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RESEARCH ARTICLE

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In vitro and in silico hormonal activity studies of di-(2-ethylhexyl)terephthalate, a di-(2-ethylhexyl)phthalate substitute used in medical devices, and its metabolites

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Abstract

Plasticizers added to polyvinylchloride used in medical devices can be released into patients' biological fluids. The substitution of di-(2-ethylhexyl)phthalate (DEHP) by alternative plasticizers is essential but their safety must be demonstrated. DEHP, di-(2-ethylhexyl)terephthalate (DEHT) and their metabolites were investigated using level 2 Organization for Economic Co-operation and Development bioassays to screen for in vitro hormonal changes. Differences between the DEHP and DEHT metabolites were observed. Albeit weak, the hormonal activities of DEHT-derived metabolites, e.g., 5-OH metabolite of mono-(ethylhexyl)terephthalate (5-OH-MEHT), were detected and the results of docking experiments performed on estrogen receptor alpha and androgen receptor agreed with the biological results. A co-stimulation of human estrogen receptor alpha and human androgen receptor was also observed. With regard to steroidogenesis, a 16-fold increase in estrogen synthesis was measured with 5-OH-MEHT. Therefore, even if DEHT remains an interesting alternative to DEHP because of its low migration from medical devices, it seems important to verify that multi-exposed patients in neonatal intensive care units do not have urinary levels of oxidized metabolites, in particular 5-OH-MEHT, suggesting a potential endocrine-disrupting effect.

KEYWORDS

DEHP, DEHT, docking, H295R steroidogenesis assay, human androgen or estrogen receptor, testosterone screen

1 | INTRODUCTION

Plasticizers are used as additives to increase the flexibility and softness of normally rigid plastics, such as polyvinylchloride (PVC). Plasticized PVC is used in medical devices such as tubings (infusors, infusion or nutrition lines, extracorporeal circuits) or blood bags. However, any additives that are not chemically bound to the polymer can be released from the material into the infused drug solutions or biological fluids and can thereby come into contact with the patient. This source of exposure presents a general public health concern. Indeed, the metabolites of these plasticizers are found in the urine of many hospitalized patients, particularly neonates in intensive care

units (Mallow & Fox, 2014; Fischer, Bickle Graz, Muehlethaler, Palmero, & Tolsa, 2013). Some of these chemicals are likely to be hazardous for patients, as has been demonstrated for di-(2-ethylhexyl) phthalate (DEHP), which is now classified as CMR 1B (carcinogenic, mutagenic or toxic for reproduction) under the CLP Regulation due to its effect on reproduction and fertility (Regulation (EU) 1272/2008, 2008). The European regulation 2017/745 of 5 April 2017 recommends that the level of DEHP be limited to 0.1% by mass in medical devices (Regulation (EU) 2017/745, 2017). Other plasticizers are recommended to soften PVC, such as di-(2-ethylhexyl)terephthalate (DEHT) (Scenihr, 2015). This additive is interesting because it has a much lower level of migration from the medical devices into the fluids infused into the patient than DEHP (Bernard et al., 2015). Moreover, it would be less toxic than DEHP. DEHT is less active in the induction of peroxisome proliferation in rats than DEHP and this is explained by a smaller amount of monoester produced during DEHT metabolism. Moreover, at equivalent doses, this monoester, mono-(ethylhexyl)terephthalate (MEHT), has a lower cytotoxicity compared to mono-(2-ethylhexyl)phthalate (MEHP). At doses where DEHP altered sexual differentiation, DEHT was inactive (Eljezi et al., 2017; Scenihr, 2015). However, its migration is not zero and toxicity data are not complete. Specifically, there is a lack of information regarding the hormonal activities of DEHT and/or its metabolites resulting from its hydrolysis and oxidation in the body. Indeed, it has been proven that the oxidized derivatives of DEHT are found in the urine, particularly in non-glucuronjugated form regarding to the carboxy (Cx) derivatives (Barber, Fox, & Giordano, 1994; Lessmann et al., 2016). Substitution of DEHP by alternative plasticizers is essential, but the safety of these substitutes must be demonstrated. In this study, we investigated the in vitro effects of DEHP and DEHT, and their metabolites, using identical bioassays and concentration ranges to check DEHT as a potential substitute. Endocrine-disrupting chemicals of estrogen receptor (ER) signaling pathways can contribute to adverse health effects on various areas of the body, such as the nervous system, heart, breast, reproductive tracts in males and females, and energetic metabolism. Endocrine-disrupting chemicals can affect the endocrine system of an organism through multiple pathways, such as mimicking natural hormones, antagonizing their action, or modifying their synthesis, metabolism and transport. In general, the main harmful effects of these compounds are due to their interaction with members of the nuclear receptor family, including the ERs (ERα, ERβ) and the androgen receptors (ARs) (Delfosse, Grimaldi, Cavaillès, Balaguer, & Bourguet, 2014). Reporter gene assays are mechanistic and sensitive tools to characterize receptor-mediated endocrine activity and are recommended in the Organization for Economic Co-operation and Development (OECD) guidelines (OECD, 2012). The action of estrogen in regulating gene transcription is mediated through specific ERs of the nuclear receptor superfamily, such as receptor alpha. To test phthalates and their metabolites, human ER (hER)α activity was measured using a stable transfected cell line (Hela 9903) and following OECD guideline TG 455 (OECD, 2016). The MDA-kb2 cell line was used for investigating the potential agonist and antagonist effects on human AR.

Docking experiments were used to assess the binding mechanism of DEHT and DEHP and to determine the potential interactions of these ligands and their metabolites with ERs and ARs. To study the effect on steroid synthesis, the H295 steroidogenesis assay was performed in accordance with OECD guideline TG 456 (OECD, 2011).

The objective of this study was therefore to investigate the potential endocrine-disrupting effects of DEHT (a promising DEHP substitute) and its metabolites on ERs and ARs, on steroid synthesis, and to compare them with those of DEHP and its metabolites.

2 | MATERIALS AND METHODS

2.1 | Plasticizers and metabolites

DEHP (ref. D201154, CAS: 117-81-7) and DEHT (ref. 525189, CAS: 6422-86-2) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Primary and secondary metabolites of DEHP and DEHT were synthesized and characterized by the IMOST team (UMR 1240, INSERM) Clermont-Ferrand, France. The compounds tested are shown in the Table 1. The purity of all our synthetized metabolites and their corresponding intermediates exceeded 95%.

2.2 | Preparation of samples

All compounds were dissolved in 100% ethanol and tested over a large range of concentrations, from 0.02 ng/mL to 200 µg/mL, depending of the assays and the quantity of synthetized metabolite powder provided by the chemists. To avoid a cytotoxic effect of the vehicle on the cell lines, the maximum concentration of ethanol in the culture medium was 1%.

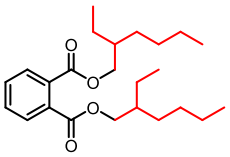
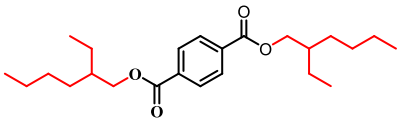
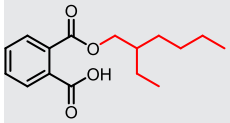
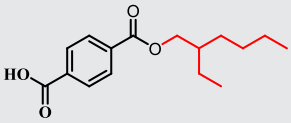
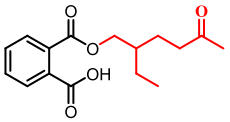
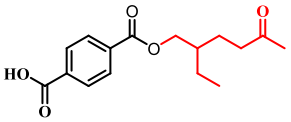
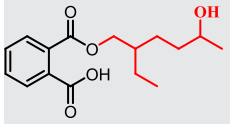
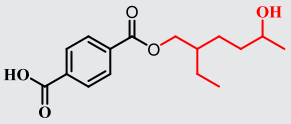
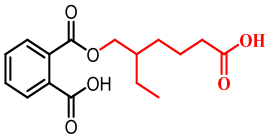
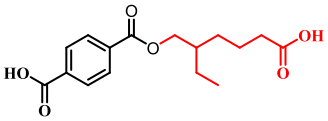
2.3 | Estrogen and androgen receptor transcriptional activation assays

2.3.1 | Cell culture

For the cell-based ER-mediated bioassay, stably transfected hERα-HeLa-9903 cells were obtained from the Japanese Collection of Research Bioresources (JCRB no. 1318) cell bank. These cells contain stable expression constructs for human ERα and firefly luciferase. The latter is under the transcriptional control of five estrogen response element promoter elements from the vitellogenin gene. Cells were maintained in Eagle's minimum essential medium without phenol red, supplemented with kanamycin (60 mg/L) and 10% (v/v) fetal bovine serum (FBS), in an incubator under 5% CO_2 at 37°C. Upon reaching 75%-90% confluency, cells were subcultured twice (not more than 10 passages) before exposure to the test chemicals.

