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1 **Human and zebrafish nuclear progesterone receptors are differently activated by**
2 **progestins**

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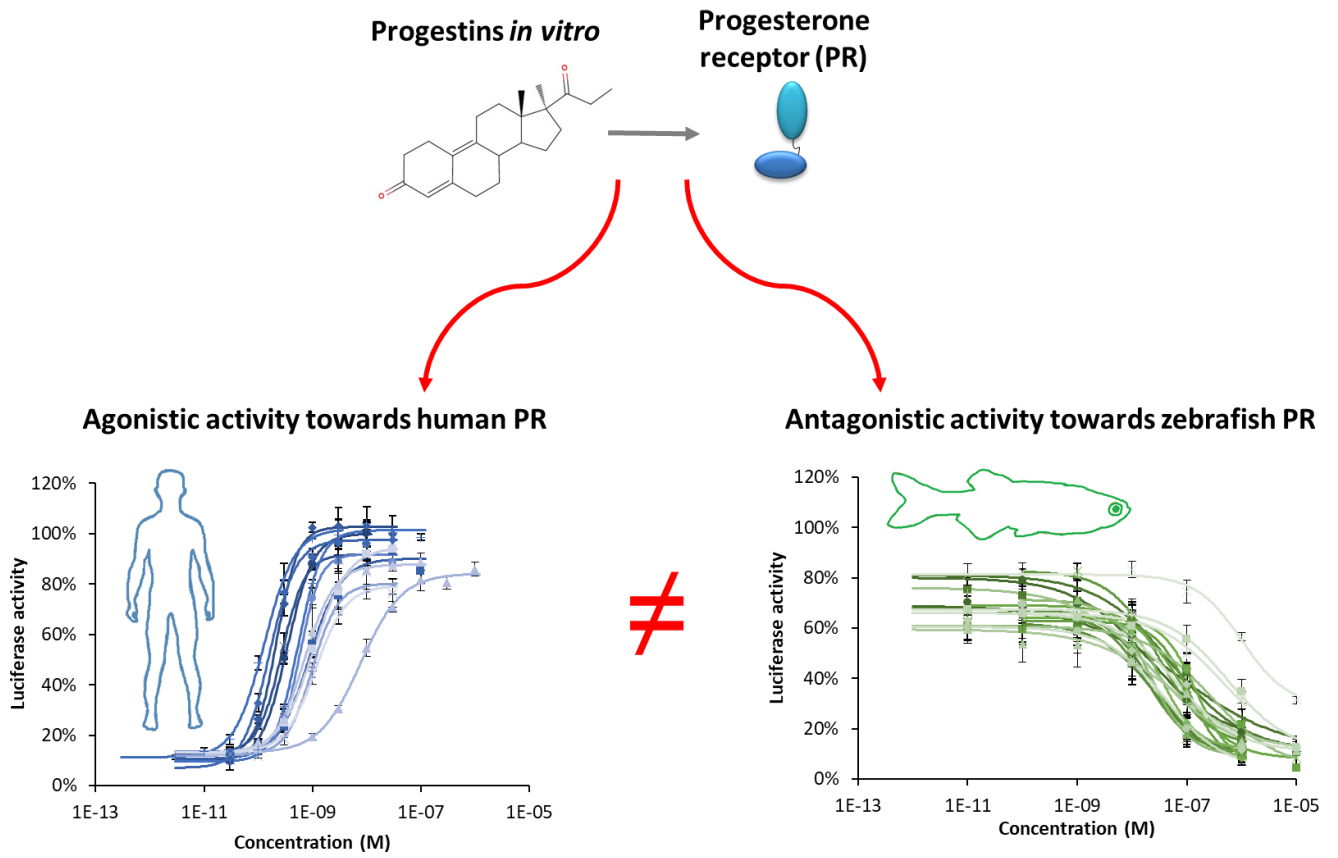
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- 21 *Keywords: synthetic progestins; 17 α ,20 β -dihydroxy-4-pregnen-3-one; human and zebrafish*
- 22 *nuclear progesterone receptor; luciferase reporter cell lines.*

23 **Graphical abstract**



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25

26 **Abstract**

27 Environmental risk of natural and synthetic ligands of the nuclear progesterone receptor
28 (nPR) has been pointed out, however there is still a lack of mechanistic information regarding
29 their ability to interact with nuclear PR in aquatic species. To identify possible interspecies
30 differences, we assessed *in vitro* the ability of a broad range of progestins to transactivate
31 zebrafish (zf) and human (h) PRs, by using two established reporter cell lines, U2OS-zfPR
32 and HELN-hPR, respectively. Reference ligands highlighted some differences between the
33 two receptors. The reference human agonist ligands promegestone (R5020) and progesterone
34 (P4) induced luciferase activity in both cell lines in a concentration-dependent manner
35 whereas the natural zebrafish progestin $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) activated
36 zfPR but not hPR. The potent human PR antagonist mifepristone (RU486) blocked PR-
37 induced luciferase in both cell models but with different potencies. In addition, a large set of
38 twenty-two synthetic progestins were screened on the two cell lines. Interestingly, all of the
39 tested compounds activated hPR in the HELN-hPR cell line whereas the majority of them
40 acted as zfPR antagonists in U2OS-zfPR. Such zfPR-specific response was further confirmed
41 in zebrafish liver cells (ZFL). This study provides novel information regarding the activity of
42 a large set of progestins on human and zebrafish PR and highlights major interspecies
43 differences in their activity, which may result in differential effects of progestins between fish
44 and humans.

