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

3
4 Short title: *In vitro* and *in silico* hormonal activity studies of DEHT

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22
23 **Keywords:** DEHT, DEHP, Human androgen or estrogen receptor, H295R steroidogenesis
24 assay, T Screen, docking

25
26
27 **ABSTRACT**

28
29 Plasticizers added to PVC used in medical devices can be released into patients' biological
30 fluids. The substitution of DEHP by alternative plasticizers is essential but their safety must
31 be demonstrated. DEHP, DEHT and their metabolites were investigated using level 2 OECD
32 bioassays to screen for *in vitro* hormonal changes. Differences between the DEHP and DEHT
33 metabolites were observed. Albeit weak, the hormonal activities of DEHT derived
34 metabolites, e.g. 5-OH-MEHT, were detected and the results of docking experiments
35 performed on ER α and AR agreed with the biological results. A co-stimulation of hER α and
36 hAR was also observed. With regard to steroidogenesis, a 16-fold increase in estrogen
37 synthesis was measured with 5-OH-MEHT. Therefore, even if DEHT remains an interesting
38 alternative to DEHP because of its low migration from medical devices, it seems important to
39 verify that multi-exposed patients in neonatal intensive care units do not have urinary levels
40 of oxidized metabolites, in particular 5OH-MEHT, suggesting a potential endocrine
41 disrupting effect.

42
43
44 **SHORT ABSTRACT**

45
46 Plasticizers as DEHT are added to PVC used in medical devices can be released into patients'
47 biological fluids. The objective of this study was to investigate the potential endocrine
48 disrupting effects of DEHT and its metabolites *in silico* and *in vitro*. The hormonal activities
49 of DEHT derived metabolites, e.g. 5-OH-MEHT, were detected and the results of docking

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

52 INTRODUCTION

53
54 Plasticizers are used as additives to increase the flexibility and softness of normally rigid
55 plastics, such as polyvinylchloride (PVC). Plasticized PVC is used in medical devices such as
56 tubings (infusors, infusion or nutrition lines, extracorporeal circuits) or blood bags. However,
57 any additives that are not chemically bound to the polymer can be released from the material
58 into the infused drug solutions or biological fluids and can thereby come into contact with the
59 patient. This source of exposure presents a general public health concern. Indeed, the
60 metabolites of these plasticizers are found in the urine of many hospitalized patients,
61 especially neonates in intensive care units (Mallow *et al.*, 2014; Fischer *et al.*, 2016). Some of
62 these chemicals are likely to be hazardous for patients, as has been demonstrated for di-(2-
63 ethylhexyl) phthalate (DEHP), which is now classified as CMR 1B (carcinogenic, mutagenic
64 or toxic for reproduction) under the CLP Regulation due to its effect on reproduction and
65 fertility (Regulation (EU) 1272/2008). European regulation 2017/745 of 5 April 2017
66 recommends that the level of DEHP be limited to 0.1% by mass in medical devices
67 (Regulation (EU) 2017/745). Other plasticizers are recommended to soften PVC, such as di-
68 (2-ethylhexyl) terephthalate (DEHT) (Scenihr, 2015). This additive is interesting because it
69 has a much lower level of migration from the medical devices into the fluids infused into the
70 patient than DEHP (Bernard *et al.*, 2015). Moreover, it would be less toxic than DEHP. DEHT
71 is less active in the induction of peroxisome-proliferation in rats than DEHP and this is
72 explained by a smaller amount of monoester produced during DEHT metabolism. Moreover,
73 at equivalent doses, this monoester (MEHT) has a lower cytotoxicity compared to MEHP. At
74 doses where DEHP altered sexual differentiation, DEHT was inactive (Scenihr, 2015, Eljezi
75 *et al.*, 2017). However, its migration is not zero and toxicity data are not complete.
76 Specifically, there is a lack of information regarding the hormonal activities of DEHT and/or
77 its metabolites resulting from its hydrolysis and oxidation in the body. Indeed, it has been
78 proven that the oxidized derivatives of DEHT are found in the urine, especially in non-
79 glucuronjugated form regarding to the carboxy derivatives (Barber *et al.* 1994; Lessmann *et*
80 *al.* 2016). Substitution of DEHP by alternative plasticizers is essential, but the safety of these
81 substitutes must be demonstrated. In this study we investigated the *in vitro* effects of DEHP
82 and DEHT, and their metabolites, using identical bioassays and concentration ranges in order
83 to check DEHT as a potential substitute. Endocrine disrupting chemicals (EDCs) of estrogen
84 ER signaling pathways can contribute to adverse health effects on various areas of the body,
85 such as the nervous system, the heart, breast, reproductive tracts in males and females and
86 energetic metabolism. EDCs can affect the endocrine system of an organism through multiple
87 pathways, such as mimicking natural hormones, antagonizing their action, or modifying their
88 synthesis, metabolism and transport. In general, the main harmful effects of these compounds
89 are due to their interaction with members of the nuclear receptor family, including the
90 estrogen (ER α ; ER β) and the androgen (AR) receptors (Delfosse *et al.* 2014). Reporter gene
91 assays are mechanistic and sensitive tools to characterize receptor mediated endocrine activity
92 and are recommended in the Organisation for Economic Co-operation and Development
93 (OECD) guidelines (OECD, 2012). The action of estrogen in regulating gene transcription is
94 mediated through specific estrogen receptors of the nuclear receptor superfamily, such as
95 receptor α . To test phthalates and their metabolites, human ER α activity was measured using
96 a stable transfected cell line (Hela 9903) and following OECD guideline TG 455 (OECD 455,
97 2016). The MDA-kb2 cell line was used for investigating the potential agonist and antagonist
98 effects on human AR.

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

103
104 The objective of this study was therefore to investigate the potential endocrine disrupting
105 effects of DEHT (a promising DEHP substitute) and its metabolites on estrogen and androgen
106 receptors, on steroid synthesis, and to compare them with those of DEHP and its metabolites.

107 **MATERIAL AND METHODS**

108 **Plasticizers and metabolites**

109
110 **DEHP** (Ref: D201154, CAS: 117-81-7) and **DEHT** (ref: 525189, CAS: 6422-86-2) were
111 purchased from Sigma Aldrich, France. Primary and secondary metabolites of DEHP and
112 DEHT were synthesized and characterized by the IMOST team (UMR 1240, INSERM)
113 Clermont-Ferrand, France. The compounds tested are shown in the Table 1. The purity of all
114 our synthesized metabolites and their corresponding intermediates exceeded 95%.

115 **Preparation of samples**

116
117 All compounds were dissolved in 100% ethanol and tested over a large range of
118 concentrations, from 0.02 ng/mL to 200 µg/mL, depending of the assays and the quantity of
119 synthesized metabolite powder provided by the chemists. In order to avoid a cytotoxic effect
120 of the vehicle on the cell lines, the maximum concentration of ethanol in the culture medium
121 was 1%.

122 **ER and AR Transcriptional Activation Assays**

123 Cell culture

124
125 For the cell-based ER-mediated bioassay, stably-transfected hER α -HeLa-9903 cells were
126 obtained from the Japanese Collection of Research Bioresources (JCRB-N^o1318) cell bank.
127 These cells contain stable expression constructs for human ER α and firefly luciferase. The
128 latter is under transcriptional control of five Estrogen Response Element (ERE) promoter
129 elements from the vitellogenin gene. Cells were maintained in Eagles Minimum Essential
130 Medium (EMEM) without phenol red, supplemented with kanamycin (60 mg/L) and 10%
131 (v/v) Foetal Bovine Serum (FBS), in an incubator under 5% CO₂ at 37°C. Upon reaching 75-
132 90% confluency, cells were subcultured twice (not more than 10 passages) prior to exposure
133 to the test chemicals.