For the cell-based AR-mediated bioassay, MDA-kb2 cells derived from the MDA-MB-453 breast cancer cell line and stably transfected with the murine mammalian tumor virus (MMTV-luciferase.neo reporter gene construct; Wilson, Bobseine, Lambright, & Gray, 2002) were obtained from the ATCC (no. CRL-2713). Cells were routinely maintained in Leibowitz-15 (L-15) medium supplemented with 10%

TABLE 1 Structures and denomination of DEHP, DEHT and metabolites

DEHP and its metabolites	DEHT and its metabolites
DEHP: di-(2-ethylhexyl)phthalate 	DEHT or DEHTP: di-(2-ethylhexyl)terephthalate 
MEHP: mono-(2-ethylhexyl)phthalate 	MEHT or MEHTP: mono-(2-ethylhexyl)terephthalate 
5-oxo-MEHP or MEOHP: mono-(2-ethyl-5-oxohexyl)phthalate 	5-oxo-MEHT or MEOHTP: mono-(2-ethyl-5-oxohexyl)terephthalate 
5-OH-MEHP or MEHHP: mono-(2-ethyl-5-hydroxyhexyl)phthalate 	5-OH-MEHT or MEHHTP: mono-(2-ethyl-5-hydroxyhexyl)terephthalate 
5-Cx-MEHP or MECPP: mono-(2-ethyl-5-carboxypentyl)phthalate 	5-Cx-MEHT or MECPTP: mono-(2-ethyl-5-carboxypentyl)terephthalate 

FBS (v/v) in a humidified incubator at 37°C without additional CO_2. Cells were subcultured when confluent over a maximum of 10 passages.

2.3.2 | Luciferase assays

The assay for (anti)estrogenic activity was performed in accordance with OECD test guideline TG455 (OECD, 2016). Before the experiments, Hela-9903 cells were maintained in culture medium supplemented with 10% (v/v) dextran-coated charcoal stripped serum (DCC-FBS) for at least two media changes. Cells were seeded at a density of 1×10^4 cells per well in 100 µL of phenol red free culture medium supplemented with 10% DCC-FBS in clear bottom white luminometer 96-well plates and allowed to attach for 3 hours.

A modified version of the original protocol by Wilson et al. (2002) was used to test compounds for (anti)androgenic activity (Ermler, Scholze, & Kortenkamp, 2010). Before the experiments, MDA-kb2 cells were maintained in L-15 medium supplemented with 10% (v/v) DCC-FBS for at least two media changes. Cells were seeded at a density of 1×10^4 cells per well in 100 µL of phenol red free L-15 medium supplemented with 10% DCC-FBS in clear bottom white luminometer 96-well plates and allowed to attach for 24 hours.

After incubation, 50 µL of a 3× dosing medium were added to the wells. The cells were exposed to the dilution series of the tested chemicals (seven different concentrations of each sample were tested), to the reference estrogen, 17β-estradiol (E_2), or reference androgen dihydrotestosterone (DHT), and to the solvent controls (0.1% v/v ethanol). DHT or E_2 (1 nM) was used as a positive control in the AR or ER agonist assay, respectively. DHT (0.25 nM) or E_2 (0.025 nM) was used as a control to establish a baseline for co-exposure to screen for AR or ER antagonism, respectively. After 24 hours of exposure, the luciferase activity was determined with Steady Glo assay reagent (Promega, Charbonières, France) as per the manufacturer's instructions.

2.3.3 | Viability

Cell viability was assessed using a resazurin-based assay performed before the determination of luciferase activity. After the exposure time and following a 4-hour (Hela-9903) or 5-hour (MDA-kb2) incubation period with 50 µL/well of 4 µg/mL resazurin (obtained from Sigma-Aldrich) in phosphate-buffered saline, cell proliferation was measured as relative fluorescence units resulting from the reduction of non-fluorescent resazurin to the fluorescent product resorufin. Fluorescence was measured at λ_{ex} = 530 nm and λ_{em} = 590 nm on a microplate reader. The average value for the vehicle control wells was used as 100% and the results for each chemical were calculated as a percentage. If the test substance showed more than 20% reduction of relative cell viability, the compound was considered cytotoxic at the tested concentration.

2.3.4 | Data analysis

Data points are representative of at least two independent experiments and three replicate wells per data point in each experiment. All values were corrected for the mean of the negative control and then related to the positive control, which was set to 100%. Average and standard deviation of the replicates were calculated. A compound was considered positive if it increased luminescence more than 10% above the blank baseline in agonist mode, or decreased luminescence by more than 20% of the maximal signal in antagonist mode.

2.4 | H295R steroidogenesis assay

2.4.1 | Cell culture and treatment

Cell culture conditions and media preparation were conducted in accordance with OECD test guideline 456 (OECD, 2011). Human H295R adrenocortical carcinoma cells (ATCC CRL-2128) were expanded for five passages and frozen in batches in liquid nitrogen. Before conducting the steroidogenesis evaluation, batches of H295R cells were thawed and passed at least four times. The maximum passage number used for steroidogenesis evaluation was 10.

Cells were routinely grown at 37°C under a 5% CO_2 atmosphere in 75 cm^2 culture flasks containing 12 mL Dulbecco minimal Eagle's medium/Ham's F12 culture medium mixture (11039021; Gibco, Gilbo, Fisher Scientific, Illkirch, France) supplemented with 1% ITS+ premix (354352; BD Bioscience, Le Pont de Claix, France) and 2.5% Nu-Serum (355100; BD Bioscience). For subculturing, the H295R cells were washed three times with phosphate-buffered saline, detached using trypsin/EDTA (0.25%/0.05% (v/v) in Hank's balanced salt solution) and seeded in a 1:3 ratio. For testing, 1 mL cell suspension containing 3×10^5 cells was seeded in each well of a 24-well plate. After 24 hours (50%-60% confluence), the medium was refreshed and compounds dissolved in ethanol were added. Exposures were performed in triplicate with a final concentration of the solvent carrier of 0.1%. Positive controls, 10 μM forskolin and 1 μM prochloraz, were included on each plate. Following 48 hours of chemical treatment, media was removed, split into two vials of approximately 500 μL media each and then stored at −80°C before E_2 and testosterone quantification.

2.4.2 | Viability

After exposure, the cells were incubated with resazurin solution to test for viability. Fluorescence was measured using a Chameleon multi-detection microplate reader (Hidex Instruments Inc., Science Tec, Courtaboeuf, France). Exposures showing a decrease in cell viability were excluded from hormone analysis.

2.4.3 | Release of hormones

Enzyme-linked immunosorbent assays were used to quantify directly the testosterone and E_2 from aliquots of the medium. The enzyme-linked immunosorbent assay kits (KGE010, KGE014) were purchased from Bio-Techne (R&D systems Europe, Lille, France). According to the manufacturer's data, the sensitivity of the testosterone assay was 0.030 ng/mL, and the intra- and interassay coefficients of variation were 4.0% and 5.6%, respectively. The sensitivity of the E_2 assay was 4.84 pg/mL, and the intra- and interassay coefficients of variation were 6.0% and 7.1%, respectively. The absorbance was determined at a wavelength of 450 nm using a Tecan (Bio-Rad, Marnes-la-Coquette, France) microplate reader.

2.4.4 | Data analysis

Fold changes in steroids levels in the H295R steroidogenesis assay were calculated by comparing the mean steroid levels of the solvent control versus the mean steroid levels in medium of H295R cells exposed to the compound under investigation.