45 **1. Introduction**

46 Progestagenic sex steroids, also called progestins or progestogens, play crucial roles in
47 development and reproduction in vertebrates, including teleosts. Progesterone (P4) is an
48 endogenous biologically active progestin in mammals and fish. However, in most teleost
49 species including zebrafish, other endogenous active progestins such as $17\alpha,20\beta$ -dihydroxy-
50 4-pregnen-3-one (DHP) and $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one (20β -S) ¹ are also
51 synthesized, P4 being considered as an intermediate. Progestins can exert their biological
52 activity by acting through the nuclear progesterone receptor (nPR), a ligand-activated
53 transcription factor that belongs to the steroid hormone receptor superfamily (Grim et al.,
54 2016). In both mammals and fish, PRs are widely distributed in different reproductive and
55 non-reproductive tissues, suggesting the widespread physiological effects of progestagenic
56 hormones ². Indeed, the central role of nPR in mediating progesterone-regulated reproductive
57 functions in diverse vertebrates, in both males and females, has been demonstrated in
58 different vertebrate models, notably by using PR knockout (PRKO) mice ³ and more recently
59 PRKO zebrafish ^{4,5}.

60 During the last few years, several studies have documented the presence of progesterone and
61 synthetic progestins in effluents of wastewater treatment plants (WWTPs) and surface waters,
62 related to their production and consumption as pharmaceuticals in a diversity of medical
63 applications such as contraception ⁶⁻¹⁰. Laboratory studies on aquatic models revealed the
64 effects of natural and synthetic progestins on fish reproduction at environmentally relevant
65 concentrations (*i.e.* in the ng/L range), thereby highlighting the risks they pose on aquatic
66 species and aquatic ecosystems ^{11,12}. Important steps forward have been recently made
67 regarding the ability of some synthetic progestins to interfere with key endocrine molecular
68 and cellular targets, such as human androgen receptor (AR)- and estrogen receptor (ER)-
69 mediated signaling pathways and steroidogenesis, which could, at least partly, explain their

70 reproductive physiological effects *in vivo*^{13–18}. However, it is noticeable that much less
71 information is available regarding the ability of progesterone and synthetic progestins to
72 interact with fish nPR^{19,20} and subsequently alter nPR-regulated physiological processes².
73 Because cross-species variations in nuclear receptor (NR) sequences can significantly
74 influence their structure, functioning and response to chemical ligands²¹, there is a need for
75 characterizing the ability of environmentally occurring progestins to interact with fish nPR to
76 assess their environmental hazard to fish species.

77 The aim of this study was therefore i) to gain knowledge on the ability of a large set of (anti)
78 progestagenic compounds to transactivate the zebrafish nPR and ii) to compare these results
79 with their activity towards the human hPR (hPR, isoform B) to identify possible interspecies
80 differences. To this end, we first developed a novel *in vitro* luciferase-reporter-gene cell line
81 expressing the zebrafish PR, namely U2OS-zfPR. The activity of four natural or synthetic
82 PR-ligands used as reference substances, *i.e.* P4, DHP, promegestone (R5020), mifepristone
83 (RU486), as well as twenty-two pharmaceutical progestins representative of different
84 chemical classes were assessed in this newly established U2OS-zfPR cell line. All the
85 substances were also tested in an already established human cell line HELN-hPRB expressing
86 hPRB²² for comparative purpose.

87 **2. Material and Methods**

88 **2.1. *Chemicals and materials***

89 Materials for cell culture are from Life Technologies (Cergy-Pontoise, France). Luciferin
90 (sodium salt) was purchased from Promega (Charbonnières, France). Chemical substances
91 used in this study are presented in Table 1. 17 β -estradiol (E2), testosterone (T), 11-
92 ketotestosterone (11-KT), pregnenolone (P5), progesterone (P4), dydrogesterone (DYD),
93 medroxyprogesterone (MEP), medroxyprogesterone acetate (MPA), megestrol acetate
94 (MGA), chlormadinone acetate (CMA), cyproterone acetate (CPA), promegestone (R5020),
95 nesterone (NES), nomegestrol acetate (NGA), ethisterone (ETH), ethynodiol diacetate (EDA),
96 lynestrenol (LYN), norethindrone acetate (NEA), norethindrone (NET), tibolone (TIB),
97 desogestrel (DSG), etonogestrel (ENG), gestodene (GES), levonorgestrel (LNG),
98 norgestimate (NTE), norgestrel (NGL), drospirenone (DRO), mifepristone (RU486), 17 α ,20 β -
99 dihydroxy-4-pregnen-3-one (DHP), spironolactone (SPIRO) and canrenone (CAN) were
100 obtained from Sigma-Aldrich Chemical Co. (St.Louis, MO). Gestonorone (GRN) was
101 obtained from Toronto Research Chemicals (Toronto, Canada). Stock solutions of chemicals
102 were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. Fresh dilutions of test
103 chemicals in medium were prepared before each experiment.

