

***In vitro* and *in silico* hormonal activity studies of DEHT, a DEHP substitute used in medical devices, and its metabolites.**

Short title: *In vitro* and *in silico* hormonal activity studies of DEHT

N. K. Kambia^a, I. Séverin^b, A. Farce^a, E. Moreau^c, L. Dahbi^b, C. Duval^a, T. Dine^a, V. Sautou^{d,*}, M.C. Chagnon^b

^a Université de Lille, EA 7365 - GRITA - Groupe de Recherche sur les formes Injectables et les Technologies Associées, F-59000 Lille, France; CHU Lille, Institut de Pharmacie, F-59 000 Lille, France

^b Université Bourgogne Franche-Comté, Derttech "Packtox", NUTOX, INSERM U1231, AgroSup Dijon, 21000 Dijon, France

^c Université Clermont Auvergne, CHU Clermont-Ferrand, CNRS, SIGMA Clermont-Ferrand, ICCF, 63000 Clermont-Ferrand, France

^d Université Clermont Auvergne, INSERM U1240, Imagerie Moléculaire et Stratégies Théranostiques, F-63000, Clermont Ferrand, France

*Corresponding author: Pharmacie, CHU Clermont-Ferrand, 58 Rue Montalembert, 63003 Clermont-Ferrand, France. Tel: +33473751758- E-mail: valerie.sautou@uca.fr

Keywords: DEHT, DEHP, Human androgen or estrogen receptor, H295R steroidogenesis assay, T Screen, docking

ABSTRACT

Plasticizers added to PVC used in medical devices can be released into patients' biological fluids. The substitution of DEHP by alternative plasticizers is essential but their safety must be demonstrated. DEHP, DEHT and their metabolites were investigated using level 2 OECD 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-MEHT, were detected and the results of docking experiments performed on ER α and AR agreed with the biological results. A co-stimulation of hER α and hAR 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 5OH-MEHT, suggesting a potential endocrine disrupting effect.

SHORT ABSTRACT

Plasticizers as DEHT are added to PVC used in medical devices can be released into patients' biological fluids. The objective of this study was to investigate the potential endocrine disrupting effects of DEHT and its metabolites *in silico* and *in vitro*. The hormonal activities of DEHT derived metabolites, e.g. 5-OH-MEHT, were detected and the results of docking

experiments performed on ER α and AR agreed with the biological results. A co-stimulation of hER α and hAR and an increase in estrogen synthesis was measured with 5-OH-MEHT

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, especially neonates in intensive care units (Mallow *et al.*, 2014; Fischer *et al.*, 2016). 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). 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). 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 (MEHT) has a lower cytotoxicity compared to MEHP. At doses where DEHP altered sexual differentiation, DEHT was inactive (Scenihr, 2015, Eljezi *et al.*, 2017). 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, especially in non-glucuronjugated form regarding to the carboxy derivatives (Barber *et al.* 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 in order to check DEHT as a potential substitute. Endocrine disrupting chemicals (EDCs) of estrogen ER signaling pathways can contribute to adverse health effects on various areas of the body, such as the nervous system, the heart, breast, reproductive tracts in males and females and energetic metabolism. EDCs 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 estrogen (ER α ; ER β) and the androgen (AR) receptors (Delfosse *et al.* 2014). Reporter gene assays are mechanistic and sensitive tools to characterize receptor mediated endocrine activity and are recommended in the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2012). The action of estrogen in regulating gene transcription is mediated through specific estrogen receptors of the nuclear receptor superfamily, such as receptor α . To test phthalates and their metabolites, human ER α activity was measured using a stable transfected cell line (Hela 9903) and following OECD guideline TG 455 (OECD 455, 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 estrogen and androgen receptors, on steroid synthesis, and to compare them with those of DEHP and its metabolites.

MATERIAL AND METHODS

Plasticizers and metabolites

DEHP (Ref: D201154, CAS: 117-81-7) and **DEHT** (ref: 525189, CAS: 6422-86-2) were purchased from Sigma Aldrich, 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 synthesized metabolites and their corresponding intermediates exceeded 95%.

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 synthesized metabolite powder provided by the chemists. In order to avoid a cytotoxic effect of the vehicle on the cell lines, the maximum concentration of ethanol in the culture medium was 1%.

ER and AR Transcriptional Activation Assays

Cell culture

For the cell-based ER-mediated bioassay, stably-transfected hER α -HeLa-9903 cells were obtained from the Japanese Collection of Research Bioresources (JCRB-N°1318) cell bank. These cells contain stable expression constructs for human ER α and firefly luciferase. The latter is under transcriptional control of five Estrogen Response Element (ERE) promoter elements from the vitellogenin gene. Cells were maintained in Eagles Minimum Essential Medium (EMEM) without phenol red, supplemented with kanamycin (60 mg/L) and 10% (v/v) Foetal Bovine Serum (FBS), in an incubator under 5% CO₂ at 37°C. Upon reaching 75-90% confluency, cells were subcultured twice (not more than 10 passages) prior to 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 *et al.*, 2002) were obtained from the ATCC (N°.CRL-2713). Cells were routinely maintained in Leibowitz-15 (L-15) medium supplemented with 10% FBS (v/v) in a humidified incubator at 37°C without additional CO₂. Cells were sub-cultured when confluent over a maximum of 10 passages.

Luciferase Assays

The assay for (anti)estrogenic activity was performed in accordance with OECD test guideline TG455 (OECD, 2016). Prior to experiments, HeLa-9903 cells were maintained in culture medium supplemented with 10% (v/v) DCC-FBS (dextran-coated charcoal stripped serum) for at least two media-changes. Cells were seeded at a density of 1×10⁴ 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 h.

A modified version of the original protocol by Wilson *et al.* (2002) was used to test compounds for (anti)androgenic activity (Ermler *et al.*, 2010). Prior to 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 μ l 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 h.