2.4.5 | Statistical analysis

Obtained data were statistically analyzed using GraphPad Prism 6.00 (GraphPad Software Incorporated, San Diego, CA, USA). Descriptive statistical characteristics (arithmetic mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance and the Dunnett's multiple comparison test were used for statistical evaluations. The level of significance was set at $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

2.5 | Docking studies

The docking of the compounds under evaluation was performed using the crystallographic coordinates 2iog for ERα (Dykstra et al., 2007) and 2 am9 for the AR (Pereira de Jesus-Tran et al., 2006). It should be noted that the docking of the compounds was performed irrespective of the pharmacological type of ligand crystallized with the receptor, which was only chosen based on the structural proximity of the crystallized ligand to the phthalate derivatives, using the lowest possible resolution. As the receptors under scrutiny are nuclear receptors, there is an adaptation of the receptor to the ligand, which has not been investigated here and mostly prevents conclusions being made on the pharmacological effect of the compounds based on these docking experiments. The co-crystallized ligand was extracted and used to define the binding site as a sphere of 10 Å using GOLD (Jones, Willett, Glen, Leach, & Taylor, 1997). The charges of the ligands and receptors were assigned using the Gasteiger-Hückel method and the geometry of each ligand configuration was optimized with the maximin2 protocol of the Sybyl 6.9.1 molecular modeling software. Thirty solutions were generated for each compound and the number of poses of each cluster gave an estimate of the particular stability of the complex compared to the other clusters. The final docking results were the most representative conformation of each cluster, in so far as it was possible to define a sensible common placement. The central

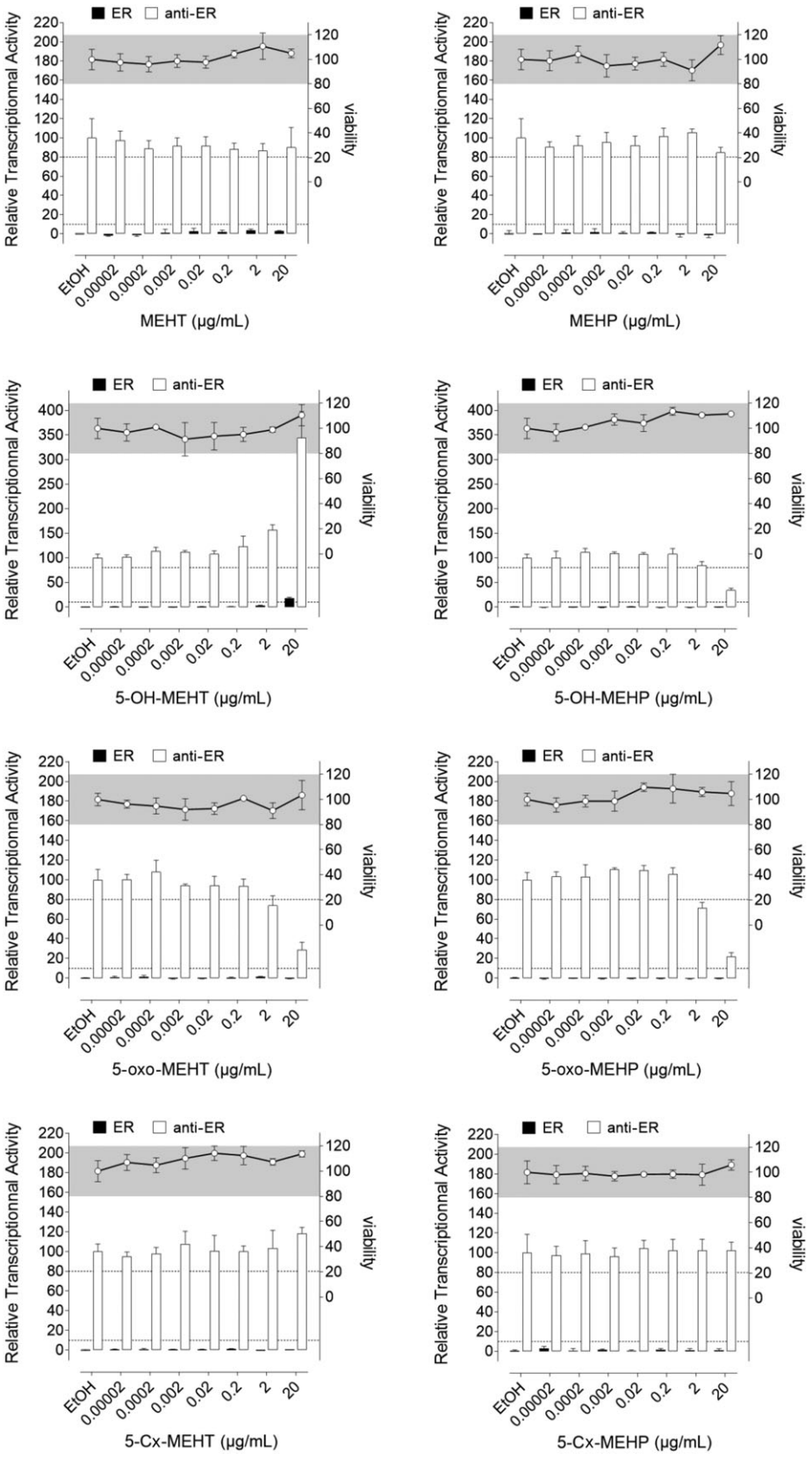


FIGURE 1 ER agonism and antagonism (anti-ER, in the presence of 0.025 nM E_2) with DEHT and DEHP metabolites in Hela-9903 transcriptional activation assays. Cell viability was evaluated by the resazurin assay. Data represents mean ± standard deviation of six data points (two experiments each in triplicate). Dotted lines (.....) highlight 10% 1 nM E_2 normalized relative transcriptional activity in the agonist mode or 80% 0.025 nM E_2 normalized relative transcriptional activity in the antagonist mode, as a threshold for categorizing positive data. E_2, 17β-estradiol; ER, estrogen receptor