104 **2.2. *Reporter gene cell lines HELN-hPR and U2OS-zfPR***

105 To assess the ability of progestins to transactivate the human PR (hPR, isoform B) or the
106 zebrafish PR (zfPR), two human luciferase-reporter-gene cell lines were established. The
107 HELN-hPR cell line has already been described²². Briefly, HELN-hPR cells were obtained
108 by stably transfection of HELN (HeLa ERE-luciferase-neomycin) cells²³ by a plasmid
109 expressing hPR where the DNA binding domain was replaced by the hER α DNA binding
110 domain (pSG5-hPR(ER α DBD)-puromycin). The U2OS-zfPR cell line was obtained by stable
111 cotransfection of the plasmid ERE-Luciferase and an expression plasmid of zfPR with the

112 hER α DNA binding domain ((pSG5-hPR(ER α DBD)-puromycin, supporting Information,
113 Figure S1). Selection of HELN-hPR cells was made with 0.5 μ g/mL puromycin and 1 mg/L
114 G418 and selection of USO2-zfPR cells was made with 0.5 μ g/mL puromycin..

115 Cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol
116 red, 1 g/L glucose, 5% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μ g/mL
117 of streptomycin. Culture medium was supplemented with 0.5 μ g/mL puromycin and 1 mg/mL
118 geneticin for HELN-hPR and 0.5 μ g/mL puromycin for U20S-zfPR cells. Cells were cultured
119 in a 5% CO₂ humidified atmosphere at 37°C. Exposure was made in phenol red-free DMEM
120 supplemented with 2% dextran-coated charcoal FBS (DCC), 100 units/mL of penicillin, and
121 100 μ g/mL of streptomycin.

122 **2.3. Reporter gene cell line ZFL-PR**

123 Zebrafish liver cells ZFL cells have been stably cotransfected by the luciferase plasmid ARE₆-
124 collagenase-Luciferase-hygromycine) and the zfPR expressing plasmid pSG5-zfPR-
125 puromycin and grown in presence of 0.5 μ g/mL puromycin and 0.25 mg/mL hygromycin
126 during 3 weeks. Among the different resistant clones, the one which expresses luciferase in
127 the most inductible manner (4 fold) was selected and called ZFL-PR.

128 The cells were grown in LDF medium (50% Leibovitz 15 culture medium L15, 35% DMEM
129 high glucose, and 15% Ham's-F12 medium) with 0.15 g/L sodium bicarbonate, 15 mM 4-(2-
130 hydroxy- ethyl)-1-piperazineethanesulfonic acid (HEPES), 0.01 mg/mL insulin, 50 ng/mL
131 epidermal growth factor (EGF), 5% fetal bovine serum (FBS), and 50 U/mL penicillin and
132 streptomycin antibiotics and supplemented with 5% vol/vol FBS. Cells were cultured in a 5%
133 CO₂ humidified atmosphere at 28°C. Exposure was made in LDF supplemented with 2%
134 dextran-coated charcoal FBS (DCC), 100 units/mL of penicillin, and 100 μ g/mL of
135 streptomycin.

136 **2.4. *In vitro* transcriptional activation bioassays**

137 HELN-hPR and U2OS-zfPR cells were seeded in 96-wells white opaque culture plates
138 (Greiner CellStar; Dutscher, Brumath, France) at a density of 7×10^4 cells per well in 100 μ L
139 culture medium. After 24h, cells were exposed by adding serial dilutions of compounds or
140 solvent control (DMSO; final concentration 0.1% v/v) in triplicates and incubated for 16h. For
141 antagonist activity assessment, cells were exposed to different concentrations of the tested
142 compounds and to a concentration of the reference compound that yield 50%-80% of the
143 maximal response, *i.e.* R5020 at 10^{-9} M for HELN-hPR and DHP at 3×10^{-9} M for U2OS-
144 zfPR. After 16h, medium was removed and replaced with 50 μ L/well of medium containing
145 0.3 mM luciferin. Luminescence signal was monitored in living cells for 2 s per well (BioTek
146 Synergy H4 Hybrid Microplate Reader). Results were expressed as the percentage of the
147 maximum luciferase activity induced by R5020 at 3×10^{-8} M for HELN-hPRB cells and DHP
148 at 3×10^{-8} M for U2OS-zfPR cells. Concentration–response curves were obtained with the Hill
149 equation model using the Regtox 7.0.6 Microsoft Excel TM macro
150 (http://www.normalesup.org/~vindimian/fr_index.html), from which effective (EC) and
151 inhibitory concentrations (IC) were derived. For a given chemical, EC₅₀ is defined as the
152 concentration inducing 50% of its maximal effect and IC₅₀ represents the concentration
153 required for 50% inhibition. Relative Effect Potencies (REPs) were calculated by dividing the
154 EC₅₀ of the reference compound by that of the tested compound.