After incubation, 50 μ l of a 3x dosing medium were added to the wells. The cells were exposed to the dilution series of the tested chemicals (7 different concentrations of each sample were tested), to the reference estrogen E2 or reference androgen DHT (dihydrotestosterone), and to the solvent controls (0.1% v/v ethanol). DHT or E2 (1 nM) was used as a positive control in the AR or ER agonist assay, respectively. DHT (0.25 nM) or E2 (0.025 nM) was used as a control in order to establish a baseline for co-exposure in order to screen for AR or ER antagonism, respectively. After 24 h of exposure, the luciferase activity was determined with Steady Glo assay reagent (Promega) as per the manufacturer's instructions.

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-h (Hela-9903) or 5-h (MDA-kb2) incubation period with 50 μ l/well of 4 μ g/mL resazurin (obtained from Sigma-Aldrich) in PBS, cell proliferation was measured as relative fluorescence units (RFUs) resulting from the reduction of non-fluorescent resazurin to the fluorescent product resorufin. Fluorescence was measured at $\lambda_{\text{ex}} = 530$ nm and $\lambda_{\text{em}} = 590$ 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.

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 per cent above the blank baseline in agonist mode, or decreased luminescence by more than 20 per cent of the maximal signal in antagonist mode.

H295R Steroidogenesis assay

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 5 passages and frozen in batches in liquid nitrogen. Prior to conducting steroidogenesis evaluation, batches of H295R cells were thawed and passed at least 4 times. The maximum passage number used for steroidogenesis evaluation was 10.

Cells were routinely grown at 37°C under a 5% CO₂ atmosphere in 75 cm² culture flasks containing 12 mL DMEM/Ham's F12 culture medium mixture (Gibco 11039021) supplemented with 1% ITS+ premix (BD Bioscience; 354352) and 2.5% Nu-Serum (BD Bioscience; 355100). For subculturing, the H295R cells were washed three times with PBS, detached using trypsin/EDTA (0.25%/0.05% (v/v) in Hank's Balanced Salt Solution (HBSS)) 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 h (50-60% confluence), the medium was refreshed and compounds dissolved in Ethanol (EtOH) were added. Exposures were performed in triplicate with a final concentration of the solvent carrier of 0.1%. Positive controls, 10 μ M forskolin (FOR) and 1 μ M prochloraz (PRO), were included on each plate.

Following 48 h of chemical treatment, media was removed, split into 2 vials of approximately 500 µl media each, and stored at -80°C prior to 17β-estradiol (E2) and testosterone (T) quantification.

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.). Exposures showing a decrease in cell viability were excluded from hormone analysis.

Release of hormones

Enzyme linked immunosorbent assays (ELISA) were used to directly quantify testosterone and 17β-estradiol from aliquots of the medium. The ELISA kits (KGE010, KGE014) were purchased from Bio-Techne (R&D systems Europe, France). According to the manufacturer's data, the sensitivity of the testosterone assay was 0.030 ng/mL, and the intra and inter-assay coefficients of variation were 4.0% and 5.6%, respectively. The sensitivity of the 17β-estradiol assay was 4.84 pg/mL, and the intra- and inter-assay coefficients of variation were 6.0% and 7.1%, respectively. The absorbance was determined at a wavelength of 450 nm using a Tecan (BioRad) microplate reader.

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.

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 (ANOVA) 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 * $p < 0.05$.

Docking studies

The docking of the compounds under evaluation was performed using the crystallographic coordinates 2iog for ERα (Dykstra *et al.*, 2007) and 2am9 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 on the basis of 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 on the basis of 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 *et al.*, 1997). The charges of the ligands and receptors were assigned using the Gasteiger-Hückel method and the geometry of the each ligand configuration was optimized with the maximin2 protocol of the Sybyl 6.9.1 molecular modeling software. 30 solutions were generated for each compound and the number of poses of each cluster gave a rough idea 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 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.

RESULTS

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

Transcriptional activity of human ER α

Agonist or antagonist activities on hER α were measured in the absence or presence of E2. Neither DEHT nor DEHP (supplementary data, figure 1) were active on hER α . The same conclusion can be drawn for their corresponding mono-esters (in terms of agonist or antagonist activities) using a large non-cytotoxic concentration range (up to 20 μ g/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 1), 5-OH-MEHT induced an agonist effect on ER α at the highest concentration and a synergic concentration dependent effect in the presence of E2 at 0.2 and up to 20 μ g/mL, while 5-OH-MEHP was an antagonist at the highest concentration without any cytotoxicity.

Oxo-derived monoester metabolites were equivalent partial antagonists of ER α with a concentration dependency effect (2 – 20 μ g/mL) without cytotoxicity (figure 1). 5-Cx-MEHT and 5-Cx-MEHP were not active in the transcriptional assay irrespective of the activity studied.

Transcriptional activity of human AR

Neither DEHT nor DEHP induced agonist or antagonist activities on AR over a large non-cytotoxic concentration range (up to 20 μ g/mL) (supplementary data, figure 1). 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 to 20 μ g/mL) when cells were co-treated with DHT. It should be noted that, under our experimental conditions, oxo-derived or carboxy-derived monoester metabolites of both phthalates had no effect on AR transcriptional activity.

Docking experiments

The co-crystallized ligands of the investigated receptors (compound 11F for ER α and testosterone for AR) were docked to validate the protocol. Both were very close to their crystallographic position and a high 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 ER α , the crystallographic conformation was found nearly exclusively, with 27 solutions out 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 ER α , 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 ER α 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 ER α . While MEHT is able to fit into the cavity of the androgen receptor 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-hydroxylated 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 most probable that the hydroxyl is binding to both residues in a mode very similar to that observed for the natural ligand (Figure 4).

Steroids synthesis assays

The H295 steroidogenesis assay was performed with derived plasticizer metabolites in order to detect substances which affect the production of E2 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.