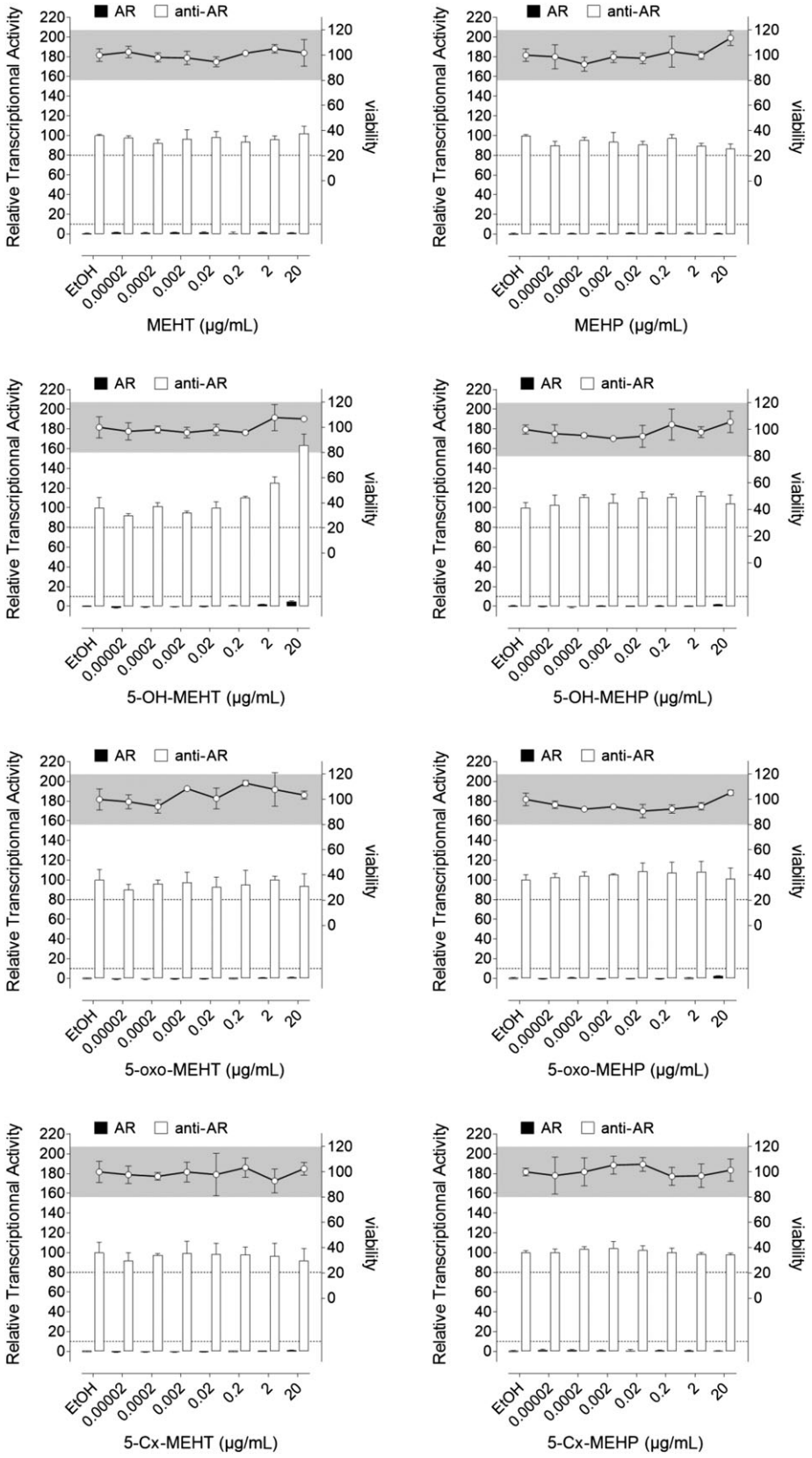


FIGURE 2 AR agonism and antagonism (anti-AR, in the presence of 0.25 nM DHT) with DEHT and DEHP metabolites in MDA-kb2 transcriptional activation assays. Cell viability was evaluated by the resazurin assay. Data represents mean ± standard deviation of six data points (two experiments each in triplicate). Dotted lines (.....) highlight 10% 1 nM DHT normalized relative transcriptional activity in the agonist mode or 80% 0.25 nM 17β-estradiol normalized relative transcriptional activity in the antagonist mode, as a threshold for categorizing positive data. AR, androgen receptor

aromatic ring was the primary structure taken into account to define a cluster. The long and flexible chains of the compounds were mostly discounted at this stage, with the exception of the oxygen atoms.

3 | RESULTS

3.1 | Agonist or antagonist activities on human nuclear receptors in gene reporter assays

3.1.1 | Transcriptional activity of human estrogen receptor alpha

Agonist or antagonist activities on hERα were measured in the absence or presence of E_2. Neither DEHT nor DEHP (Figure S1) were active on hERα. The same conclusion can be drawn for their corresponding monoesters (in terms of agonist or antagonist activities) using a large non-cytotoxic concentration range (up to 20 μg/mL), with the exception of a weak antagonist activity of MEHP at the highest concentration but without any cytotoxicity (Figure 1).

With regard to the hydroxylated monoesters (Figure S1), 5-OH-MEHT induced an agonist effect on ERα at the highest concentration and a synergic concentration-dependent effect in the presence of E_2 at 0.2 and up to 20 μg/mL, while 5-OH-MEHP was an antagonist at the highest concentration without any cytotoxicity.

Oxo-derived monoester metabolites were equivalent partial antagonists of ERα with a concentration dependency effect (2-20 μg/mL) without cytotoxicity (Figure S1). 5-Cx-MEHT and 5-Cx-MEHP were not active in the transcriptional assay irrespective of the activity studied.

3.1.2 | Transcriptional activity of human androgen receptor

Neither DEHT- nor DEHP-induced agonist or antagonist activities on AR over a large non-cytotoxic concentration range (up to 20 μg/mL) (Figure S1). The same conclusion can be drawn with regard to the respective monoester and derived metabolites (Figure 2).

Concerning DEHT metabolites, 5-OH-MEHT was the only metabolite active on AR, with a synergic concentration-dependent effect (0.2-20 μg/mL) when cells were co-treated with DHT. It should be noted that, under our experimental conditions, oxo-derived or Cx-derived monoester metabolites of both phthalates had no effect on AR transcriptional activity.

3.2 | Docking experiments

The co-crystallized ligands of the investigated receptors (compound 11F for ERα and testosterone for AR) were docked to validate the protocol. Both were very close to their crystallographic position and a large majority of their 30 conformations were in this single conformation. For AR, testosterone was less univocal in its binding mode than the other co-crystallized molecule, as it could be placed in either its crystallographic position for about two-thirds of the poses, or exchange its extremities for the remaining third. Both conformations were nonetheless strongly bound to the receptor via hydrogen bonds with Thr 877 and Arg 752. As expected, the second hydrogen bond formed between the hydroxyl group and Asn 705 was only found in its crystallographic position. For ERα, the crystallographic conformation was found nearly exclusively, with 27 solutions of 30. The three other positions were mostly different orientations of the side chains, and in one case was an inversion of the positions of the chains on each side of the amide linkage. The strong ionic interaction with Asp 351 was maintained in all but two cases, while the hydrogen bonds with Glu 353 and Arg 394 were only lost in a single case of chain inversion. These results agree with those obtained by Delfosse et al. (2014).

MEHT binds sufficiently with ERα, irrespective of the configuration of its branched ester chain. The free acid interacts strongly with Arg 334, putting the benzene ring in a good position for stacking with the nearby Phe 404. These are the two main interactions of the co-crystallized ligand. The other end of the compound is less fixed and fluctuates in the wide binding site, as the ester is much smaller than the original ligand. There is, therefore, a wide range of conformations from a single common point of interaction rather than a well-defined cluster, which may indicate that, apart from this single ionic interaction, MEHT is not able to find a favorable binding environment. MEHT binds well with AR with about three-fourths of the 30 solutions in a single cluster irrespective of the configuration. Again, the free acid forms a strong interaction with Arg 752, and it is most probable that the nearby Phe 764 would reorient slightly to form a stacking. Quite unsurprisingly, these are the main interactions of testosterone. The ester chain is mostly rolled up toward the aromatic ring, in a conformation that is not very energetically favorable for the interaction with the receptor (Figure 3).

5-OH-MEHT is placed in ERα in much the same way, with a conserved interaction with Arg 394 at the acid end. The hydroxyl group at the other end forms a near constant hydrogen bond with Thr 347. It forms a fan that is bound by the acid and spreads at the ester end. On the contrary, in AR, 5-OH-MEHT occupies only two positions, both very close to that of its parent molecule and forming interactions at both extremities, as in ERα. While MEHT is able to fit into the cavity of the AR and form an ionic interaction with Arg 752, it lacks the rear side interaction formed by testosterone, and its long ester chain is not stabilized in a particular conformation. On the contrary, the 5-OH-MEHT congener, while assuming the same position for the central block and the interaction with Arg 752, also keeps a hydrogen bond at the rear, formed by its hydroxyl moiety either with Asn 752, Thr 877 or both. Keeping in mind that the side chains of the residues were kept rigid, it is probable that the hydroxyl is binding to both residues in a mode similar to that observed for the natural ligand (Figure 4).

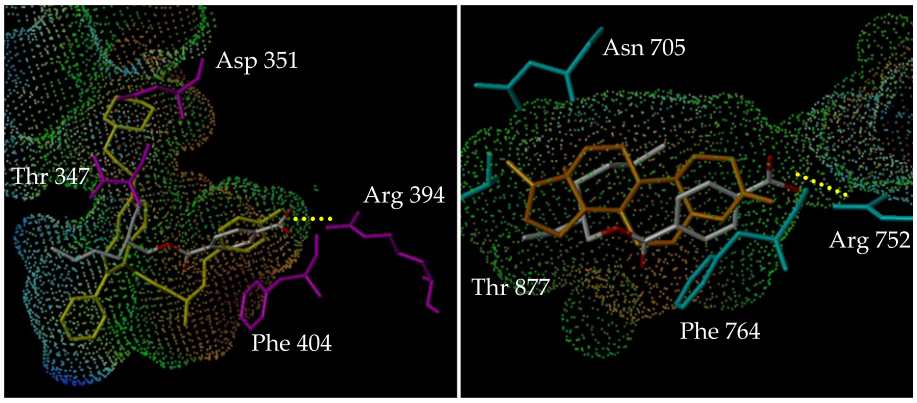


FIGURE 3 Docking of MEHT in estrogen receptor α (left panel, reference ligand in yellow) and androgen receptor (right panel, reference ligand in orange)

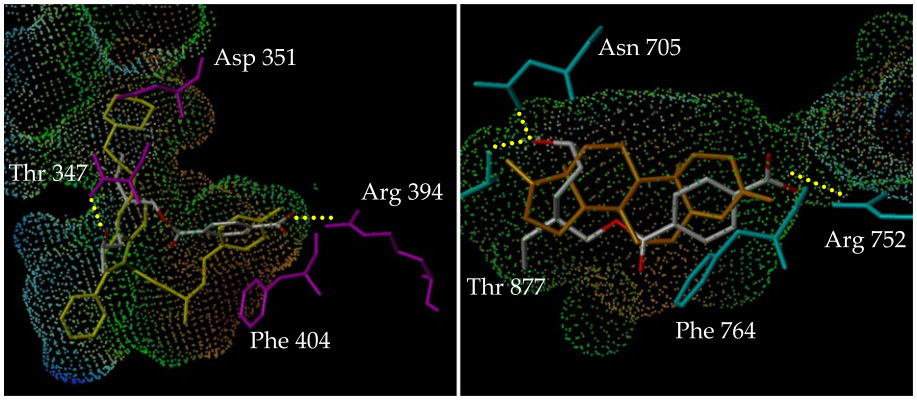


FIGURE 4 Docking of 5-OH-MEHT in estrogen receptor α (left panel, reference ligand in yellow) and androgen receptor (right panel, reference ligand in orange)

3.3 | Steroid synthesis assays

The H295 steroidogenesis assay was performed with derived plasticizer metabolites to detect substances that affect the production of E_2 or testosterone and to understand if an indirect mechanism, such as enzyme inhibition or induction, could occur. Figure 5 shows statistical fold changes in hormone synthesis with the tested metabolites.