155 Chemicals were tested in at least two independent experiments when inactive (*i.e.* maximal
156 luciferase induction or inhibition below 20%) and three independent experiments when active
157 (*i.e.* luciferase induction or inhibition above 20 %). Data are expressed as the percentage of
158 the maximum luciferase activity induced by 3×10^{-8} M R5020 or 3×10^{-8} M DHP \pm standard
159 deviation (SD).

160 **3. Results and discussion**

161 **3.1. *hPR and zfPR transactivation by PR, ER and AR reference ligands***

162 P4, R5020, RU486, and DHP were first tested to characterize the response of the established
163 cell lines to reference PR ligands. Some marked differences were revealed in terms of
164 transactivation efficacies and potencies depending of the cell model (Figure 1, Table 2).

165 In HELN-hPR, R5020 was the most efficient ligand with a full agonist response profile and a
166 low EC₅₀ (in the sub-nM range) while it only partially induced luciferase activity in U2OS-
167 zfPR. P4 was active in both cell models but was less efficient to induce luciferase activity as
168 compared to R5020 in HELN-hPR and to DHP in U2OS-zfPR. For DHP, a major difference
169 was noticed as it was the most active and efficient steroid in U2OS-zfPR but was unable to
170 induce any luciferase activity in HELN-hPR (Figures 1A, 1B; Table 2). The higher efficacy of
171 DHP to transactivate fish PR as compared to P4 confirms previous *in vitro* data using
172 transactivation assays for teleost nPR including the zebrafish ², the rainbow fish ¹⁹ and the
173 fathead minnow ²⁰. The species-specificity of DHP to induce zfPR and not hPR likely reflects
174 its crucial role in reproductive endocrinology in most teleost species as maturation-inducing
175 substance (MIS) while in mammals P4 is the endogenous progestagenic hormone. It is
176 however noteworthy that DHP was able to inhibit R5020-induced hPR activity in HELN-hPR
177 cells, but at a quite high concentration (*i.e.* IC₅₀ 576 nM, Table S1), suggesting that the fish
178 progestin DHP is able to bind to the human nPR but with a much lower affinity than for zfPR.

179 A marked difference was also noticed regarding the efficacy of RU486 to antagonize human
180 and zebrafish PRs (Figure 1C, D). In both cell lines, RU486 behaved as an antagonist ligand
181 but with a 2000-fold higher efficacy in HELN-hPR than in U2OS-zfPR (Table 2), which
182 could reflect a higher binding affinity of RU486 for hPR than zfPR.

183 Importantly, none of the other steroidal hormones tested, *i.e.* 17 β -estradiol, testosterone and
184 11-ketotestosterone were able to transactivate either hPR or zfPR (Figure S2). In addition,
185 P4, R5020 and DHP were all inactive in HELN cells that express luciferase but no functional
186 receptor (data not shown).

187 As the cellular context may influence the transcriptional activity of liganded nuclear receptors
188 ²⁴, the reference PR ligands were assessed in U2OS and HeLa cells that were transiently
189 transfected with hPR and zfPR, respectively. The response profiles obtained in transient
190 transfection experiments were very similar to that obtained with U2OS-zfPR and HELN-hPR
191 stable cell lines (Figure S3). These results demonstrate that in our study the cellular context
192 did not influence the biological response and the observed differences between the two cell
193 lines were related to the different PRs.

194 Altogether, these data highlight that both models respond to reference PR ligands in a
195 sensitive and PR-specific manner, revealing differential transactivation properties depending
196 on the ligands and the origin species of the nPR.

197 **3.2. Synthetic progestins transactivate hPR**

198 The twenty-two pharmaceutical progestins (Table 1) were able to activate luciferase activity
199 in a concentration-dependent manner in HELN-hPR (Figure 2A and 2B) with EC₅₀ values
200 ranging from 0.14 nM for GES to 249 nM for MEP (Table 2). All the tested progestins,
201 except LYN and MEP, were equally or more potent than the endogenous ligand P4, and five
202 of them (*i.e.* MPA, NES, ENG, GES and LNG) had higher relative progestagenic potency
203 than the prototypical synthetic ligand R5020. Interestingly, drospirenone, an analogue of
204 spironolactone, had potent progestagenic activity in HELN-hPR (EC₅₀ 5 nM, Tables 2 and S1,
205 Figure 2B), which was significantly higher than spironolactone and its metabolite canrenone
206 that were previously described as hPR agonists ¹⁰ with EC₅₀ of 1008 nM and 657 nM,
207 respectively (Figure S4).