Estradiol synthesis

MEHT and MEHP were weakly active, with for MEHP a significant change between 2 and 40 $\mu\text{g/mL}$ and a 6-fold induction at 40 $\mu\text{g/mL}$ for MEHP.

Unfortunately MEHT was cytotoxic for the cells above 10 $\mu\text{g/mL}$. Hydroxylated derived metabolites were more potent, with a concentration dependent increase in estradiol synthesis. A maximum 12-fold increase was seen at 80 $\mu\text{g/mL}$. This significant effect started at a lower concentration with 5-OH-MEHP (0.2 $\mu\text{g/mL}$) compared to 10 $\mu\text{g/mL}$ with 5-OH-MEHT. Oxo-derived monoesters were the most active metabolites, with an induction starting at 10 $\mu\text{g/mL}$, and reaching a 14 to 16-fold induction at the highest concentration (80 $\mu\text{g/mL}$). Carboxy-derived metabolites started to be significant agonists at 40 $\mu\text{g/mL}$, but with only a weak effect (around 2-fold).

Testosterone synthesis

A similar and statistically significant decrease of testosterone (2-fold) was observed with OH-derived metabolites. 5-OH-MEHT had an effect at a lower concentration (20 $\mu\text{g/mL}$) than 5-OH-MEHP (40 $\mu\text{g/mL}$). It should be noted that at 10 $\mu\text{g/mL}$, MEHT also decreased testosterone synthesis. Figure 3 shows the same tendency with the oxo-derived metabolites at 40 and 80 $\mu\text{g/mL}$, with a change observed at a lower oxo-MEHT concentration (10 $\mu\text{g/mL}$ instead of 40 $\mu\text{g/mL}$ with oxo-MEHP). 5-Cx-derived metabolites had no effect on testosterone synthesis.

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

Impact on sexual hormones

DEHP and DEHP metabolites

We found that, when a transcriptional effect was observed on ER α , 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 agree with Shen *et al* (2009), Jobling *et al*, 1995 and Zacharewski *et al.*, 1998 who showed no ER transactivity and no capacity for DEHP to compete with E2-ER binding *in vitro*. In contrast, Takeuchi *et al* (2005), who tested DEHP and its corresponding monoester MEHP, observed a weak activation of hER α with 5.5 μ M 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 μ M. 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 E2 at a very high concentration (100 μ M). More recently, Yang *et al* (2018) used nanoMolar concentrations to demonstrate that MEHP can trigger the proliferation of cervical cancer cells *via* the activation of the G-protein coupled estrogen receptor (GPER) rather than ER α . 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 *et al.*, 1999). It is important to note that DEHP has to be metabolized to MEHP and its derived metabolites in order to be bioactive both *in vivo* or *in vitro* (Gray *et al.*, 1986; Koch *et al.*, 2005; Chauvigne *et al.*, 2009) and the differences observed in *in vitro* models may also 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 μ M. However, the same authors observed an AR inhibition at 50 μ M, a two-fold 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 in order to avoid false positives data. It is essential to test substances at non-cytotoxic concentrations, especially 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 EC50 (concentration which gives a half-maximum response) or IC50 (concentration which inhibits the response by half) exceeding 10^{-4} M, which is a very high concentration. Araki *et al.* (2005) also demonstrated an antagonist effect of DEHP on AR. However, this was not seen by Kruger *et al.* (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 effect in reporter gene was observed only with DEHP metabolites.

Using the steroidogenesis synthesis assay, which gives information on another mode of ED action (not genomic), we have shown that MEHP is also active at 40 µg/mL, with an increase of E2 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 *et al.* (2013), who demonstrated that hormone synthesis was affected by DEHP concentrations of 10 µg/mL, resulting in a greater production of E2 (4-fold) 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 4 and 5 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 to also reduce testosterone production *in vitro* after 48 h, associating phthalate exposure with the impairment of the androgynous status.

DEHT and DEHT metabolites

5-OH-MEHT showed an agonist effect at the highest concentration and, interestingly, a synergism in the presence of E2, with a concentration dependency effect on ER (from 0.2 up to 20 µg/mL). However, when expressed as Eq/L E2 (supplementary data, figure 2), the agonist effect of 5-OH-MEHT is weak, with a relative potency 3.5×10^{-6} fold lower than E2. 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 E2 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 estradiol level as demonstrated *in vitro* by Lovekamp and Davis (2001) with MEHP.

Docking

Efforts were limited to the estrogen receptor α due to the fact that ER β 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 Defosse *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 ER α 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 4 conformations in ER and 2 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 ER α and AR in a mode very similar to that of the natural ligands.

In vitro data versus biomonitoring values

In neonatal intensive care units (NICU), 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. Strommen *et al.* (2016) showed that the urinary concentration of 5-oxo and 5-OH-MEHP could reach 1 $\mu\text{g/mL}$. The cohort studied by Demirel *et al.* (2016) presented even higher values with maximum limits in the order of 5 $\mu\text{g/mL}$ for these two oxidized metabolites. Our study shows that at these concentrations there is an antagonistic effect on estrogen receptors. Moreover, the effects of 5-OH-MEHP on the synthesis of estradiol are observed from 0.2 $\mu\text{g/mL}$, which is close to the median concentration observed in these newborns. In intensive care, extracorporeal membrane oxygenation (ECMO) 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 ECMO had urinary 5-OH-MEHP concentrations of more than 5 $\mu\text{g/mL}$ and blood concentrations of more than 0.8 $\mu\text{g/mL}$ (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 to 17 years. The maximum levels observed were 0.06 $\mu\text{g/mL}$ for 5-oxo-MEHT, 0.18 $\mu\text{g/mL}$ for 5-OH-MEHT and 0.34 $\mu\text{g/mL}$ for 5-cx-MEHT. Even if the median concentration of 5-OH-MEHT was much lower (0.045 $\mu\text{g/mL}$), the maximum concentration observed corresponds to the concentration showing the first synergistic effect with E2 on hER α and agonist effects on AR receptors. 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 further assess the risk. Experiments are on-going 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 (Ghisary *et al.*, 2009; Kortenkamp *et al.*, 1998). Therefore *in vitro* experiments are important in order 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 *et al.*, 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 5-cx-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 to 15% whereas 5-cx-MEHP accounts for 60 to 83% of all metabolites (Strommen *et al.*, 2016, Stroustrup *et al.*, 2018). In adults, 5-OH-MEHP is present in greater quantity than 5-cx-MEHP (around 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).