3.3.1 | Estradiol synthesis

MEHT and MEHP were weakly active, with for MEHP a significant change between 2 and 40 μg/mL and a sixfold induction at 40 μg/mL for MEHP.

Unfortunately, MEHT was cytotoxic for the cells above 10 μg/mL. Hydroxylated derived metabolites were more potent, with a concentration-dependent increase in E_2 synthesis. A maximum 12-fold increase was seen at 80 μg/mL. This significant effect started at a lower concentration with 5-OH-MEHP (0.2 μg/mL) compared to 10 μg/mL with 5-OH-MEHT. Oxo-derived monoesters were the most active metabolites, with an induction starting at 10 μg/mL, and reaching a 14-16-fold induction at the highest concentration (80 μg/mL). Cx-derived metabolites started to be significant agonists at 40 μg/mL, but with only a weak effect (about twofold).

3.3.2 | Testosterone synthesis

A similar and statistically significant decrease of testosterone (twofold) was observed with OH-derived metabolites. 5-OH-MEHT had an effect at a lower concentration (20 μg/mL) than 5-OH-MEHP (40 μg/mL). It should be noted that at 10 μg/mL, MEHT also decreased testosterone synthesis. Figure 3 shows the same tendency with the oxo-derived metabolites at 40 and 80 μg/mL, with a change observed at a lower oxo-MEHT concentration (10 μg/mL instead of 40 μg/mL with oxo-MEHP). 5-Cx-derived metabolites had no effect on testosterone synthesis.

4 | DISCUSSION

We used the reporter gene assays recommended by the OECD (level 2) to screen for hormonal activities, with the corresponding absence or presence of the reference hormone, and to test the agonist, antagonist and synergic properties of DEHP and DEHT and their

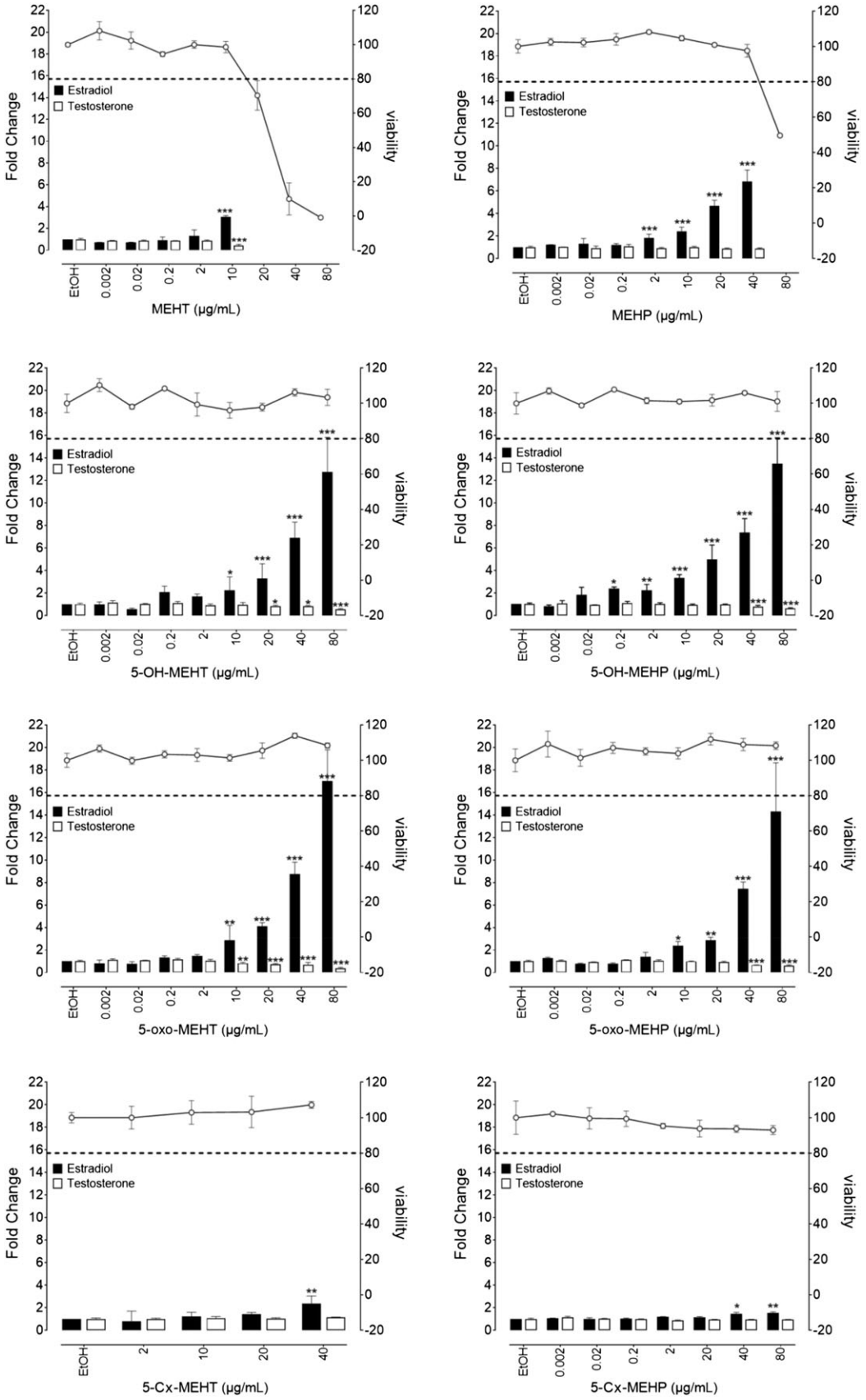


FIGURE 5 Changes in hormone levels (estradiol and testosterone) in H295R cell medium after 48 h of exposure to DEHT and DEHP metabolites. Changes in hormone levels are expressed taking into account the effect of the ethanol solvent (mean ± SD, $n = 3$). Statistical significance $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

metabolites (Satya et al., 2012). The compounds were also docked to assess their binding affinity with ER and AR.