208 Further characterization was achieved by coexposing progestins with R5020 1 nM to assess
209 hPR antagonistic activity. The full PR agonists (*i.e.* inducing 80 - 100% of maximum activity,
210 Figure 2A) were unable to inhibit R5020-induced luciferase (data not shown). Conversely, all
211 the progestins that partially induced luciferase activity (Figure 2B) were also able to inhibit
212 R5020-induced luciferase in a concentration-dependent manner to reach the activity level
213 obtained with the compound alone, which is the typical response profile of a partial agonistic
214 ligand (Table 2 and Table S1). For this second category, the calculated IC₅₀ values ranged
215 from 4.70 nM for MGA to 156 nM for EDA.

216 The progestagenic activities reported herein agree with EC₅₀ values derived from *in vitro* cell-
217 based transactivation assays for 8 pharmaceuticals common to our study using either HELN-
218 hPRB²² or U2OS-hPR cells¹⁹ albeit the EC₅₀ reported for tibolone in HG5LN-Gal4-hPR
219 cells was ten times higher than in our study²⁵.

220

221 **3.3. Progestins modulated zfPR activity but most of them were antagonists**

222 The *in vitro* response profiles of progestins were dramatically different when assessing zfPR
223 activity. In contrast to the results obtained on hPR, very few progestins induced zfPR activity
224 (Figure 2C). Only NES and DRO transactivated zfPR, with EC₅₀ values of 1.4 nM and 14.1
225 nM, respectively (Table 2). In coexposure experiments, both compounds antagonized DHP-
226 induced luciferase activity to reach the level of activity obtained with the compound alone and
227 were therefore considered as partial zfPR agonists (Figure 2D, Tables 2 and S1). Interestingly,
228 the two compounds spironolactone (SPI) and its metabolite canrenone (CAN), were also able
229 to transactivate zfPR to induce luciferase activity in U2OS-zfPR by 10- and 2-fold higher
230 levels than in HELN-hPRB, respectively (Figure S4). Our results thus confirm the study of
231 Ellestad et al., 2014 who demonstrated that DRO activates fathead minnow PR *in vitro* and

232 newly reports an *in vitro* progestagenic activity of NES, SPI (the parent compound of DRO),
233 and CAN toward a fish PR.

234 None of the other pharmaceutical progestins were able to induce luciferase activity in U2OS-
235 zfPR at the concentrations tested (Table S1). Very few studies investigated the interaction of
236 progestins towards fish PR and the number of investigated progestins was limited to few of
237 them. Notwithstanding, the lack of progestagenic activity of progestins toward zfPR reported
238 herein agrees with available *in vitro* fish PR transactivation assays for the rainbowfish¹⁹ and
239 the fathead minnow²⁰. Therefore, our study confirms the lack of progestagenic activity of
240 some progestins and extends this knowledge to a broad range of pharmaceutical progestins in
241 another teleost fish species, the zebrafish. Hence, it suggests that the lack of progestagenic
242 activity of progestins is a common feature in teleost.

243 A major finding of our study was to demonstrate that all the progestins were able to fully
244 antagonize DHP-induced luciferase activity in a concentration-dependent manner in the zfPR
245 assay. The IC₅₀ ranged from 14 nM for LNG to 1002 nM for DSG. Remarkably, RU486, a
246 potent antagonist compound on hPR (Figure 1C, Table 2), had a much weaker anti-
247 progestagenic activity in U2OS-zfPR, *i.e.* by 2000-fold, and was the least active anti-
248 progestagenic compound in U2OS-zfPR.

249 To ensure that the human cellular context did not influence the biological response measured
250 on zfPR in U2OS, we tested the interaction of selected ligands with the full length zfPR in a
251 fish hepatic cell context, the zebrafish liver cell line (ZFL). The ZFL cell line has been shown
252 to be metabolically competent possessing both phase I and phase II enzymes^{26,27} and is a
253 relevant *in vitro* zebrafish model to study the interactions of EDCs with NR-regulated
254 signaling pathways such as AhR²⁷ or ERs²⁸⁻³⁰. In ZFL-zfPR cells, the reference agonists
255 DHP, R5020 and P4 were all active, with similar patterns in terms of both effective
256 concentrations and response intensity as compared to the U2OS-zfPR cells (Figure S5-A),

257 which validates the relevance of the established zebrafish cell model. RU486 had no agonistic
258 activity but inhibited DHP-induced luciferase activity at high concentration, which reflects its
259 weak anti-progestagenic activity observed in U2OS-zfPR (Figure S5-B). Most importantly,
260 the ZFL-zfPR assay confirmed the lack of progestagenic activity of MPA and ENG while
261 they antagonized DHP-induced luciferase (Figures S5-C, S5-D). It should be pointed out that
262 the anti-progestagenic activities of MPA and ENG are less pronounced in the ZFL context
263 compared to U2OS-zfPR. These differences might be attributed to the higher metabolic
264 activity of the hepatic cell line compared to U2OS cells, which is important to consider given
265 that progestins are known to be metabolized into active metabolites possessing diverse
266 endocrine properties^{17,31}. Based on these data, it can be concluded that the *in vitro* anti-
267 progestagenic activity of progestins was not specific to the cellular context, but on the
268 contrary reflected the intrinsic properties of progestins on zfPR.