134
135 For the cell-based AR-mediated bioassay, MDA-kb2 cells derived from the MDA-MB-453
136 breast cancer cell line and stably transfected with the murine mammalian tumor virus
137 (MMTV-luciferase.neo reporter gene construct, Wilson *et al.*, 2002) were obtained from the
138 ATCC (N^o.CRL-2713). Cells were routinely maintained in Leibowitz-15 (L-15) medium
139 supplemented with 10% FBS (v/v) in a humidified incubator at 37°C without additional CO₂.
140 Cells were sub-cultured when confluent over a maximum of 10 passages.

141 Luciferase Assays

142 The assay for (anti)estrogenic activity was performed in accordance with OECD test guideline
143 TG455 (OECD, 2016). Prior to experiments, HeLa-9903 cells were maintained in culture
144 medium supplemented with 10% (v/v) DCC-FBS (dextran-coated charcoal stripped serum)
145 for at least two media-changes. Cells were seeded at a density of 1×10⁴ cells per well in 100 µl
146 of phenol red free culture medium supplemented with 10% DCC-FBS in clear bottom white
147 luminometer 96-well plates and allowed to attach for 3 h.

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

154 After incubation, 50 μ l of a 3x dosing medium were added to the wells. The cells were
155 exposed to the dilution series of the tested chemicals (7 different concentrations of each
156 sample were tested), to the reference estrogen E2 or reference androgen DHT
157 (dihydrotestosterone), and to the solvent controls (0.1% v/v ethanol). DHT or E2 (1 nM) was
158 used as a positive control in the AR or ER agonist assay, respectively. DHT (0.25 nM) or E2
159 (0.025 nM) was used as a control in order to establish a baseline for co-exposure in order to
160 screen for AR or ER antagonism, respectively. After 24 h of exposure, the luciferase activity
161 was determined with Steady Glo assay reagent (Promega) as per the manufacturer's
162 instructions.

163 Viability

164 Cell viability was assessed using a resazurin-based assay performed before the determination
165 of luciferase activity. After the exposure time and following a 4-h (Hela-9903) or 5-h (MDA-
166 kb2) incubation period with 50 μ l/well of 4 μ g/mL resazurin (obtained from Sigma-Aldrich)
167 in PBS, cell proliferation was measured as relative fluorescence units (RFUs) resulting from
168 the reduction of non-fluorescent resazurin to the fluorescent product resorufin. Fluorescence
169 was measured at $\lambda_{\text{ex}} = 530$ nm and $\lambda_{\text{em}} = 590$ nm on a microplate reader. The average value
170 for the vehicle control wells was used as 100% and the results for each chemical were
171 calculated as a percentage. If the test substance showed more than 20% reduction of relative
172 cell viability, the compound was considered cytotoxic at the tested concentration.

173 Data analysis

174 Data points are representative of at least two independent experiments and three replicate
175 wells per data point in each experiment. All values were corrected for the mean of the
176 negative control and then related to the positive control, which was set to 100%. Average and
177 standard deviation of the replicates were calculated. A compound was considered positive if it
178 increased luminescence more than 10 per cent above the blank baseline in agonist mode, or
179 decreased luminescence by more than 20 per cent of the maximal signal in antagonist mode.
180

181 **H295R Steroidogenesis assay**

182 Cell culture and treatment

183 Cell culture conditions and media preparation were conducted in accordance with OECD test
184 guideline 456 (OECD, 2011). Human H295R adrenocortical carcinoma cells (ATCC CRL-
185 2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to
186 conducting steroidogenesis evaluation, batches of H295R cells were thawed and passed at
187 least 4 times. The maximum passage number used for steroidogenesis evaluation was 10.

188 Cells were routinely grown at 37°C under a 5% CO₂ atmosphere in 75 cm² culture flasks
189 containing 12 mL DMEM/Ham's F12 culture medium mixture (Gibco 11039021)
190 supplemented with 1% ITS+ premix (BD Bioscience; 354352) and 2.5% Nu-Serum (BD
191 Bioscience; 355100). For subculturing, the H295R cells were washed three times with PBS,
192 detached using trypsin/EDTA (0.25%/0.05% (v/v) in Hank's Balanced Salt Solution (HBSS))
193 and seeded in a 1:3 ratio. For testing, 1 mL cell suspension containing 3×10^5 cells was
194 seeded in each well of a 24-well plate. After 24 h (50-60% confluence), the medium was
195 refreshed and compounds dissolved in Ethanol (EtOH) were added. Exposures were
196 performed in triplicate with a final concentration of the solvent carrier of 0.1%. Positive
197 controls, 10 μ M forskolin (FOR) and 1 μ M prochloraz (PRO), were included on each plate.

198 Following 48 h of chemical treatment, media was removed, split into 2 vials of approximately
199 500 µl media each, and stored at -80°C prior to 17β-estradiol (E2) and testosterone (T)
200 quantification.

201 **Viability**

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

206 **Release of hormones**

207 Enzyme linked immunosorbent assays (ELISA) were used to directly quantify testosterone
208 and 17β-estradiol from aliquots of the medium. The ELISA kits (KGE010, KGE014) were
209 purchased from Bio-Techne (R&D systems Europe, France). According to the manufacturer's
210 data, the sensitivity of the testosterone assay was 0.030 ng/mL, and the intra and inter-assay
211 coefficients of variation were 4.0% and 5.6%, respectively. The sensitivity of the 17β-
212 estradiol assay was 4.84 pg/mL, and the intra- and inter-assay coefficients of variation were
213 6.0% and 7.1%, respectively. The absorbance was determined at a wavelength of 450 nm
214 using a Tecan (BioRad) microplate reader.

216 **Data analysis**

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

220 **Statistical analysis**

221 Obtained data were statistically analyzed using GraphPad Prism 6.00 (GraphPad Software
222 Incorporated, San Diego, CA, USA). Descriptive statistical characteristics (arithmetic mean,
223 minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-
224 way analysis of variance (ANOVA) and the Dunnett's multiple comparison test were used for
225 statistical evaluations. The level of significance was set at ***p < 0.001; **p < 0.01 and *p <
226 0.05.

228 **Docking studies**

229
230 The docking of the compounds under evaluation was performed using the crystallographic
231 coordinates 2iog for ERα (Dykstra *et al.*, 2007) and 2am9 for the AR (Pereira de Jesus-Tran *et al.*,
232 2006). It should be noted that the docking of the compounds was performed irrespective of
233 the pharmacological type of ligand crystallized with the receptor, which was only chosen on the
234 basis of the structural proximity of the crystallized ligand to the phthalate derivatives, using the
235 lowest possible resolution. As the receptors under scrutiny are nuclear receptors, there is an
236 adaptation of the receptor to the ligand which has not been investigated here and mostly prevents
237 conclusions being made on the pharmacological effect of the compounds on the basis of these
238 docking experiments. The co-crystallized ligand was extracted and used to define the binding
239 site as a sphere of 10 Å using GOLD (Jones *et al.*, 1997). The charges of the ligands and
240 receptors were assigned using the Gasteiger-Hückel method and the geometry of the each ligand
241 configuration was optimized with the maximin2 protocol of the Sybyl 6.9.1 molecular modeling
242 software. 30 solutions were generated for each compound and the number of poses of each
243 cluster gave a rough idea of the particular stability of the complex compared to the other clusters.
244 The final docking results were the most representative conformation of each cluster, in so far as
245 it was possible to define a sensible common placement. The central aromatic ring was the
246 primary structure taken into account to define a cluster. The long and flexible chains of the
247 compounds were mostly discounted at this stage, with the exception of the oxygen atoms.