4.1 | Impact on sexual hormones

4.1.1 | DEHP and DEHP metabolites

We found that, when a transcriptional effect was observed on ERα, it was mainly due to the oxidized metabolites of DEHP, such as 5-OH-MEHP. Indeed, 5-OH-MEHP was an antagonist at the highest concentration, with the effect being more pronounced when the cell line was exposed to non-cytotoxic concentrations. Our data on the absence of estrogenic agonist activity with DEHP agreed with Shen et al. (2009), Jobling, Reynolds, White, Parker and Sumpter (1995) and Zacharewski et al. (1998) who showed no ER transactivity and no capacity for DEHP to compete with E_2-ER binding in vitro. In contrast, Takeuchi et al. (2005), who tested DEHP and its corresponding monoester MEHP, observed a weak activation of hERα with 5.5 μM DEHP in a transiently transfected cell line (CHO K1 cells). Our data do not agree with the study by Engel et al. (2017) who demonstrated, using stably transfected human embryonic cells (HEK293), that DEHP metabolites were never active up to a concentration of 100 μM. Their data also proved that the absence of an effect was not due to a lack of cellular uptake of the metabolites in their model. Furthermore, Engel et al. only noted an inhibition with DEHP when using a co-treatment of E_2 at high concentration (100 μM). More recently, Yang et al. (2018) used nanomolar concentrations to demonstrate that MEHP can trigger the proliferation of cervical cancer cells via activation of the G-protein coupled ER rather than ERα. These discrepancies in the literature data could be due to the different cell lines used and to differences in experimental setups, such as the reporter gene constructs (Jones, Parrott, & White, 1999). It is important to note that DEHP has to be metabolized to MEHP and its derived metabolites to be bioactive both in vivo or in vitro (Chauvigne et al., 2009; Gray & Gangolli, 1986; Koch, Bolt, Preuss, & Angerer, 2005) and the differences observed in in vitro models may be due to the presence or absence of enzymatic activities in the cell lines used. Moreover, DEHP is known to be greatly metabolized in vivo after oral exposure, suggesting a low probability of a direct tissue exposure to the parent substances. Furthermore, long chain phthalates are converted to oxidized metabolites by hepatic enzymes, then at the molecular level the adverse effects of phthalates may be in fact due to effects mediated by phthalates metabolites (Kluwe, 1982). Concerning the transcriptional activity of AR, our data agree with those found by Engel et al. (2017) where the authors did not observe any agonist effect on AR in the presence of DEHP or its derived metabolites up to a concentration of 25 μM. However, the same authors observed an AR inhibition at 50 μM, a twofold higher concentration compared to our study. This could be due to some cytotoxic response not displayed in the cytotoxicity assay used. A crucial parameter when performing in vitro tests is the use of the proper concentrations to avoid false positive data. It is essential to test substances at non-cytotoxic concentrations, particularly when an antagonist effect is observed.

In the study by Shen et al. (2009), both mixed androgenic and anti-androgenic effects were observed with DEHP on the same cell line (MDA-kb2), with an EC_{50} (concentration that gives a half-maximum response) or IC_{50} (concentration that inhibits the response by half) exceeding 10^{-4} M, which is a high concentration. Araki, Ohno, Nakai, Takeyoshi and Lida (2005) also demonstrated an antagonist effect of DEHP on AR. However, this was not seen by Krüger, Long, and Bonefeld-Jørgensen (2008). Again the sensitivity of the cell line and consequent variant sensitivity could be at the origin of the differences between the data. It should be noted that the cell line we used was probably not able to metabolize DEHP until ultimate active metabolites such as hydroxylated/oxidized metabolites as the effect in the reporter gene was observed only with DEHP metabolites.

Using the steroidogenesis synthesis assay, which gives information on another mode of Endocrine Disruptor action (not genomic), we have shown that MEHP is also active at 40 μg/mL, with an increase of E_2 and a decrease of testosterone. The effect was even more pronounced with its derived hydroxylated monoester (5-OH and 5-oxo-MEHP). The effects observed at the concentration range used in this study agree with those seen by Mankidy, Wiseman, Ma and Giesy (2013), who demonstrated that hormone synthesis was affected by DEHP concentrations of 10 μg/mL, resulting in a greater production of E_2 (fourfold) and a concurrent reduction of testosterone concentration. However, they did not test DEHP metabolites. In our study, the induction observed with DEHP metabolites was even more pronounced (up to 15-fold). Interestingly, Desdoits-Lethimonier et al. (2012) used human testis explants to demonstrate that phthalates affect human testis steroidogenesis but that DEHP has to be metabolized to MEHP to be bioactive. MEHP metabolites, including 5-OH-MEHP, also display anti-androgenic activities. Production of all testosterone precursors of the four and five pathways was inhibited by MEHP. Using NCI-H295 cells over concentration ranges found in men in recent epidemiological studies, DEHP and MEHP have been shown also to reduce testosterone production in vitro after 48 hours, associating phthalate exposure with the impairment of the androgynous status.

4.1.2 | DEHT and DEHT metabolites

5-OH-MEHT showed an agonist effect at the highest concentration and, interestingly, a synergism in the presence of E_2, with a concentration dependency effect on ER (from 0.2 up to 20 μg/mL). However, when expressed as Eq/L E_2 (Figure S2), the agonist effect of 5-OH-MEHT is weak, with a relative potency 3.5×10^{-6}-fold lower than E_2.

Furthermore, only 5-OH-MEHT was active on AR, with again a synergic concentration-dependent effect when cells were co-treated with DHT. Using the Wilson model, reporter gene induction may be triggered via GR or AR activation (Wilson et al., 2002). However, as a synergic effect was observed with DHT, we can conclude that AR was involved.

To date, co-stimulation by 5-OH-MEHT and E_2 or DHT has never been observed in in vitro studies. With regard to steroid synthesis, estrogen synthesis could increase up to 16-fold and Cx-derived metabolites had a very weak effect. In terms of estrogen synthesis,

the rank order potency was as follows: MEHT < corresponding OH metabolite < corresponding OXO metabolite. With regard to testosterone levels, a weak but significant decrease was noted with no difference between the metabolites.

Concerning DEHT, we lack information on this endpoint. However, it is interesting to note that DEHT metabolites were more active in the steroidogenesis assay compared to DEHP metabolites. Experiments are ongoing in the lab on the effect of DEHT and/or its metabolites on the aromatase level, which could be involved in the changes in E_2 level as demonstrated in vitro by Lovekamp and Davis (2001) with MEHP.

4.2 | Docking

Efforts were limited to the ERα due to the fact that ERβ has a low number of different residues in its binding site, the most notable of which is a valine in place of a leucine at position 487, at the entry of the pocket. However, the overall difference is a slight movement of the C-terminal loop-helix-loop assembly, resulting in a slightly different spatial arrangement of this residue. These observations are consistent with those of Delfosse et al. (2014). The phthalate metabolites were further investigated due to their potential for hydrogen bond formation with the free acid group. It should be kept in mind that docking only investigates the direct interactions with the receptor, without taking into account accessibility to the binding site. In particular, the high flexibility of MEHT's long ester chain may mask the free acid or get entangled in the entry of the pocket and inhibit its binding. Both phenomena are beyond the scope of the in silico tool employed here, and may thus explain the observed differences between the docking results and the biological results for MEHT. The monoesters, MEHT and MEHP, behave differently. MEHT strongly binds to the arginine of both receptors but with no anchorage; its second ester adopts a large number of possible conformations in the pocket, which may relate to a poor fit for the binding sites, despite the ionic bond with the arginine. MEHP has no interaction with ER; the free acid is clearly being screened by the large ester chain. The same is true for AR. As a result, this metabolite has apparently a very low possibility of being a ligand for ERα or AR. Among the oxidized metabolites, the 5-OH-MEHT is able to bind quite well to both receptors, with well-kept interactions at both the free acid, pointing toward an arginine, and the ester chain hydroxyl group, which readily forms hydrogen bonds. It also fits into both receptors in a single conformation. Inversely, the other metabolites of this series do not show the same binding capacity and have several different conformations (data not shown). The MEHP congener behaves differently, with four conformations in ER and two in AR. The 5-oxo-MEHT and -MEHP can both bind to the two studied receptors in two or more different conformations, even lacking any full interaction for the latter in AR. The addition of another acid group on the ester chain is not optimal. 5-Cx-MEHT is able to bind to the arginine of both receptors but not in a well-defined conformation. There is a slightly better docking with AR than ER. 5-Cx-MEHP forms a large number of small size clusters in the two receptors studied, indicating an unstable docking and therefore hinting at a low potential affinity, if any. Overall, the best binder is clearly 5-OH-MEHT, which readily binds to ERα and AR in a mode very similar to that of the natural ligands.

4.3 | In vitro data versus biomonitoring values

In neonatal intensive care units, neonates are particularly exposed to plasticizers released from PVC medical devices. Biomonitoring studies have allowed the measurement of the urinary levels of DEHP metabolites in neonates hospitalized in these units. Strømmen et al. (2016) showed that the urinary concentration of 5-oxo and 5-OH-MEHP could reach 1 μg/mL. The cohort studied by Demirel et al. (2016) presented even higher values with maximum limits in the order of 5 μg/mL for these two oxidized metabolites. Our study shows that at these concentrations there is an antagonistic effect on ERs. Moreover, the effects of 5-OH-MEHP on the synthesis of E_2 are observed from 0.2 μg/mL, which is close to the median concentration observed in these newborns. In intensive care, extracorporeal membrane oxygenation is one of the primary medical situations that exposes patients to DEHP for several days or even weeks. In particular, a study in adults has shown that patients on extracorporeal membrane oxygenation had urinary 5-OH-MEHP concentrations of more than 5 μg/mL and blood concentrations of more than 0.8 μg/mL (Huygh et al., 2015).