CONCLUSION

This study presents biological hormonal activities of the derived metabolites of DEHP and DEHT, involving carboxy-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. This is the first time that a co-stimulation of hER α and hAR has been observed with 5-OH-MEHT. *In silico* results for ER α 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 better protect vulnerable populations to DEHP substitutes.

Our study shows that investigations concerning the hazard of DEHT during exposition of neonates to medical devices must be monitored before attesting to its safety. Several elements play in favor of this plasticizer as an alternative to DEHP: its weak diffusion towards the liquids in contact with the medical devices limiting the exposure of the patients, its 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 estrogen receptors, and an increase in estrogen synthesis.

ACKNOWLEDGMENTS:

This study is a part of the ARMED-NEO project and received financial support from the French National Agency for the Safety of Medicines and Health Products (ANSM).

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Figure Legends

Figure 1: Estrogen receptor agonism (ER) and antagonism (anti-ER, in the presence of 0.025 nM E2) with DEHT- and DEHP-metabolites in Hela-9903 transcriptional activation assays. Cell viability was evaluated by the resazurin assay. Data represents mean \pm standard deviation of six data points (two experiments each in triplicate).

The dotted lines (.....) highlight 10% 1nM E2 normalized RTA (Relative Transcriptional Activity) in the agonist mode or 80% 0.025nM E2 normalized RTA in the antagonist mode, as a threshold for categorizing positive data.

Figure 2: Androgen receptor agonism (AR) 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 \pm standard deviation of six data points (two experiments each in triplicate).

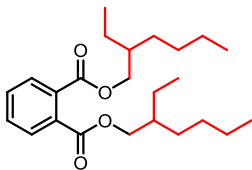
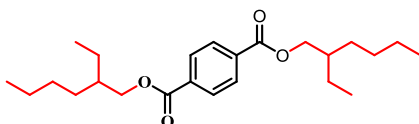
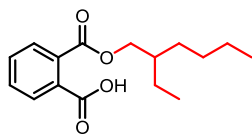
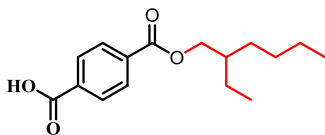
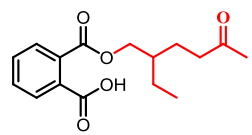
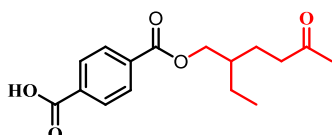
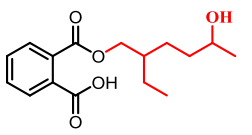
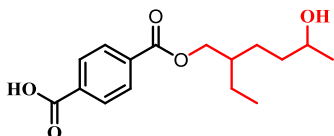
The dotted lines (.....) highlight 10% 1nM DHT normalized RTA (Relative Transcriptional Activity) in the agonist mode or 80% 0.25nM E2 normalized RTA in the antagonist mode, as a threshold for categorizing positive data.

Figure 3: Docking of MEHT in ER α (left panel, reference ligand in yellow) and AR (right panel, reference ligand in orange)

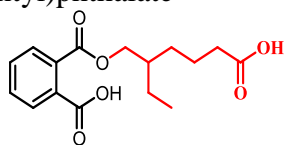
Figure 4: Docking of 5-OH-MEHT in ER α (left panel, reference ligand in yellow) and AR (right panel, reference ligand in orange)

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 \pm SD, n=3). Statistical significance *p<0.05, **p<0.01 and ***p<0.001.

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DEHP and its metabolites	DEHT and its metabolites
<p>DEHP: di-(2-ethylhexyl)phthalate</p> 	<p>DEHT or DEHTP: di-(2-ethylhexyl)terephthalate</p> 
<p>MEHP: mono-(2-ethylhexyl)phthalate</p> 	<p>MEHT or MEHTP: mono-(2-ethylhexyl)terephthalate</p> 
<p>5-oxo-MEHP or MEOHP: mono-(2-ethyl-5-oxohexyl)phthalate</p> 	<p>5-oxo-MEHT or MEOHTP: mono-(2-ethyl-5-oxohexyl)terephthalate</p> 
<p>5-OH-MEHP or MEHHP: mono-(2-ethyl-5-hydroxyhexyl)phthalate</p> 	<p>5-OH-MEHT or MEHHTP: mono-(2-ethyl-5-hydroxyhexyl)terephthalate</p> 