248

249 **RESULTS**

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

251 **Transcriptional activity of human ER α**

252 Agonist or antagonist activities on hER α were measured in the absence or presence of E2.
253 Neither DEHT nor DEHP (supplementary data, figure 1) were active on hER α . The same
254 conclusion can be drawn for their corresponding mono-esters (in terms of agonist or
255 antagonist activities) using a large non- cytotoxic concentration range (up to 20 $\mu\text{g/mL}$), with
256 the exception of a weak antagonist activity of MEHP at the highest concentration but without
257 any cytotoxicity (figure 1).

258 With regard to the hydroxylated monoesters (figure 1), 5-OH-MEHT induced an agonist
259 effect on ER α at the highest concentration and a synergic concentration dependent effect in
260 the presence of E2 at 0.2 and up to 20 $\mu\text{g/mL}$, while 5-OH-MEHP was an antagonist at the
261 highest concentration without any cytotoxicity.

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

266 **Transcriptional activity of human AR**

268 Neither DEHT nor DEHP induced agonist or antagonist activities on AR over a large non-
269 cytotoxic concentration range (up to 20 $\mu\text{g/mL}$) (supplementary data, figure 1). The same
270 conclusion can be drawn with regard to the respective monoester and derived metabolites
271 (figure 2).

272 Concerning DEHT metabolites, 5-OH-MEHT was the only metabolite active on AR, with a
273 synergic concentration dependent effect (0.2 to 20 $\mu\text{g/mL}$) when cells were co-treated with
274 DHT. It should be noted that, under our experimental conditions, oxo-derived or carboxy-
275 derived monoester metabolites of both phthalates had no effect on AR transcriptional activity.

276 **Docking experiments**

278 The co-crystallized ligands of the investigated receptors (compound 11F for ER α and
279 testosterone for AR) were docked to validate the protocol. Both were very close to their
280 crystallographic position and a high majority of their 30 conformations were in this single
281 conformation. For AR, testosterone was less univocal in its binding mode than the other co-
282 crystallized molecule, as it could be placed in either its crystallographic position for about two
283 thirds of the poses, or exchange its extremities for the remaining third. Both conformations
284 were nonetheless strongly bound to the receptor via hydrogen bonds with Thr 877 and Arg
285 752. As expected, the second hydrogen bond formed between the hydroxyl group and Asn
286 705 was only found in its crystallographic position. For ER α , the crystallographic
287 conformation was found nearly exclusively, with 27 solutions out of 30. The three other
288 positions were mostly different orientations of the side chains, and in one case was an
289 inversion of the positions of the chains on each side of the amide linkage. The strong ionic
290 interaction with Asp 351 was maintained in all but two cases, while the hydrogen bonds with
291 Glu 353 and Arg 394 were only lost in a single case of chain inversion. These results agree
292 with those obtained by Delfosse *et al.*, 2014.

293 MEHT binds sufficiently with ER α , irrespective of the configuration of its branched ester
294 chain. The free acid interacts strongly with Arg 334, putting the benzene ring in a good
295 position for stacking with the nearby Phe 404. These are the two main interactions of the co-
296 crystallized ligand. The other end of the compound is less fixed and fluctuates in the wide
297 binding site, as the ester is much smaller than the original ligand. There is, therefore, a wide

298 range of conformations from a single common point of interaction rather than a well-defined
299 cluster, which may indicate that, apart from this single ionic interaction, MEHT is not able to
300 find a favorable binding environment. MEHT binds well with AR with about three-fourths of
301 the 30 solutions in a single cluster irrespective of the configuration. Again, the free acid forms
302 a strong interaction with Arg 752, and it is most probable that the nearby Phe 764 would
303 reorient slightly to form a stacking. Quite unsurprisingly, these are the main interactions of
304 testosterone. The ester chain is mostly rolled up toward the aromatic ring, in a conformation
305 that is not very energetically favorable for the interaction with the receptor (Figure 3).
306 5-OH-MEHT is placed in ER α in much the same way, with a conserved interaction with Arg
307 394 at the acid end. The hydroxyl group at the other end forms a near constant hydrogen bond
308 with Thr 347. It forms a fan that is bound by the acid and spreads at the ester end. On the
309 contrary, in AR, 5-OH-MEHT occupies only two positions, both very close to that of its
310 parent molecule and forming interactions at both extremities, as in ER α . While MEHT is able
311 to fit into the cavity of the androgen receptor and form an ionic interaction with Arg 752, it
312 lacks the rear side interaction formed by testosterone, and its long ester chain is not stabilized
313 in a particular conformation. On the contrary, the 5-hydroxylated congener, while assuming
314 the same position for the central block and the interaction with Arg 752, also keeps a
315 hydrogen bond at the rear, formed by its hydroxyl moiety either with Asn 752, Thr 877 or
316 both. Keeping in mind that the side chains of the residues were kept rigid, it is most probable
317 that the hydroxyl is binding to both residues in a mode very similar to that observed for the
318 natural ligand (Figure 4).

319

320 **Steroids synthesis assays**

321 The H295 steroidogenesis assay was performed with derived plasticizer metabolites in order
322 to detect substances which affect the production of E2 or testosterone and to understand if an
323 indirect mechanism, such as enzyme inhibition or induction, could occur. Figure 5 shows
324 statistical fold changes in hormone synthesis with the tested metabolites.

325 **Estradiol synthesis**

326 MEHT and MEHP were weakly active, with for MEHP a significant change between 2 and
327 40 $\mu\text{g}/\text{mL}$ and a 6-fold induction at 40 $\mu\text{g}/\text{mL}$ for MEHP.

328 Unfortunately MEHT was cytotoxic for the cells above 10 $\mu\text{g}/\text{mL}$. Hydroxylated derived
329 metabolites were more potent, with a concentration dependent increase in estradiol synthesis.
330 A maximum 12-fold increase was seen at 80 $\mu\text{g}/\text{mL}$. This significant effect started at a lower
331 concentration with 5-OH-MEHP (0.2 $\mu\text{g}/\text{mL}$) compared to 10 $\mu\text{g}/\text{mL}$ with 5-OH-MEHT.
332 Oxo-derived monoesters were the most active metabolites, with an induction starting at
333 10 $\mu\text{g}/\text{mL}$, and reaching a 14 to 16-fold induction at the highest concentration (80 $\mu\text{g}/\text{mL}$).
334 Carboxy-derived metabolites started to be significant agonists at 40 $\mu\text{g}/\text{mL}$, but with only a
335 weak effect (around 2-fold).

336 **Testosterone synthesis**

337 A similar and statistically significant decrease of testosterone (2-fold) was observed with OH-
338 derived metabolites. 5-OH-MEHT had an effect at a lower concentration (20 $\mu\text{g}/\text{mL}$) than 5-
339 OH-MEHP (40 $\mu\text{g}/\text{mL}$). It should be noted that at 10 $\mu\text{g}/\text{mL}$, MEHT also decreased
340 testosterone synthesis. Figure 3 shows the same tendency with the oxo-derived metabolites at
341 40 and 80 $\mu\text{g}/\text{mL}$, with a change observed at a lower oxo-MEHT concentration (10 $\mu\text{g}/\text{mL}$
342 instead of 40 $\mu\text{g}/\text{mL}$ with oxo-MEHP). 5-Cx-derived metabolites had no effect on
343 testosterone synthesis.

344

345

346 **DISCUSSION**

347 We used the reporter gene assays recommended by the OECD (level 2) to screen for
348 hormonal activities, with the corresponding absence or presence of the reference hormone,
349 and to test the agonist, antagonist and synergic properties of DEHP and DEHT and their
350 metabolites (Sataya *et al.*, 2012). The compounds were also docked to assess their binding
351 affinity with ER and AR.