269 Altogether, the *in vitro* comparative data demonstrate that a great majority of the tested
270 synthetic steroidal progestins have potent progestagenic activity towards hPR but anti-
271 progestagenic activity towards zfPR (except DRO and NES).

272 **3.4. *Species-specificity of the nuclear progesterone receptor transactivation by*** 273 ***progestins***

274 We highlighted major differences regarding the progestagenic activity of progestins towards
275 human and zebrafish PR. Previous studies investigating cross-species differences between
276 mammalian and fish models for several nuclear receptors provided evidence that there exist
277 differential binding affinity, transactivation properties and/or selectivity of ligands and
278 environmental xenobiotics depending on the nuclear receptor studied and the origin species of
279 NR^{28,32,33}. Among them, the pregnane X receptor (PXR) showed the most significant
280 differences in ligand specificity across species^{34,35}. Our study further documents new
281 evidence on such interspecies differences between human and fish for the PR. Comparative

282 analysis of the DBD/LBD amino acid sequences of human NRs and the corresponding
283 orthologs in fish could provide relevant insights into differential transactivation patterns
284 between human and fish ³⁵. For the PR, a single *prg* gene is transcribed in human into two
285 isoforms, PRA and PRB, by alternate initiation of transcription of two distinct promoters ³⁶
286 while in zebrafish, one *prg* gene coding for one PR have been cloned and characterized ². As
287 for other NRs, the DBD region of the PR is very well conserved between human and fish with
288 90% of sequence similarities while the LBD displayed more variation with only 67% of
289 similarities between amino acid sequences in human and zebrafish ^{2,35,37}. Similar differences
290 in amino acid sequences in the LBD domain between human and fish have been reported for
291 other NRs such as ERs and PPARs for which differences in transactivation properties by
292 environmental contaminants is documented ³⁸. However, none of these latter studies reported
293 such an ambivalent biological activity for a given substance, *i.e.* which behave as an agonist
294 in human and as an antagonist in fish as in our study for the progestins.

295 In order to explore the differential activity of PR ligands between h and zfPR, a structural
296 analysis and molecular modeling was done (Figure 3, Figure S6). It revealed several amino
297 acid differences within the ligand-binding pockets (LBPs) of zfPR and hPR that are likely to
298 account for their ligand-binding specificities. Using the modeling and docking server EDMon
299 (<http://edmon.cbs.cnrs.fr/>) ³⁹, we generated homology models of the PR ligand-binding
300 domains (LBDs) of the two species in complex with RU486 or DHP, two ligands displaying
301 distinct species preferences (Table S1, Figure 1). A visual inspection of the h- and zf-models
302 reveals essentially five residue substitutions of amino acids in contact or in proximity to the
303 ligands (G722/C406, V760/L444, S793/P477, F794/I478, L887/V571). Interestingly, the
304 V760/L444 and L887/V571 compensatory mutations allow the preservation of the stabilizing
305 interaction between helix H5 and helix H11 (black dotted lines in Figure 3). In the human
306 receptor, V760 (H5) and L887 (H11) make moderate van der Waals interactions with both

307 ligands. In contrast, our model of the zfPR suggests tighter contacts of L444 (H5) and V571
308 (H11) with the ligands (magenta dotted lines in Figure 3) which may account, at least in part,
309 for the higher affinity of DHP for zfPR. Another key substitution is the replacement of the
310 glycine residue G722 in the human receptor by cysteine C406 in zfPR. The additional carbon
311 (C β) and sulfur atoms are very likely to provide additional contact points with DHP, thereby
312 participating to increasing further the affinity of this ligand for the zfPR relative to its human
313 homolog (green dotted lines in Figure 3D). In contrast, the side chain of C406 generates a
314 strong steric clash with the antagonist extension of RU486 (red asterisk in Figure 3B).
315 Because the position of the bulky RU486 is very much constrained by the amino acid
316 environment, it is very unlikely that the LBP can accommodate the cysteine side-chain
317 without a drastic loss in the binding affinity of RU486.

318 Overall, the comparison of the LBP and LBD sequences and modeling inform on the
319 differential affinity of investigated ligands between h and zfPR but is not informative enough
320 to explain the observed differences in term of activity. This should be further investigated, for
321 instance by using in depth analysis of the crystal structure of the purified human and zebrafish
322 PR.

323 **3.5. (Eco)toxicological relevance of these findings**

324 The observed differences between the *in vitro* progestagenic activities of the tested substances
325 on human and zebrafish PR raise the question of the relevance of the toxicological
326 information provided using mammalian models to predict possible hazard in other species
327 such as zebrafish. Based on our study, it seems that extrapolation of data from mammalian
328 pharmacology and toxicology to fish species may not be helpful. This point is particularly
329 important in the context of environmental hazard assessment of endocrine disrupting
330 substances. In this respect, our results argue for the development of species-specific strategies

331 to reliably inform on the endocrine mechanism, properties of environmental chemicals to fish,
332 such approaches being currently lacking from a regulatory point of view.