5-cx-MEHP or MECPP: mono-(2-ethyl-5-carboxypentyl)phthalate



5-cx-MEHT or MECPTP: mono-(2-ethyl-5-carboxypentyl)terephthalate

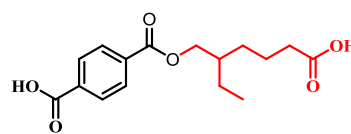
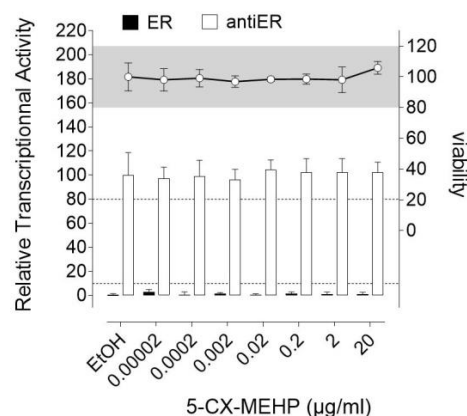
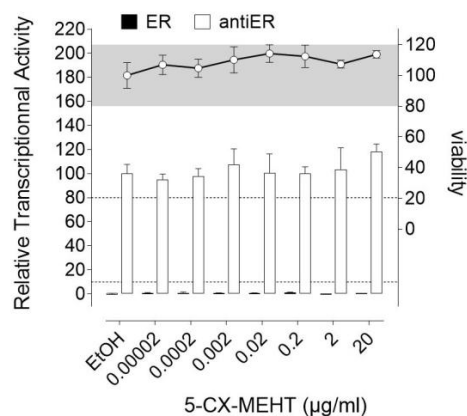
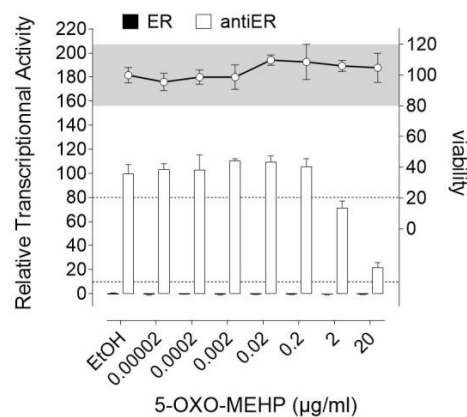
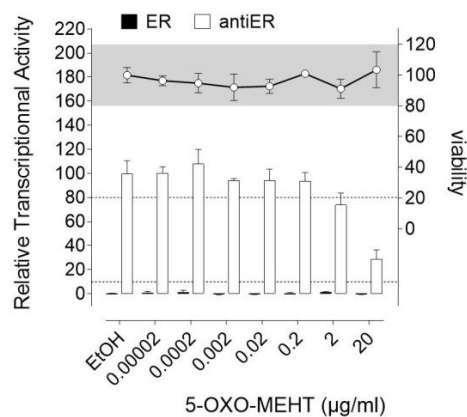
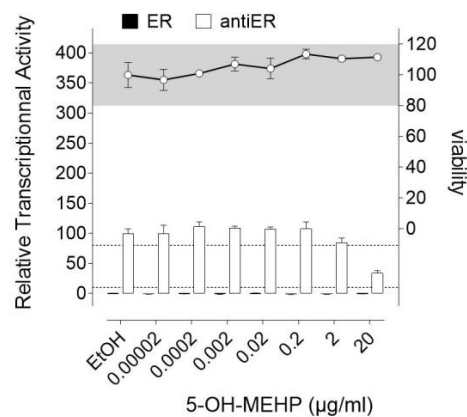
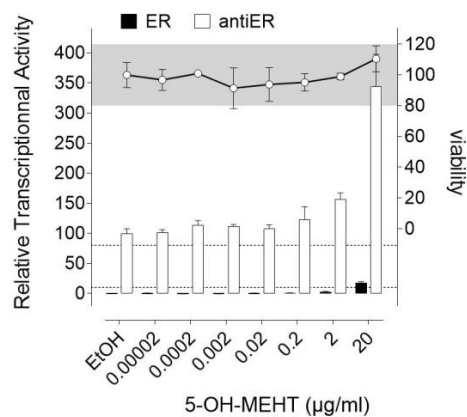