352

353 **Impact on sexual hormones**

354 **DEHP and DEHP metabolites**

355 We found that, when a transcriptional effect was observed on ER α , it was mainly due to the
356 oxidized metabolites of DEHP, such as 5-OH-MEHP. Indeed, 5-OH-MEHP was an antagonist
357 at the highest concentration, with the effect being more pronounced when the cell line was
358 exposed to non-cytotoxic concentrations. Our data on the absence of estrogenic agonist
359 activity with DEHP agree with Shen *et al* (2009), Jobling *et al*, 1995 and Zacharewski *et al.*,
360 1998 who showed no ER transactivity and no capacity for DEHP to compete with E2-ER
361 binding *in vitro*. In contrast, Takeuchy *et al* (2005), who tested DEHP and its corresponding
362 monoester MEHP, observed a weak activation of hER α with 5.5 μ M DEHP in a transiently
363 transfected cell line (CHO K1 cells). Our data do not agree with the study by Engel *et al*
364 (2017) who demonstrated, using stably transfected human embryonic cells (HEK293), that
365 DEHP metabolites were never active up to a concentration of 100 μ M. Their data also proved
366 that the absence of an effect was not due to a lack of cellular uptake of the metabolites in their
367 model. Furthermore, Engel *et al.* only noted an inhibition with DEHP when using a co-
368 treatment of E2 at a very high concentration (100 μ M). More recently, Yang *et al* (2018) used
369 nanoMolar concentrations to demonstrate that MEHP can trigger the proliferation of cervical
370 cancer cells *via* the activation of the G-protein coupled estrogen receptor (GPER) rather than
371 ER α . These discrepancies in the literature data could be due to the different cell lines used and
372 to differences in experimental setups, such as the reporter gene constructs (Jones *et al.*, 1999).
373 It is important to note that DEHP has to be metabolized to MEHP and its derived metabolites
374 in order to be bioactive both *in vivo* or *in vitro* (Gray *et al.*, 1986; Koch *et al.*, 2005;
375 Chauvigne *et al.*, 2009) and the differences observed in *in vitro* models may also be due to the
376 presence or absence of enzymatic activities in the cell lines used. Moreover, DEHP is known
377 to be greatly metabolized *in vivo* after oral exposure, suggesting a low probability of a direct
378 tissue exposure to the parent substances. Furthermore, long chain phthalates are converted to
379 oxidized metabolites by hepatic enzymes, then at the molecular level the adverse effects of
380 phthalates may be in fact due to effects mediated by phthalates metabolites (Kluwe, 1982).
381 Concerning the transcriptional activity of AR, our data agree with those found by Engel *et al.*
382 (2017) where the authors did not observe any agonist effect on AR in the presence of DEHP
383 or its derived metabolites up to a concentration of 25 μ M. However, the same authors
384 observed an AR inhibition at 50 μ M, a two-fold higher concentration compared to our study.
385 This could be due to some cytotoxic response not displayed in the cytotoxicity assay used. A
386 crucial parameter when performing *in vitro* tests is the use of the proper concentrations in
387 order to avoid false positives data. It is essential to test substances at non-cytotoxic
388 concentrations, especially when an antagonist effect is observed.

389 In the study by Shen *et al.* (2009), both mixed androgenic and anti-androgenic effects were
390 observed with DEHP on the same cell line (MDA-kb2), with an EC50 (concentration which
391 gives a half-maximum response) or IC50 (concentration which inhibits the response by half)
392 exceeding 10^{-4} M, which is a very high concentration. Araki *et al.* (2005) also demonstrated
393 an antagonist effect of DEHP on AR. However, this was not seen by Kruger *et al.* (2008).
394 Again the sensitivity of the cell line and consequent variant sensitivity could be at the origin
395 of the differences between the data. It should be noted that the cell line we used was probably

396 not able to metabolize DEHP until ultimate active metabolites such as hydroxylated/oxidized
397 metabolites as effect in reporter gene was observed only with DEHP metabolites.

398
399 Using the steroidogenesis synthesis assay, which gives information on another mode of ED
400 action (not genomic), we have shown that MEHP is also active at 40 µg/mL, with an increase
401 of E2 and a decrease of testosterone. The effect was even more pronounced with its derived
402 hydroxylated monoester (5-OH and 5-oxo-MEHP). The effects observed at the concentration
403 range used in this study agree with those seen by Mankidy *et al.* (2013), who demonstrated
404 that hormone synthesis was affected by DEHP concentrations of 10 µg/mL, resulting in a
405 greater production of E2 (4-fold) and a concurrent reduction of testosterone concentration.
406 However, they did not test DEHP metabolites. In our study, the induction observed with
407 DEHP metabolites was even more pronounced (up to 15-fold). Interestingly, Desdoits-
408 Lethimonier *et al.* (2012) used human testis explants to demonstrate that phthalates affect
409 human testis steroidogenesis but that DEHP has to be metabolized to MEHP to be bioactive.
410 MEHP metabolites, including 5-OH-MEHP, also display anti-androgenic activities.
411 Production of all testosterone precursors of the 4 and 5 pathways was inhibited by MEHP.
412 Using NCI-H295 cells over concentration ranges found in men in recent epidemiological
413 studies, DEHP and MEHP have been shown to also reduce testosterone production *in vitro*
414 after 48 h, associating phthalate exposure with the impairment of the androgynous status.

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417 **DEHT and DEHT metabolites**

418 5-OH-MEHT showed an agonist effect at the highest concentration and, interestingly, a
419 synergism in the presence of E2, with a concentration dependency effect on ER (from 0.2 up
420 to 20 µg/mL). However, when expressed as Eq/L E2 (supplementary data, figure 2), the
421 agonist effect of 5-OH-MEHT is weak, with a relative potency 3.5×10^{-6} fold lower than E2.

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

426 To date, co-stimulation by 5-OH-MEHT and E2 or DHT has never been observed in *in vitro*
427 studies. With regard to steroid synthesis, estrogen synthesis could increase up to 16-fold and
428 Cx derived metabolites had a very weak effect. In terms of estrogen synthesis, the rank order
429 potency was as follows: MEHT < corresponding OH metabolite < corresponding OXO
430 metabolite. With regard to testosterone levels, a weak but significant decrease was noted with
431 no difference between the metabolites.

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

437

438 **Docking**

439 Efforts were limited to the estrogen receptor α due to the fact that ER β has a low number of
440 different residues in its binding site, the most notable of which is a valine in place of a leucine
441 at position 487, at the entry of the pocket. However, the overall difference is a slight
442 movement of the C-terminal loop-helix-loop assembly, resulting in a slightly different spatial
443 arrangement of this residue. These observations are consistent with those of Defosse *et al.*
444 2014. The phthalate metabolites were further investigated due to their potential for hydrogen
445 bond formation with the free acid group. It should be kept in mind that docking only