Concerning DEHT, there is currently no biomonitoring study performed in a medical environment while this plasticizer was identified in medical devices used in pediatric intensive care units (Malarvannan et al., 2019). The study by Lessmann et al. (2017) gives urinary concentrations of DEHT metabolites in a population of children aged 4-17 years. The maximum levels observed were 0.06 μg/mL for 5-oxo-MEHT, 0.18 μg/mL for 5-OH-MEHT and 0.34 μg/mL for 5-Cx-MEHT. Even if the median concentration of 5-OH-MEHT was much lower (0.045 μg/mL), the maximum concentration observed corresponds to the concentration showing the first synergistic effect with E_2 on hERα and agonist effects on ARs. Therefore, the question arises regarding the level of exposure of patients using medical devices containing DEHT and the potential endocrine-disrupting effect. The ongoing biomonitoring study under the Armed-Neo project should provide us with the necessary elements to assess further the risk. Experiments are ongoing to check hormonal activities of neonatal urine extracts.

In this study, the biological effects of single tested metabolites appear to be weak and far less potent than natural hormones. However, an observed synergic effect at low levels must be taken into account and not be considered as insignificant as the human population is continuously exposed to complex mixtures of chemicals in the presence of natural hormones (Ghisari & Bonefeld-Jorgensen, 2009; Kortenkamp & Altenburger, 1998). Therefore in vitro experiments are important to monitor the effects of metabolites and can be relevant to in vivo situations, at least for people with higher exposure

levels, such as neonates exposed to medical devices in neonatal intensive care units (Calafat, Needham, Silva, & Lambert, 2004).

However, it is reassuring that the main oxidized metabolite found in the urine of newborns exposed to DEHP or DEHT is the carboxylated metabolite. Our work highlights that 5-Cx-MEHP and -MEHT derivatives are not active in vitro whatever the hormonal activity studied. Indeed, biomonitoring studies in neonates exposed to DEHP by medical devices have shown an urinary level of 5OH-MEHP of 5%-15% whereas 5-Cx-MEHP accounts for 60%-83% of all metabolites (Strømmen et al., 2016; Stroustrup et al., 2018). In adults, 5-OH-MEHP is present in greater quantity than 5-Cx-MEHP (about 40% of each of these two metabolites, 20% of 5-Oxo-MEHP). Concerning the metabolites of DEHT, a study on a children population not exposed to medical devices has shown a similar distribution in favor of 5-Cx-MEHT (85% 5-Cx-MEHT, 9% 5-OH-MEHT and 6% 5-oxo-MEHT) (Lessmann et al., 2017).

5 | CONCLUSION

This study presents biological hormonal activities of the derived metabolites of DEHP and DEHT, involving Cx metabolites and demonstrates, at a molecular level, the different mechanisms of action of phthalate metabolites compared to the respective parent molecules, as well as the differences between DEHP and DEHT. The effects observed were more important for steroidogenesis synthesis, suggesting an indirect mode of action for DEHP or DEHT metabolites. To the best of our knowledge, this is the first time that a co-stimulation of hERα and hAR has been observed with 5-OH-MEHT. In silico results for ERα and AR are in good agreement with the observed biological results for 5-OH-MEHT and MEHP, while the docking of MEHT is less conclusive. This compound maintains an interaction with the arginines but lacks other interactions, and its ester is unfavorably constrained to fit into the pockets.

These data, taken together with the phthalate exposure levels of neonates via medical devices, demonstrate the relevance and the sensitivity of bioassays to detect hormonal activities, as recommended by the level 2 OECD guidelines. They also show the importance of monitoring the hormonal activities, such as antagonism or synergism, at the molecular level and their use as a screening step to protect better the vulnerable populations to DEHP substitutes.

Our study shows that investigations concerning the hazard of DEHT during the exposure of neonates to medical devices must be monitored before attesting to its safety. Several elements are at play in favor of this plasticizer as an alternative to DEHP: weak diffusion towards the liquids in contact with the medical devices limiting the exposure of the patients, and less toxicity compared to the DEHP (cytotoxicity, carcinotoxicity, reprotoxicity). However, the results of our study lead to caution with respect to the potential endocrine-disrupting effect of the hydroxylated metabolite (5-OH-MEHT). It should be ensured that the urinary levels of this metabolite are lower than the concentrations that have shown co-stimulation of ERs, and an increase in estrogen synthesis.

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REFERENCES

Araki, N., Ohno, K., Nakai, M., Takeyoshi, M., & Lida, M. (2005). Screening for androgen receptor activities in 253 industrial chemicals by in vitro reporter gene assays using AR-EcoScreen cells. *Toxicology In Vitro*, *19*(6), 831–842. https://doi.org/10.1016/j.tiv.2005.04.009

Barber, E. D., Fox, J. A., & Giordano, C. J. (1994). Hydrolysis, absorption and metabolism of di(2-ethylhexyl) terephthalate in the rat. *Xenobiotica*, *24*(5), 441–450. https://doi.org/10.3109/00498259409043247

Bernard, L., Cueff, R., Chagnon, M. C., Abdoulouhab, F., Décaudin, B., Breysse, C., … Sautou, V. (2015). Migration of plasticizers from PVC medical devices: Development of an infusion model. *International Journal of Pharmaceutics*, *494*(1), 136–145. https://doi.org/10.1016/j.ijpharm.2015.08.033

Calafat, A. M., Needham, L. L., Silva, M. J., & Lambert, G. (2004). Exposure to di-(2-ethylhexyl) phthalate among premature neonates in a neonatal intensive care unit. *Pediatrics*, *113*(5), 429–434.

Chauvigne, F., Menuet, A., Lesne, L., Chagnon, M. C., Chevrier, C., Regnier, J. F., … Jegou, B. (2009). Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis in vitro. *Environmental Health Perspectives*, *117*(4), 515–521. https://doi.org/10.1289/ehp.11870

Delfosse, V., Grimaldi, M., Cavaillès, V., Balaguer, P., & Bourguet, W. (2014). Structural and functional profiling of environmental ligands for estrogen receptors. *Environmental Health Perspectives*, *122*(12), 1306–1313. https://doi.org/10.1289/ehp.1408453

Demirel, A., Çoban, A., Yıldırım, S., Doğan, C., Sancı, R., & İnce, Z. (2016). Hidden toxicity in neonatal intensive care units: phthalate exposure in very low birth weight infants. *Journal of Clinical Research in Pediatric Endocrinology*, *8*(3), 298–304. https://doi.org/10.1016/j.envint.2016.01.024

Desdoits-Lethimonier, C., Albert, O., Le, B. B., Perdu, E., Zalko, D., Courant, F., … Jegou, B. (2012). Human testis steroidogenesis is inhibited by phthalates. *Human Reproduction*, *27*(5), 1451–1459. https://doi.org/10.1093/humrep/dex093

Dykstra, K. D., Guo, L., Birzin, E. T., Chan, W., Yang, Y. T., Hayes, E. C., … Hammond, M. L. (2007). Estrogen receptor ligands. Part 16: 2-Aryl indoles as highly subtype selective ligands for ERalpha. *Bioorganic & Medicinal Chemistry Letters*, *17*(8), 2322–2328. https://doi.org/10.1016/j.bmcl.2007.01.054

Eljezi, T., Pinta, P., Richard, D., Pinguet, J., Chezal, J. M., Chagnon, M. C., … Moreau, E. (2017). In vitro cytotoxic effects of DEHP-alternative plasticizers and their primary metabolites on a L929 cell line. *Chemosphere*, *173*, 452–459. https://doi.org/10.1016/j.chemosphere.2017.01.026.