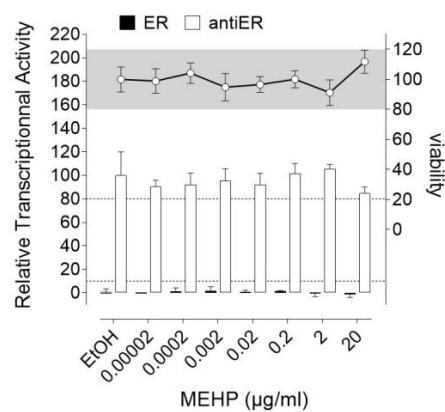
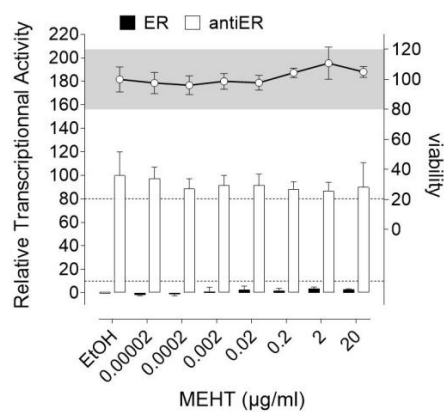
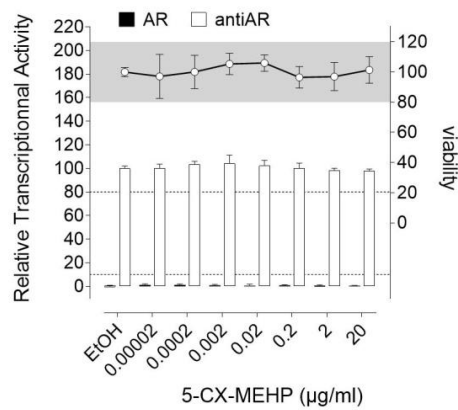
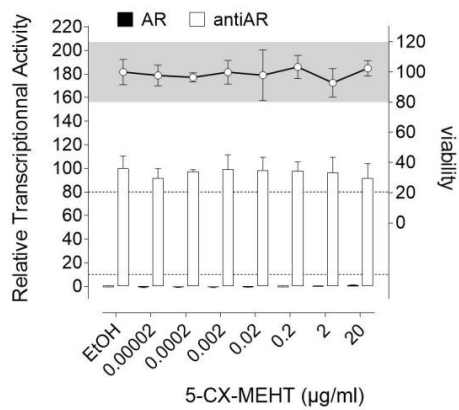
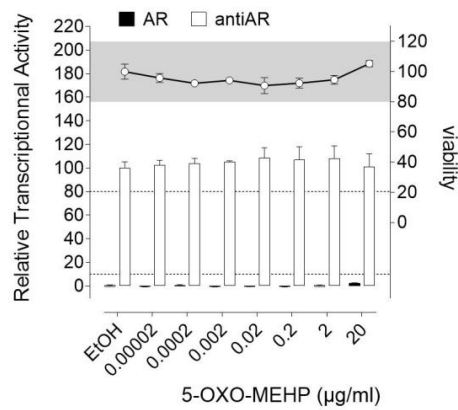
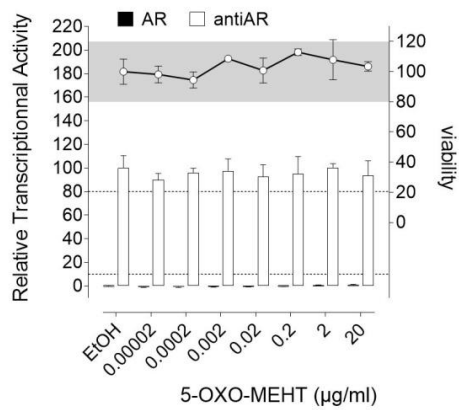
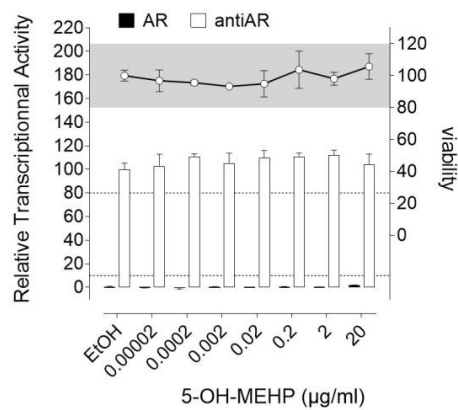
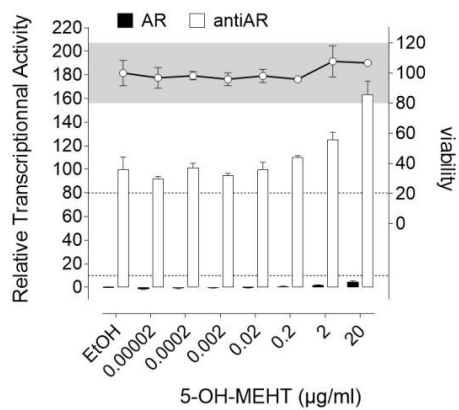
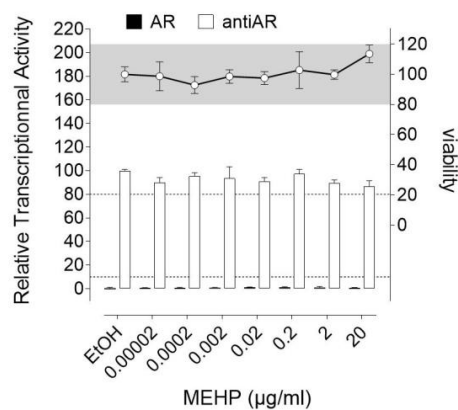
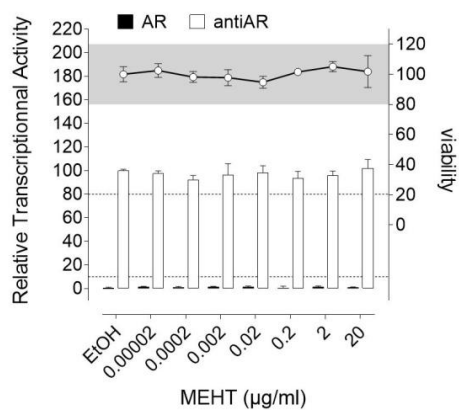