446 investigates the direct interactions with the receptor, without taking into account accessibility
447 to the binding site. In particular, the high flexibility of MEHT's long ester chain may mask
448 the free acid or get entangled in the entry of the pocket and inhibit its binding. Both
449 phenomena are beyond the scope of the *in silico* tool employed here, and may thus explain the
450 observed differences between the docking results and the biological results for MEHT. The
451 monoesters, MEHT and MEHP, behave differently. MEHT strongly binds to the arginine of
452 both receptors but with no anchorage; its second ester adopts a large number of possible
453 conformations in the pocket, which may relate to a poor fit for the binding sites despite the
454 ionic bond with the arginine. MEHP has no interaction with ER; the free acid is clearly being
455 screened by the large ester chain. The same is true for AR. As a result, this metabolite has
456 apparently a very low possibility of being a ligand for ER α or AR. Among the oxidized
457 metabolites, the 5-OH-MEHT is able to bind quite well to both receptors, with well-kept
458 interactions at both the free acid, pointing toward an arginine, and the ester chain hydroxyl
459 group, which readily forms hydrogen bonds. It also fits into both receptors in a single
460 conformation. Inversely, the other metabolites of this series do not show the same binding
461 capacity and have several different conformations (data not shown). The MEHP congener
462 behaves differently, with 4 conformations in ER and 2 in AR. The 5-oxo-MEHT and -MEHP
463 can both bind to the two studied receptors in two or more different conformations, even
464 lacking any full interaction for the latter in AR. The addition of another acid group on the
465 ester chain is not optimal. 5-cx-MEHT is able to bind to the arginine of both receptors but not
466 in a well-defined conformation. There is a slightly better docking with AR than ER. 5-cx-
467 MEHP forms a large number of small size clusters in the two receptors studied, indicating an
468 unstable docking and therefore hinting at a low potential affinity, if any. Overall, the best
469 binder is clearly 5-OH-MEHT, which readily binds to ER α and AR in a mode very similar to
470 that of the natural ligands.

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473 **In vitro data versus biomonitoring values**

474 In neonatal intensive care units (NICU), neonates are particularly exposed to plasticizers
475 released from PVC medical devices. Biomonitoring studies have allowed the measurement of
476 the urinary levels of DEHP metabolites in neonates hospitalized in these units. Strommen *et*
477 *al.* (2016) showed that the urinary concentration of 5-oxo and 5-OH-MEHP could reach
478 1 $\mu\text{g/mL}$. The cohort studied by Demirel *et al.* (2016) presented even higher values with
479 maximum limits in the order of 5 $\mu\text{g/mL}$ for these two oxidized metabolites. Our study shows
480 that at these concentrations there is an antagonistic effect on estrogen receptors. Moreover, the
481 effects of 5-OH-MEHP on the synthesis of estradiol are observed from 0.2 $\mu\text{g/mL}$, which is
482 close to the median concentration observed in these newborns. In intensive care,
483 extracorporeal membrane oxygenation (ECMO) is one of the primary medical situations that
484 exposes patients to DEHP for several days or even weeks. In particular, a study in adults has
485 shown that patients on ECMO had urinary 5-OH-MEHP concentrations of more than 5 $\mu\text{g/mL}$
486 and blood concentrations of more than 0.8 $\mu\text{g/mL}$ (Huygh *et al.*, 2015).

487 Concerning DEHT, there is currently no biomonitoring study performed in a medical
488 environment while this plasticizer was identified in medical devices used in pediatric
489 intensive care units (Malarvannan *et al.*, 2019). The study by Lessmann *et al.* (2017) gives
490 urinary concentrations of DEHT metabolites in a population of children aged 4 to 17 years.
491 The maximum levels observed were 0.06 $\mu\text{g/mL}$ for 5-oxo-MEHT, 0.18 $\mu\text{g/mL}$ for 5-OH-
492 MEHT and 0.34 $\mu\text{g/mL}$ for 5-cx-MEHT. Even if the median concentration of 5-OH-MEHT
493 was much lower (0.045 $\mu\text{g/mL}$), the maximum concentration observed corresponds to the
494 concentration showing the first synergistic effect with E2 on hER alpha and agonist effects on
495 AR receptors. Therefore, the question arises regarding the level of exposure of patients using

496 medical devices containing DEHT and the potential endocrine disrupting effect. The ongoing
497 biomonitoring study under the Armed-Neo project should provide us with the necessary
498 elements to further assess the risk. Experiments are on-going to check hormonal activities of
499 neonatal urine extracts.

500 In this study, the biological effects of single tested metabolites appear to be weak and
501 far less potent than natural hormones. However, an observed synergic effect at low levels
502 must be taken into account and not be considered as insignificant as the human population is
503 continuously exposed to complex mixtures of chemicals in the presence of natural hormones
504 (Ghisary *et al.*, 2009; Kortenkamp *et al.*, 1998). Therefore *in vitro* experiments are important
505 in order to monitor the effects of metabolites and can be relevant to *in vivo* situations, at least
506 for people with higher exposure levels, such as neonates exposed to medical devices in
507 neonatal intensive care units (Calafat *et al.*, 2004).

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

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521 CONCLUSION

522 This study presents biological hormonal activities of the derived metabolites of DEHP and
523 DEHT, involving carboxy-metabolites, and demonstrates, at a molecular level, the different
524 mechanisms of action of phthalate metabolites compared to the respective parent molecules,
525 as well as the differences between DEHP and DEHT. The effects observed were more
526 important for steroidogenesis synthesis, suggesting an indirect mode of action for DEHP or
527 DEHT metabolites. This is the first time that a co-stimulation of hER α and hAR has been
528 observed with 5-OH-MEHT. *In silico* results for ER α and AR are in good agreement with the
529 observed biological results for 5-OH-MEHT and MEHP, while the docking of MEHT is less
530 conclusive. This compound maintains an interaction with the arginines but lacks other
531 interactions, and its ester is unfavorably constrained to fit into the pockets.

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

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

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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).

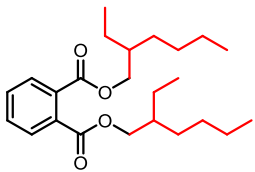
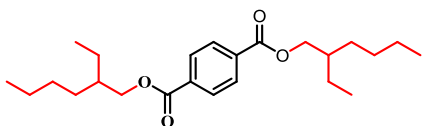
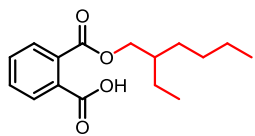
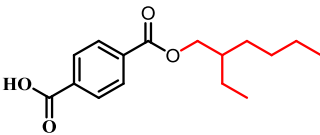
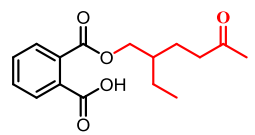
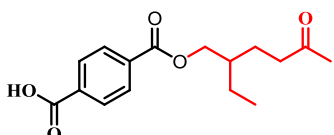
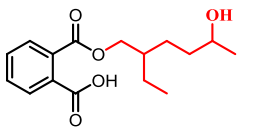
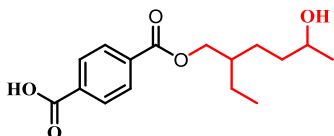
The dotted lines (.....) highlight 10% 1nM DHT normalized RTA (Relative Transcriptional Activity) in the agonist mode or 80% 0.25nM DHT normalized RTA in the antagonist mode, as a threshold for categorizing positive data.

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

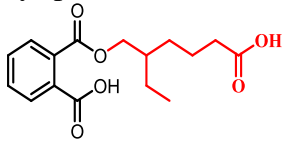
Figure 4: Docking of 5-OH-MEHT in ER α (left panel, reference ligand in yellow) and AR (right panel, reference ligand in orange)

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.