333 Progestins are now well-recognized as environmental contaminants occurring at low but
334 active concentrations, hence posing a risk to aquatic species such as fish ¹¹. The anti-
335 progestagenic activity reported for most of the investigated progestins as well as the
336 progestagenic activity for NES, DRO and the two spironolactone steroids, SPI and CAN on
337 zfPR, may have consequences on multiple physiological functions mediated by the PR
338 signaling pathway in exposed fish. An increasing number of studies reports multiple effects of
339 progestins in fish notably on reproductive functions with decreases in egg productions and/or
340 cessation of egg laying ¹⁴. Although these *in vivo* effects are often interpreted with regard to
341 the other endocrine properties of progestins, notably their interaction with the androgen
342 receptor, the *in vitro* anti-progestagenic activities reported herein may also contribute to the
343 observed effects. Indeed, the inhibition of the DHP-induced zfPR signaling agrees with recent
344 data in homozygous knockout zebrafish for PR females that are infertile due to ovulation
345 defects (anovulation), a physiological response directly attributed to the role of PR in
346 ovulation process ^{4,5}. Remarkably, the inability of females to ovulate, resulting in abdominal
347 swelling, has been observed in a wild population of gudgeon exposed to a pharmaceutical
348 manufacture effluent releasing synthetic steroids, including high concentrations of synthetic
349 progestins ⁴⁰. While the anti-progestagenic activity could provide a new molecular basis to
350 explain the inhibition of eggs released in exposed-female fish to progestins, direct
351 extrapolation of *in vitro* data to the *in vivo* situation should however consider
352 pharmacodynamics of progestins that are currently lacking for fish. Whatever, our data stress
353 the need for a better characterization of environmental contaminants towards fish NRs in
354 general, particularly fish PR and to further investigate the effects of progestins on key PR-
355 dependent physiological processes.

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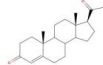
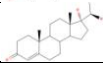
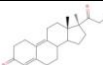
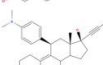



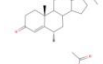
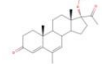
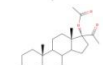

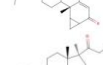


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Table 1: Classification of natural hormones and natural and synthetic nPR ligands tested on HELN-hPRB and U2OS-zfPR cells.

Classification		Compound	Abbreviation	Uses	Molecular weight (g/mol)	CAS	Structure
Natural progestin in human and fish		Progesterone	P4	Clinical use	314.46	57-83-0	
Natural progestin in fish		17 α ,20 β -dihydroxy-4-pregnen-3-one	DHP		332.48	1662-06-2	
Synthetic progestin derived from 19-norprogesterone		Promegestone	R5020	Clinical use	326.47	34184-77-5	
PR antagonist		Mifepristone	RU486	Clinical use	429.60	84371-65-3	
Synthetic progestin retroprogesterone, isomer of P4		Dydrogesterone	DYD	Clinical use	312.45	152-62-5	
Progestins structurally related to progesterone	Derived from 17 α -hydroxyprogesterone	Medroxyprogesterone	MEP	Clinical use	344.49	520-85-4	
		Medroxyprogesterone acetate	MPA	Clinical use	386.52	71-58-9	
		Megestrol acetate	MGA	Clinical use	384.51	595-33-5	
		Chlormadinone acetate	CMA	Clinical use	406.94	302-22-7	
		Cyproterone acetate	CPA	Clinical use	416.94	427-51-0	
	Derived from 19-norprogesterone	Promegestone	R5020	Clinical use	326.47	34184-77-5	
		Nestorone	NES	Clinical use	370.48	7759-35-5	
		Nomegestrol acetate	NGA	Clinical use	370.48	58652-20-3	
	Derived from 17 α -hydroxy-19-norprogesterone	Gestonorone	GRN	Clinical use of gestonorone caproate	316.43	2137-18-0	

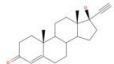
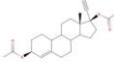
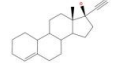
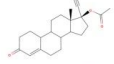
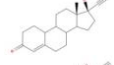

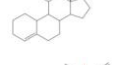
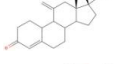
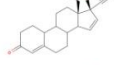
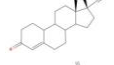