Table 1: Structures and denomination of DEHP, DEHT and metabolites



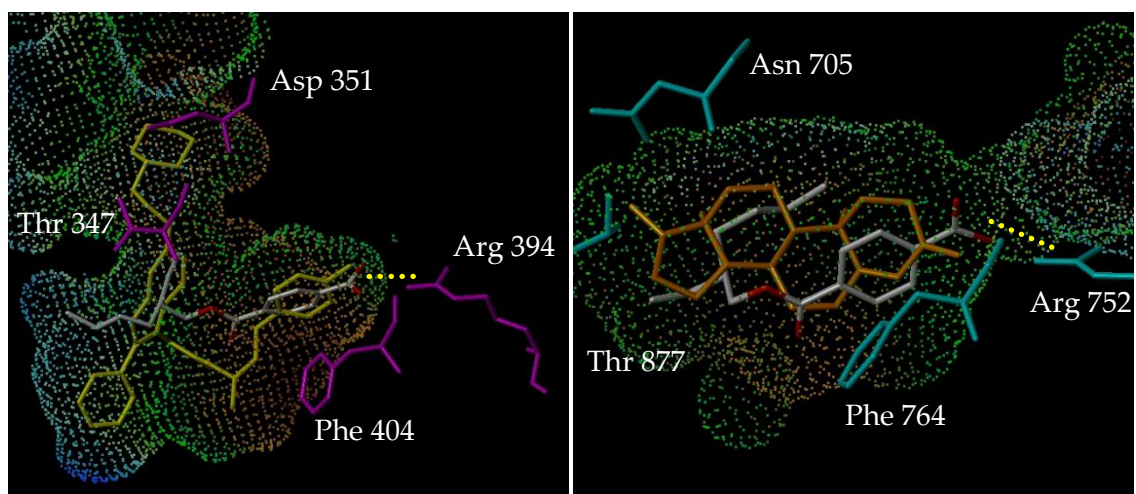
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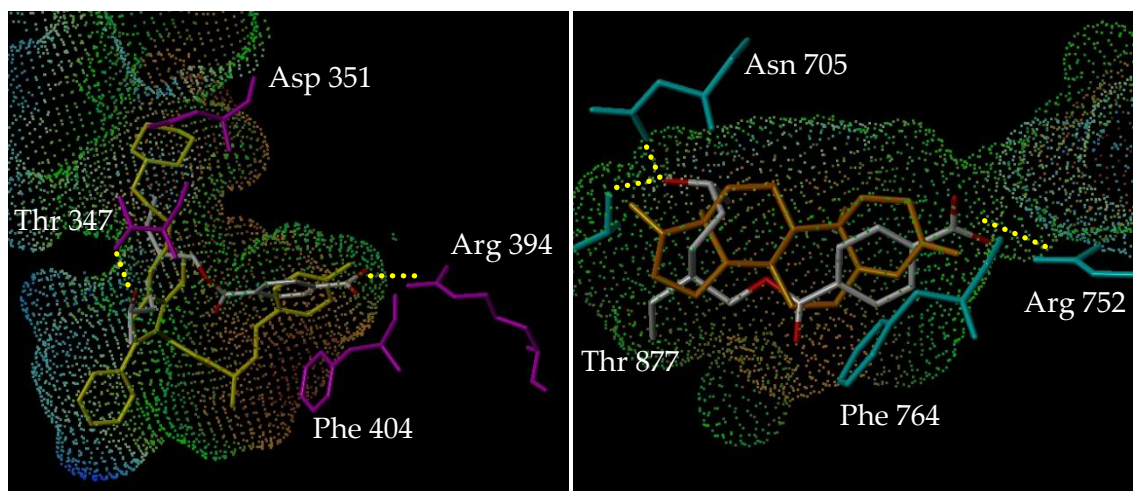
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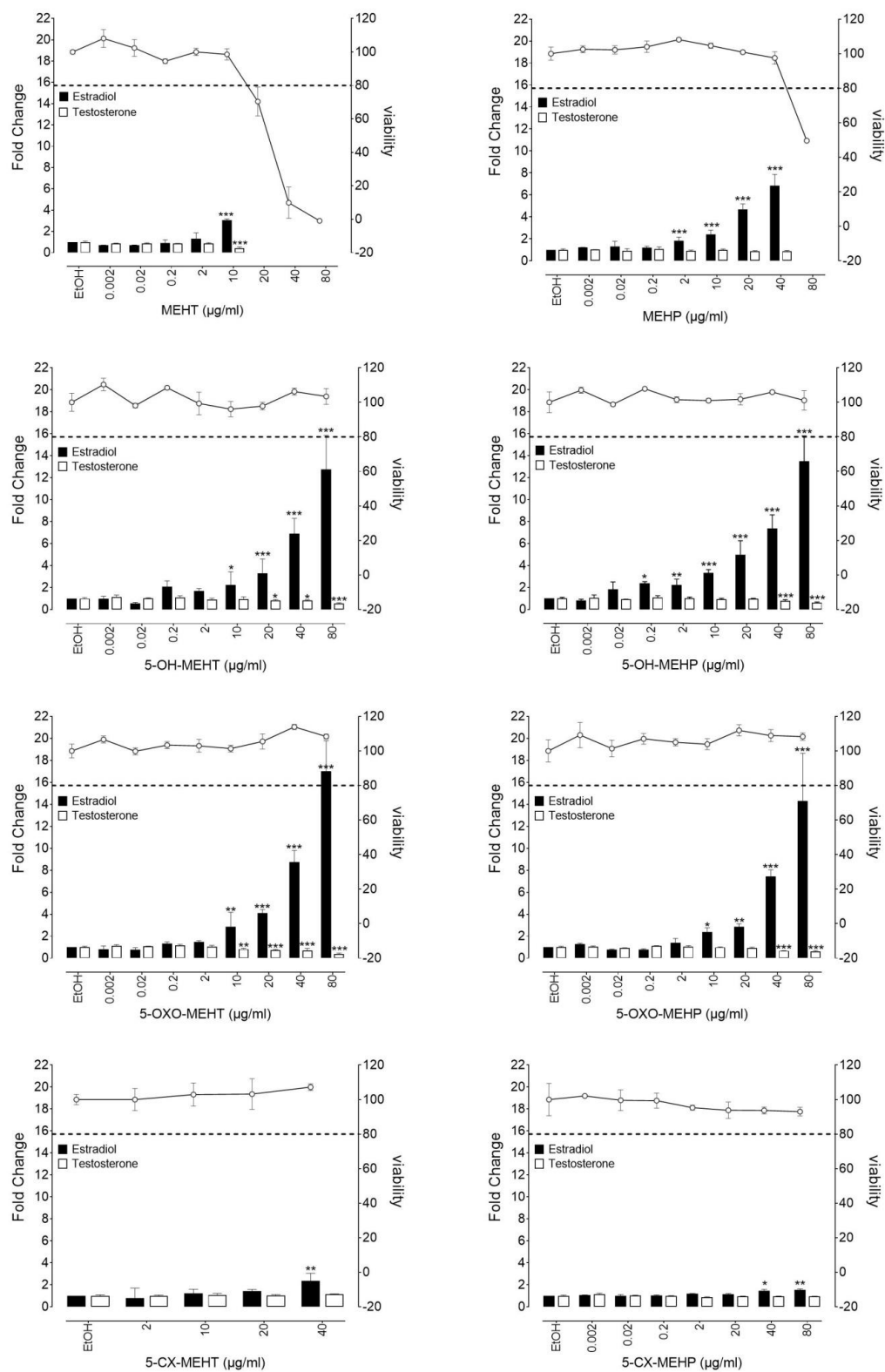