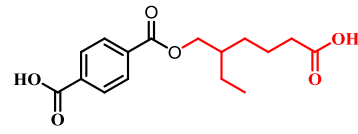
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<p>DEHP and its metabolites</p>	<p>DEHT and its metabolites</p>
<p>DEHP: di-(2-ethylhexyl)phthalate</p> 	<p>DEHT or DEHTP: di-(2-ethylhexyl)terephthalate</p> 
<p>MEHP: mono-(2-ethylhexyl)phthalate</p> 	<p>MEHT or MEHTP: mono-(2-ethylhexyl)terephthalate</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



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

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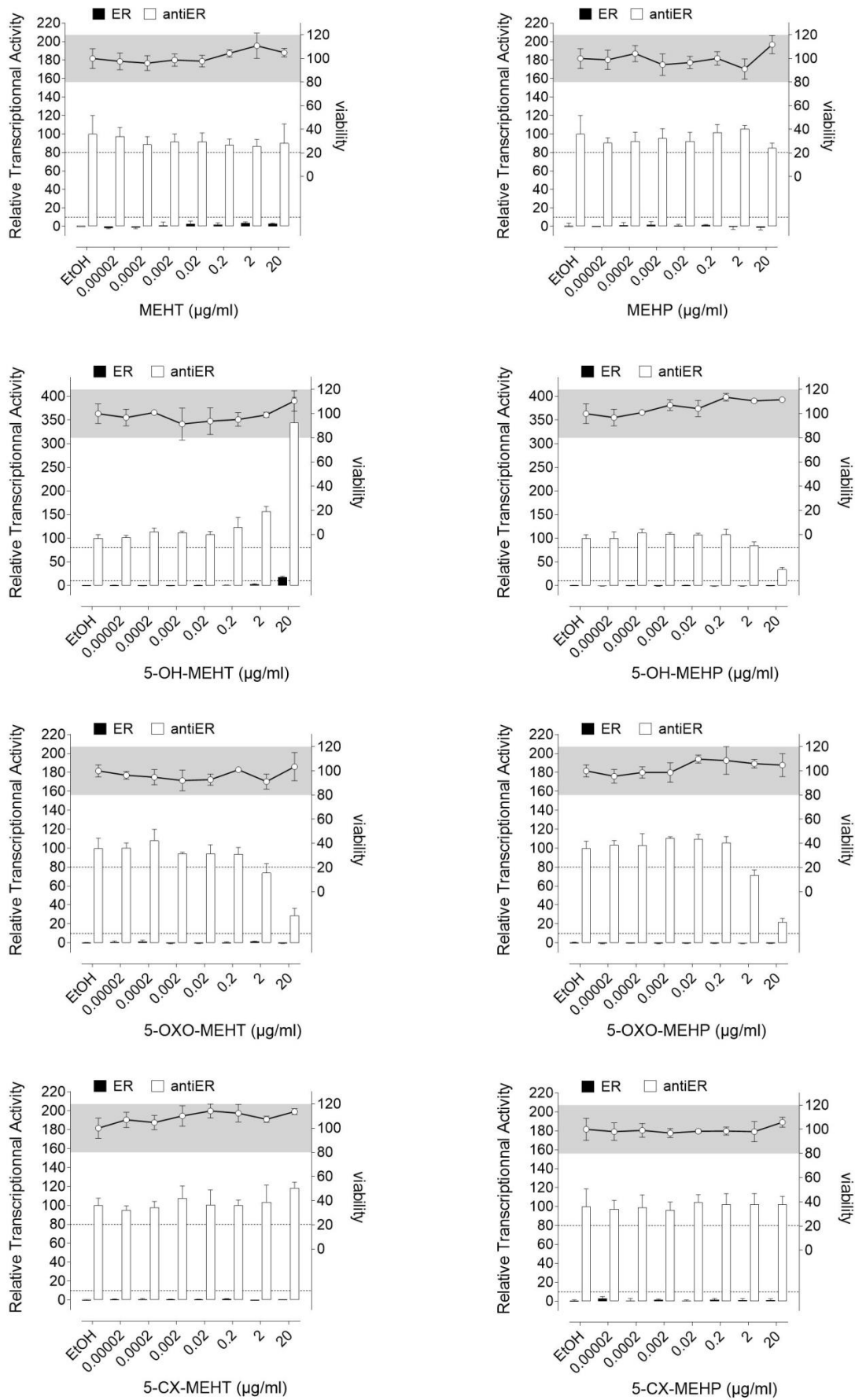
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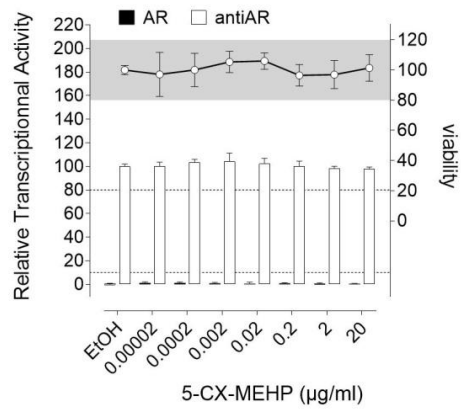
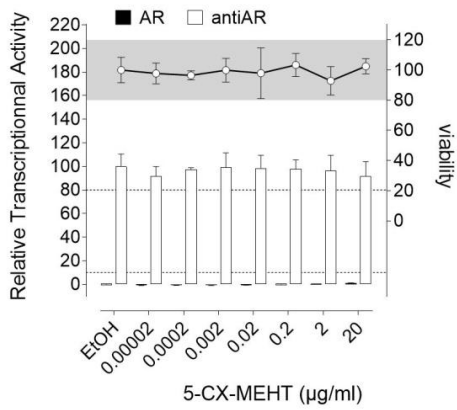
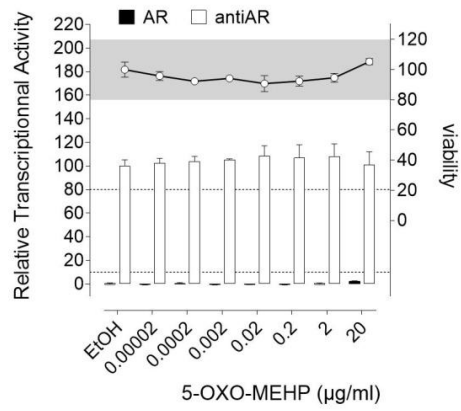
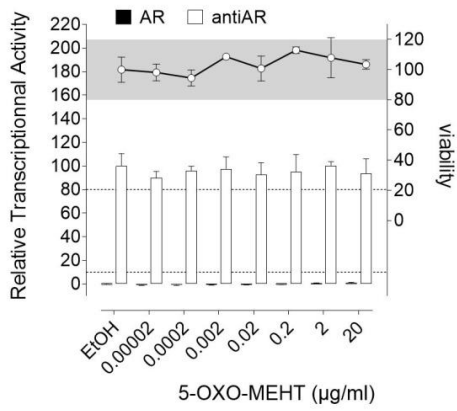
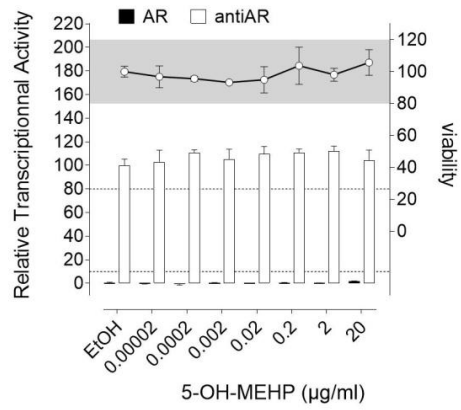
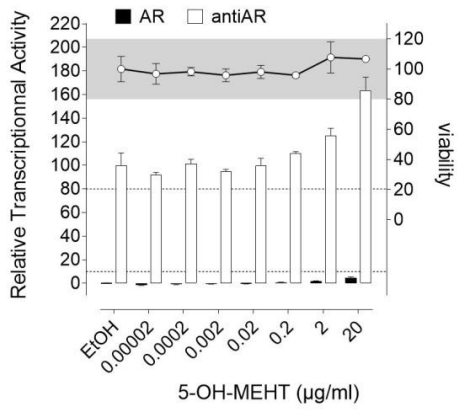
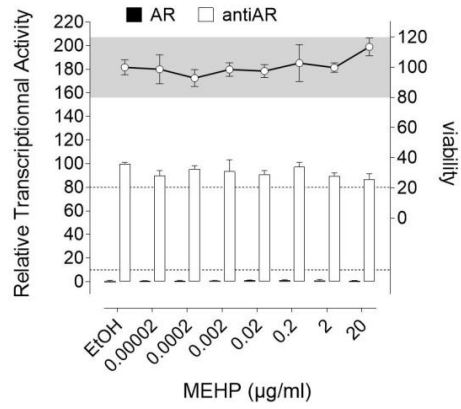
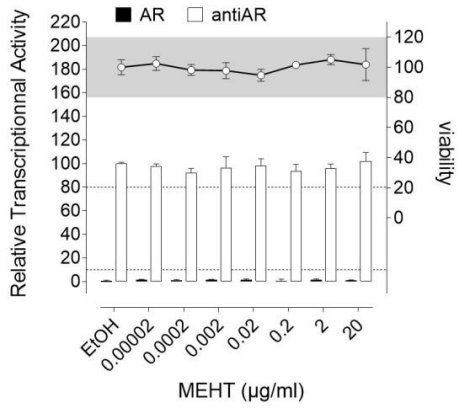
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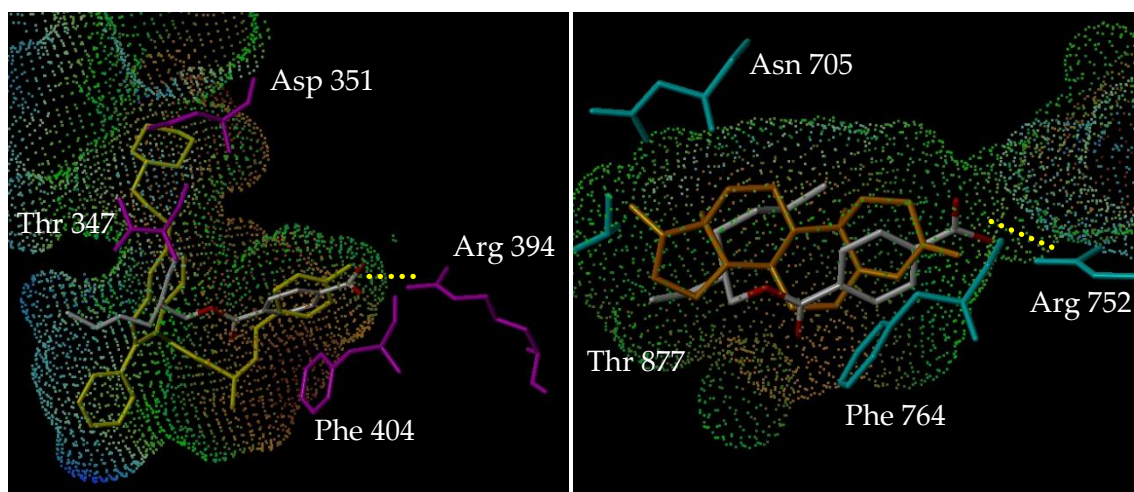
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828 Figure 1