Engel, A., Buhrke, T., Imber, F., Jessel, S., Seidel, A., Volkel, W., & Lampen, A. (2017). Agonistic and antagonistic effects of phthalates and their urinary metabolites on the steroid hormone receptors ERα, ERβ, and AR. *Toxicology Letters*, *277*, 54–63. https://doi.org/10.1016/j.toxlet.2017.05.028

Ermler, S., Scholze, M., & Kortenkamp, A. (2010). The sensitivity of the MDA-kb2 cell in vitro assay in detecting anti-androgenic chemicals—identification of sources of variability and estimation of statistical power. *Toxicology In Vitro*, *24*(6), 1845–1853. https://doi.org/10.1016/j.tiv.2010.05.007

Fischer, C. J., Bickle Graz, M., Muehlethaler, V., Palmero, D., & Tolsa, J. F. (2013). Phthalates in the NICU: is it safe? *Journal of Paediatrics and Child Health*, *49*(9), E413–E419. https://doi.org/10.1111/jpc.12244

Ghisari, M., & Bonefeld-Jorgensen, E. C. (2009). Effects of plasticizers and their mixtures on estrogen receptor and thyroid hormone functions. *Toxicology Letters*, *189*(1), 67–77. https://doi.org/10.1016/j.toxlet.2009.05.004

Gray, T. J., & Gangolli, S. D. (1986). Aspects of the testicular toxicity of phthalate esters. *Environmental Health Perspectives*, *65*, 229–235. https://doi.org/10.1289/ehp.8665229

Huygh, J., Clotman, K., Malarvannan, G., Covaci, A., Schepens, E., Verbrugghe, W., … Jorens, P. G. (2015). Considerable exposure to the endocrine disrupting chemicals phthalates and bisphenol-A in intensive care unit (ICU) patients. *Environmental International*, *81*, 64–72. https://doi.org/10.1016/j.envint.2015.04.008

Jobling, S., Reynolds, T., White, R., Parker, M. G., & Sumpter, J. P. (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environmental Health Perspectives*, *103*(6), 582–587. https://doi.org/10.1289/ehp.95103582

Jones, G., Willett, P., Glen, R. C., Leach, A. R., & Taylor, R. (1997). Development and validation of a genetic algorithm for flexible docking. *Journal of Biomolecular Biology*, *267*(3), 717–748.

Jones, P. S., Parrott, E., & White, I. N. H. (1999). Activation of transcription by estrogen receptor and is cell type- and promoter dependent. *Journal of Biological Chemistry*, *274*(45), 32008–32014.

Kluwe, W. M. (1982). Overview of phthalate ester pharmacokinetics in mammalian species. *Environmental Health Perspectives*, *45*, 3–10. https://doi.org/10.1289/ehp.82453

Koch, H. M., Bolt, H. M., Preuss, R., & Angerer, J. (2005). New metabolites of di(2-ethylhexyl)phthalate (DEHP) in human urine and serum after single oral doses of deuterium-labelled DEHP. *Archives of Toxicology*, *79*(5), 367–376. https://doi.org/10.1007/s00204-004-0642-4

Kortenkamp, A., & Altenburger, R. (1998). Synergisms with mixtures of xenoestrogens: a reevaluation using the method of isoboles. *The Science of the Total Environment*, *221*(1), 59–73. https://doi.org/10.1016/S0048-9697(98)00261-7

Krüger, T., Long, M., & Bonefeld-Jørgensen, E. C. (2008). Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. *Toxicology*, *246*(2–3), 112–123. https://doi.org/10.1016/j.tox.2007.12.028

Lessmann, F., Correia-Sá, L., Calhau, C., Domingues, V. F., Weiss, T., Brüning, T., & Koch, H. M. (2017). Exposure to the plasticizer di(2-ethylhexyl) terephthalate (DEHTP) in Portuguese children—urinary metabolite levels and estimated daily intakes. *Environmental International*, *104*, 25–32. https://doi.org/10.1016/j.envint.2017.03.028

Lessmann, F., Schtüze, A., Weiss, T., Langsch, A., Otter, R., Brünning, T., & Koch, H. M. (2016). Metabolism and urinary excretion kinetics of di(2-ethylhexyl) terephthalate (DEHTP) in three male volunteers after oral dosage. *Archives of Toxicology*, *90*(7), 1659–1667. https://doi.org/10.1007/s00204-016-1715-x

Lovekamp, T. N., & Davis, B. J. (2001). Mono-(2-ethylhexyl)phthalate suppresses aromatase transcripts levels and oestradiol production in cultured rat granulosa cells. *Toxicology and Applied Pharmacology*, *172*(3), 217–224. https://doi.org/10.1006/taap.2001.9156

Malarvannan, G., Onghena, M., Verstraete, S., Van Puffelen, E., Jacobs, A., Vanhorebeek, I., … Covaci, A. (2019). Phthalate and alternative plasticizers in indwelling medical devices in pediatric intensive care units. *Journal of Hazardous Materials*, *5*(363), 64–72. https://doi.org/10.1016/j.jhazmat.2018.09.087

Mallow, E. B., & Fox, M. A. (2014). Phthalates and critically ill neonates: device-related exposures and non-endocrine toxic risks. *Journal of Perinatology*, *34*(12), 892–897. https://doi.org/10.1038/jp.2014.157

Mankidy, R., Wiseman, S., Ma, H., & Giesy, J. P. (2013). Biological impact of phthalates. *Toxicology Letters*, *217*, 50–58. https://doi.org/10.1016/j.toxlet.2012.11.025

OECD. (2012). Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. Paris: OECD.

OECD. (2016). Test no. 455: The Stably Transfected Human Estrogen Receptor-alpha Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals. Paris: OECD.

OECD 456. (2011). Test no. 456: H295R Steroidogenesis Assay. Paris: OECD.

Pereira de Jesus-Tran, K., Cote, P. L., Cantin, L., Blanchet, J., Labrie, F., & Breton, R. (2006). Comparison of crystal structures of human androgen receptor ligand-binding domain complexed with various agonists reveals molecular determinants responsible for binding affinity. *Protein Science*, *15*(5), 987–999. https://doi.org/10.1110/ps.051905906

Regulation (EU) (2008). 1272/2008 of the European parliament and of the council of 16 December 2008 on classification, labelling and Packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation EC No 1907/2006.

Regulation (EU) (2017). 2017/745 of the European parliament and of the council of 5 April 2017 on medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC.

Satya, S., Wade, M., Hass, U., Holbech, H., Lofstedt, M., Vinggaard, A. M., Tyle, K. H., … Christiansen, S. (2012). Guidance document on standardized test guidelines for evaluating chemicals for endocrine disruption. OECD, ENV/JM/MONO. 22.

SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks) (2015 update). Opinion on the safety of medical devices containing dehp- plasticized pvc or other plasticizers on Neonates and other groups possibly at risk.

Shen, O., Du, G., Sun, H., Wu, W., Jiang, Y., Song, L., & Wang, X. (2009). Comparison of in vitro hormone activities of selected phthalates using reporter gene assays. *Toxicology Letters*, *191*(1), 9–14. https://doi.org/10.1016/j.toxlet.2009.07.019

Strømmen, K., Lyche, J. L., Blakstad, E. W., Moltu, S. J., Veierød, M. B., Almaas, A. N., … Iversen, P. O. (2016). Increased levels of phthalates in very low birth weight infants with septicemia and bronchopulmonary dysplasia. *Environmental International*, *89-90*, 228–234. https://doi.org/10.1016/j.envint.2016.01.024

Stroustrup, A., Bragg, J. B., Busgang, S. A., Andra, S. S., Curtin, P., Spear, E. A., … Gennings, C. (2018). Sources of clinically significant neonatal intensive care unit phthalate exposure. *Journal of Exposure Science and Environmental Epidemiology*. https://doi.org/10.1038/s41370-018-0069-2

Takeuchi, S., Iida, M., Kobayashi, S., Jin, K., Matsuda, T., & Kojima, H. (2005). Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor. *Toxicology*, *210*(2–3), 223–233. https://doi.org/10.1016/j.tox.2005.02.002

Wilson, V. S., Bobseine, K., Lambright, C. R., & Gray, L. E. (2002). A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid- responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicological Sciences*, *66*(1), 69–81. https://doi.org/10.1093/toxsci/66.1.69

Yang, W., Tan, W., Zheng, J., Zhang, B., Li, H., & Li, X. (2018). MEHP promotes the proliferation of cervical cancer via GPER mediated

activation of Akt. *European Journal of Pharmacology*, *824*, 11–16. https://doi.org/10.1016/j.ejphar.2018.01.040

Zacharewski, T. R., Meek, M. D., Clemons, J. H., Wu, Z. F., Fielden, M. R., & Matthews, J. B. (1998). Examination of the in vitro and in vivo estrogenic activities of eight commercial phthalate esters. *Toxicological Science*, *46*(2), 282–293.

SUPPORTING INFORMATION

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