Figure 5

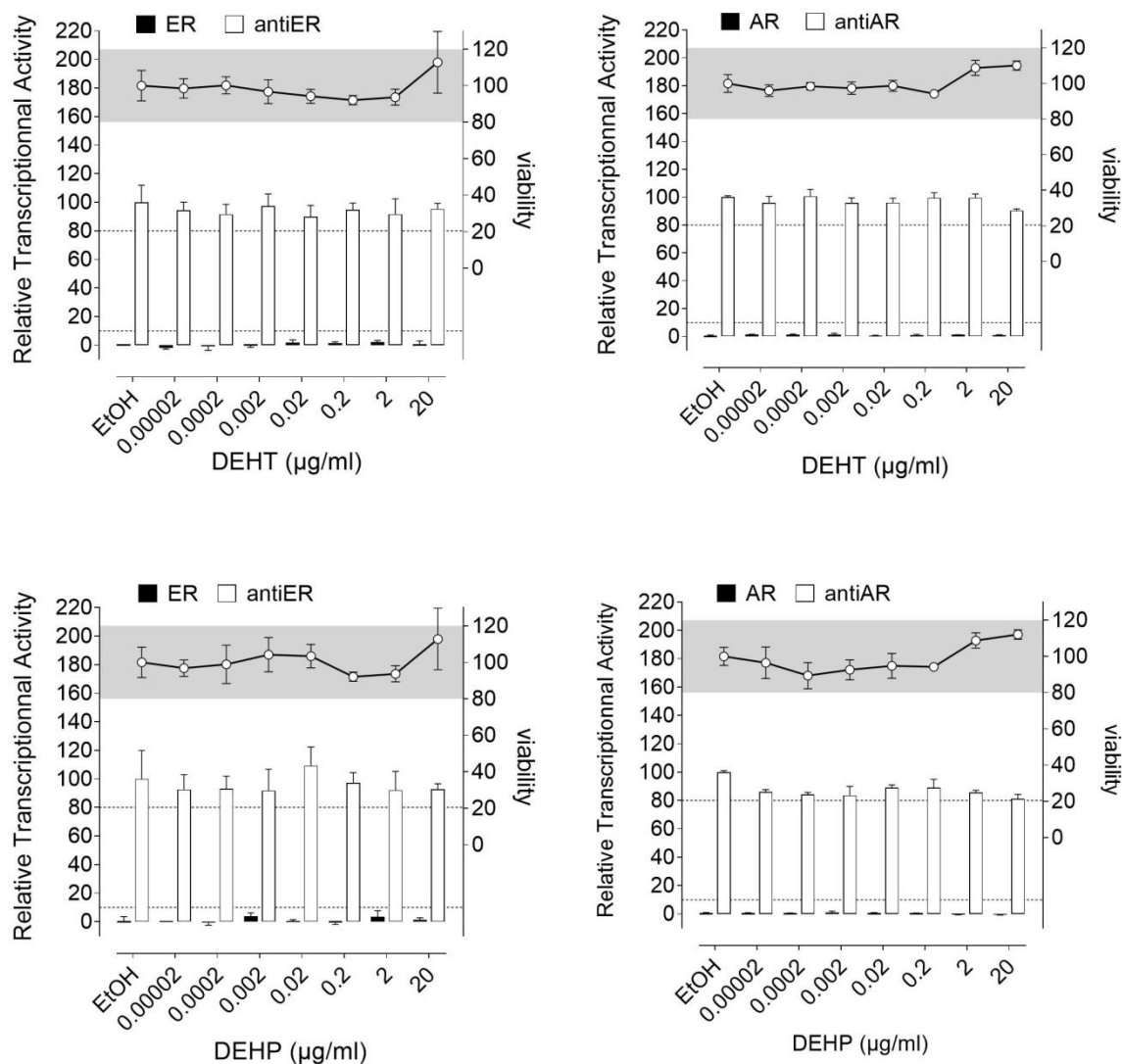
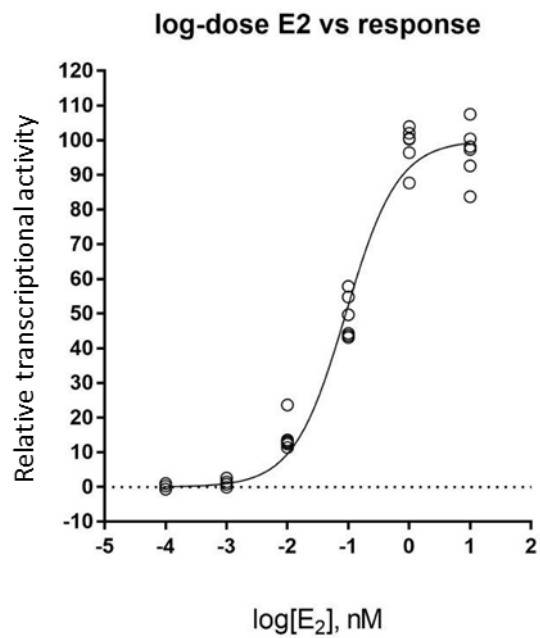


Figure 1s: Estrogen or androgen receptor agonism and antagonism with DEHT and DEHP in Hela-9903 or MDA-kb2 transcriptional activation assays, respectively. Cell viability was evaluated by the resazurin assay. Data represents mean \pm SD of two independent experiments (performed in triplicate). The dotted lines (.....) highlight 10% 1nM E2 or 1nM DHT normalized RTA (Relative Transcriptional Activity) in the agonist mode or 80% 0.025nM E2 or 0.25nM DHT normalized RTA in the antagonist mode, as a threshold for categorizing positive data.

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Figure 2s: Dose response of the estrogen reference (estradiol, E2) in HeLa-9903 transcriptional activation assays for the determination of estradiol equivalent activity of phthalate metabolites