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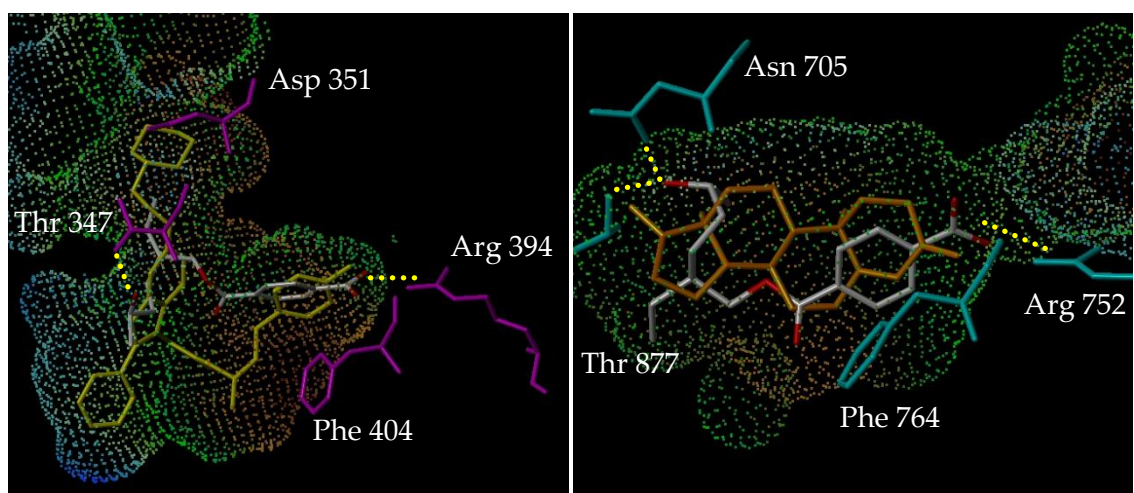
830 Figure 2

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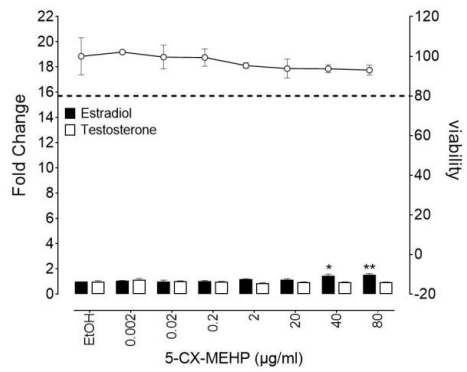
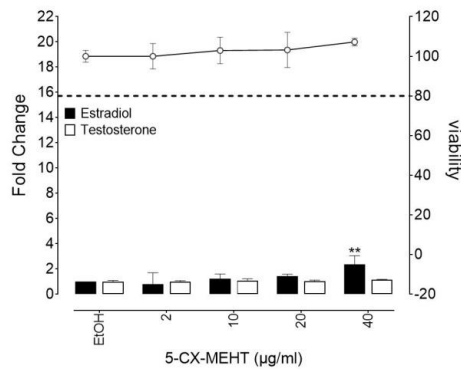
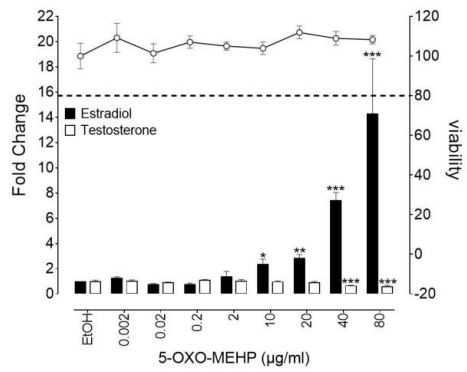
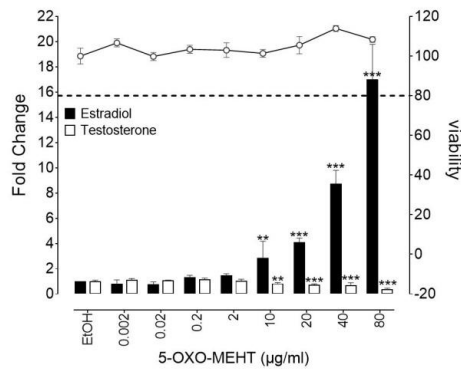
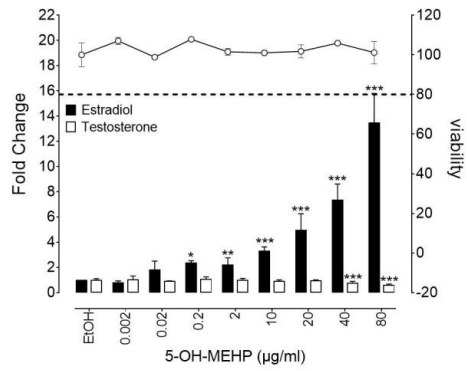
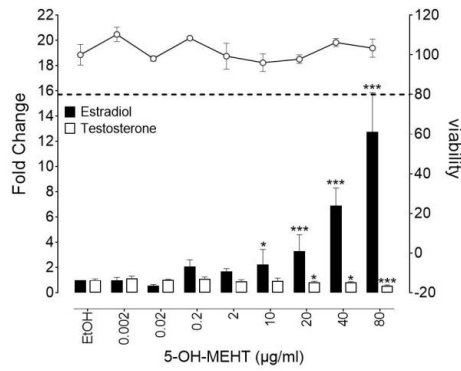
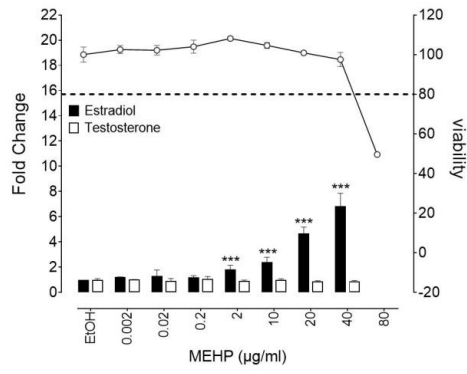
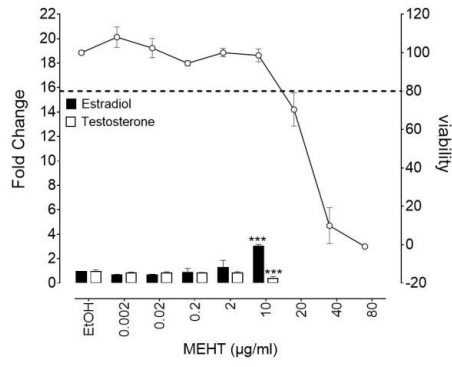
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Figure 3



