (Davis and Fenton 2013). These methods can be used in repeated-dose toxicity studies to detect mammary gland effects (Tucker et al. 2018). Developmental studies rarely evaluate the mammary gland, and it is not a required end point in test guideline studies, despite the fact that mammary gland development—as revealed by mammary gland whole mounts—has been shown to be altered following developmental exposure to some EDCs (reviewed by Rudel et al. 2011).

Similarly, it is possible that effects on lactation could be detected with greater sensitivity than in current study designs, where reduced pup BW may indicate problems with lactation (Makris 2011). In addition, guideline toxicity studies rarely use sensitive measures of circulating E2 or P4 concentrations and never measure localized concentrations or aromatase activity in mammary tissue (OECD 2018b; Schwarzman et al. 2015), so effects of chemicals that increase these hormones are likely missed. Thus, in guideline studies currently, the ability to detect effects on mammary gland is limited. As a result of these limitations in toxicology study design and interpretation, there may be underreporting of relevant *in vivo* effects, including in authoritative chemical risk evaluations and databases, such as the U.S. EPA pesticide registrations and ToxValDB (Judson 2019; Williams et al. 2017), which are commonly used to identify effects of chemicals. The review of existing toxicity studies would be facilitated if the many studies submitted to the U.S. EPA for pesticide registrations were readily accessible and searchable—currently Freedom of Information Act requests are required to access many documents (<https://iaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:1:0::NO:1>).

Additional research is needed to better understand dose–response relationships between these chemicals and effects such as serum or tissue hormone concentrations and altered mammary gland structure, development, histopathology, lactation, and cancer. The dose–response implications of co-exposures to chemicals that activate multiple important cancer pathways should also be investigated given that we expect strong interactions between agents that induce genomic damage and those that increase cell proliferation (Helm and Rudel 2020), such as these E2- and P4-up chemicals. Previous research has shown similar interactions between inflammation and DNA damage, for example, with a much higher number of mutated cells when the two stimuli are given together (Kiraly et al. 2015). It is also important that these chemicals be tested during different windows of susceptibility (WOS)—such as the prenatal, pubertal, pregnancy, and menopausal transitions—because exposures to these hormones during WOS may be especially consequential and important for later mammary carcinogenesis or lactation effects. For example, studies have shown that exposure of rodents to EDCs—including E2, P4, or both—in critical time periods of mammary gland development leads to changes in later development, structure, and function; these alterations may in turn increase susceptibility to breast cancer (Gore et al. 2015; Rudel et al. 2011).

The data presented here also support reconsidering the uses of these chemicals in light of the many common exposure sources, including pesticides and food and consumer products, and the potential for cumulative exposures, given that people are usually exposed to chemicals in mixtures. It is possible that E2- and P4-up chemicals may act additively with each other and with other chemicals active at the ER or in other biological pathways because there is ample evidence that EDCs can act cumulatively, even by varied mechanisms (Bois et al. 2017; National Research Council 2008). In our opinion, risk assessment for combined exposures is a priority, although it is rarely performed. In one notable mixtures risk assessment model, Bois et al. (2017) considered combined exposures to environmental chemicals that inhibit aromatase to predict the chance of effects on ovulation and fertility (Bois et al. 2017). This study found that although predicted exposures to individual chemicals were not expected to be high enough to cause effects on ovulation, simulations of exposures to mixtures of these chemicals predicted there would be effects on ovulation in a portion of the general population (Bois et al. 2017). Our findings in this study suggest that it is a priority to extend these risk assessment approaches to mixtures of chemicals that increase E2 and P4, especially in connection with their

potential effects on breast cancer. In addition, existing risk assessments likely need to be modified to better consider cumulative exposures. Additional work is also needed to develop biomonitoring methods to guide exposure reduction and to use in epidemiologic studies. We propose that advances in analytical chemistry and data analysis for the epidemiologic study of mixtures as well as efforts to develop functional assays for integrated measurements of exposure, and to better understand the biological pathways, will contribute to this effort.

This analysis presents several hundred endocrine active chemicals that should be considered as potential risk factors for breast cancer on the basis of their ability to increase E2 or P4 synthesis and demonstrates use of the HT-H295R *in vitro* steroidogenesis screening assay for chemical screening. We demonstrate that many of these chemicals are likely or possible carcinogens and/or repro/dev toxicants and that about half of mammary carcinogens that were tested increased E2 or P4 synthesis. In addition, we found that toxicity testing is missing or incomplete for many chemicals that increased E2 and P4 steroidogenesis, and assessment of mammary gland effects is especially limited. Exposure to many of these chemicals is likely ubiquitous, based on exposure prediction models. We conclude that these EDCs are priorities for biomonitoring and exposure reduction as well as for additional study to better understand potential effects on breast cancer and other reproductive and developmental effects.

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