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Figure 4



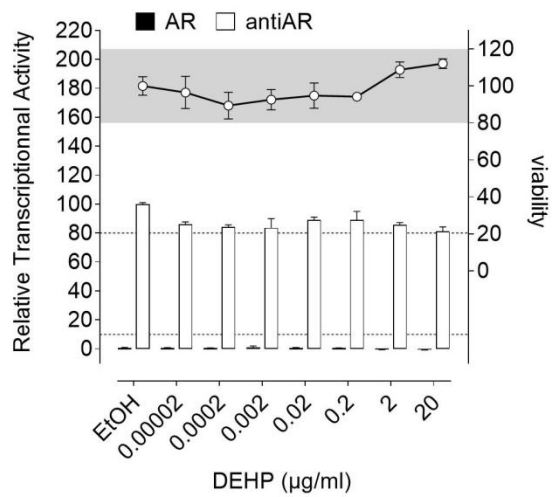
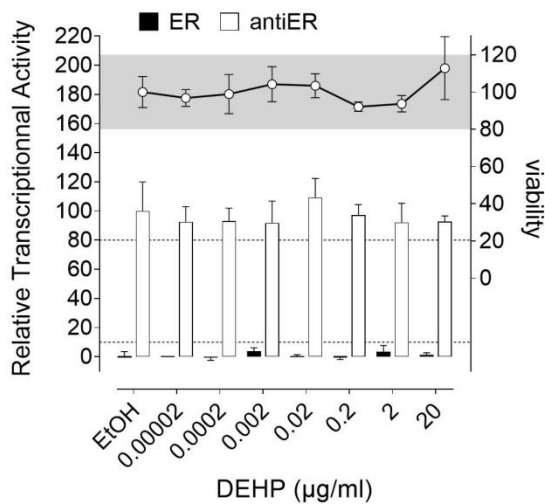
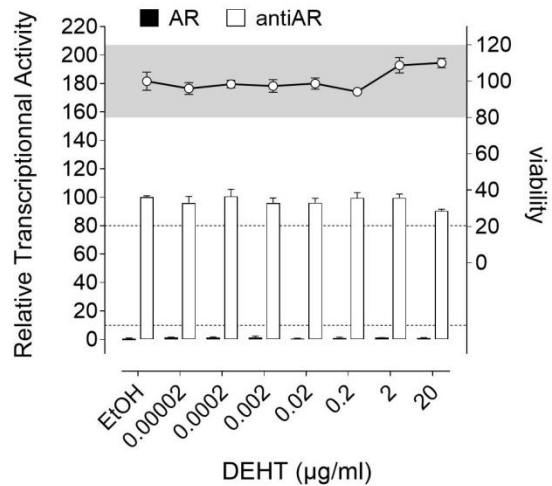
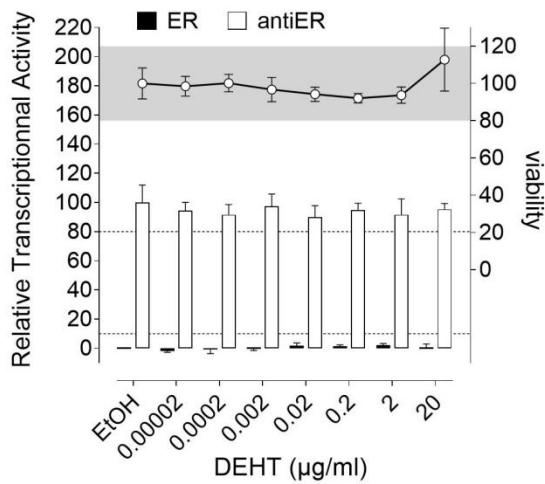
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850 Figure 5

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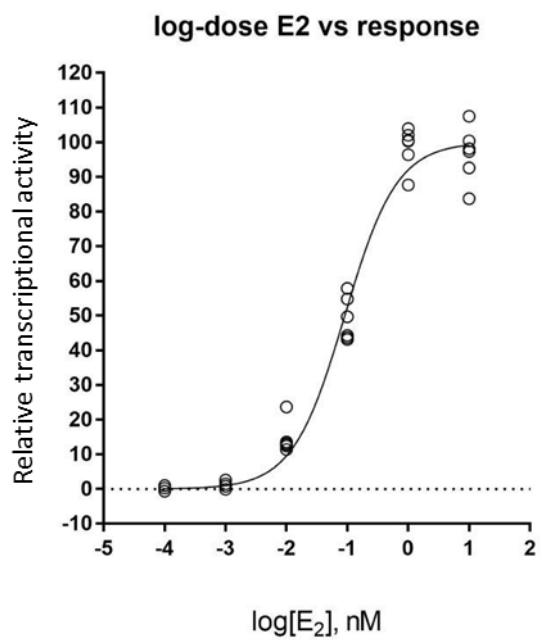
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857 **Figure 1s:** Estrogen or androgen receptor agonism and antagonism with DEHT and DEHP in Hela-9903
858 or MDA-kb2 transcriptional activation assays, respectively. Cell viability was evaluated by the
859 resazurin assay. Data represents mean \pm SD of two independent experiments (performed in
860 triplicate).

861 The dotted lines (.....) highlight 10% 1nM E2 or 1nM DHT normalized RTA (Relative Transcriptional
862 Activity) in the agonist mode or 80% 0.025nM E2 or 0.25nM DHT normalized RTA in the antagonist
863 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