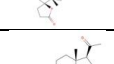
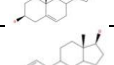
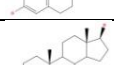
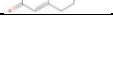
Progestins structurally related to testosterone: derived from 19-nortestosterone	Estranes	Ethisterone	ETH	Stopped	312.45	434-03-7	
		Ethinodiol diacetate	EDA	Clinical use	384.51	297-76-7	
		Lynestrenol	LYN	Clinical use	284.44	52-76-6	
		Norethindrone acetate	NEA	Clinical use	340.46	51-98-9	
		Norethindrone	NET	Clinical use	298.42	68-22-4	
		Tibolone	TIB	Clinical use	312.4	5630-53-5	
	Gonanes	Desogestrel	DSG	Clinical use	310.47	54024-22-5	
		Etonogestrel	ENG	Clinical use, metabolite of desogestrel	324.45	54048-10-1	
		Gestodene	GES	Clinical use	310.43	60282-87-3	
		Levonorgestrel	LNG	Clinical use	312.45	797-63-7	
		Norgestimate	NTE	Clinical use	369.50	35189-28-7	
		Norgestrel	NGL	Clinical use	312.45	6533-00-2	
Progestin structurally related to spironolactone	Drospirenone	DRO	Clinical use	366.49	67392-87-4		
Natural hormones and precursors	Pregnenolone (progesterone precursor)	P5	Clinical use	316.48	145-13-1		
	17 β -estradiol	E2	Clinical use	272.38	50-28-2		
	Testosterone	T	Clinical use	288,42	58-22-0		

Table 2: Calculated EC₅₀ and IC₅₀ (nM) in HELN-hPRB and U2OS-zfPR cells.

Classification	Abb	HELN-PRB			U2OS-zfPR				
		Agonism		Antagonism	Agonism			Antagonism	
		EC ₅₀ ± sd (nM)	max ± sd (%)	REP	IC ₅₀ ± sd (nM)	EC ₅₀ ± sd (nM)	max ± sd (%)	REP	IC ₅₀ ± sd (nM)
Reference compounds	P4	13.5 ± 7.14	70 ± 9	0.02	17.1 ± 15.7	1.97 ± 0.57	40 ± 7	0.95	2.09 ± 0.33
	R5020	0.33 ± 0.10	100	1	-	1.93 ± 0.60	64 ± 11	0.97	-
	DHP	ne			576 ± 319	1.87 ± 0.44	100	1	-
	RU486	ne			1.04 ± 0.28	ne			2253 ± 292
Other progestins	DYD	8.03 ± 3.28	66 ± 13	0.04	14.5	ne			24.9 ± 6.38
	MEP	249 ± 105	37 ± 8	0.001	39.6 ± 23.4	ne			91.4 ± 55.9
	MPA	0.20 ± 0.05	68 ± 15	1.7	8.08 ± 5.74	ne			30.0 ± 7.86
	MGA	0.57 ± 0.04	74 ± 14	0.58	4.70 ± 3.87	ne			95.2 ± 55.5
	CMA	0.65 ± 0.07	84 ± 7	0.50	ne	ne			125 ± 22
	CPA	1.15 ± 0.16	79 ± 0	0.29	ne	ne			244 ± 93.3
	NES	0.25 ± 0.03	90 ± 5	1.34	ne	1.41 ± 0.41	40 ± 2	1.33	6.39 ± 4.00
	NGA	0.60 ± 0.22	92 ± 14	0.55	ne	ne			17.8 ± 6.99
	GRN	18.4 ± 5.11	50 ± 15	0.02	34.9 ± 15.0	ne			26.2 ± 11.3
	ETH	6.20 ± 0.61	55 ± 12	0.05	14.1 ± 4.42	ne			69.5 ± 8.17
	EDA	15.3 ± 4.80	62 ± 19	0.02	156 ± 117	ne			354 ± 295
	LYN	25.7 ± 10.8	56 ± 2	0.01	61.9 ± 13.2	ne			503 ± 110
	NEA	0.85 ± 0.57	90 ± 12	0.39	ne	ne			22.2 ± 0.55
	NET	0.93 ± 0.27	83 ± 4	0.35	ne	ne			34.3 ± 1.66
	TIB	9.97 ± 1.33	85 ± 6	0.03	ne	ne			146
	DSG	14.1 ± 6.80	72 ± 24	0.02	76.0 ± 51.2	ne			1002
	ENG	0.18 ± 0.04	94 ± 7	1.77	ne	ne			148 ± 98
	GES	0.14 ± 0.05	95 ± 5	2.43	ne	ne			15.2 ± 16.2
LNG	0.16 ± 0.04	96 ± 6	2.08	ne	ne			14.0 ± 7.41	
NTE	1.11 ± 0.26	78 ± 10	0.30	ne	ne			77.4 ± 4.16	
NGL	0.47 ± 0.12	101 ± 1	0.70	ne	ne			43.6 ± 17.2	
DRO	5.72 ± 1.38	60 ± 14	0.06	58.0 ± 66.1	14.1 ± 4.49	30±6	0.13	16.1 ± 1.44	

REP: Relative potency in HELN-hPRB cells (EC₅₀(R5020)/EC₅₀(chemical)) and U2OS-zfPR cells (EC₅₀(DHP)/EC₅₀(chemical)). Standard deviations (sd); ne: no effect.

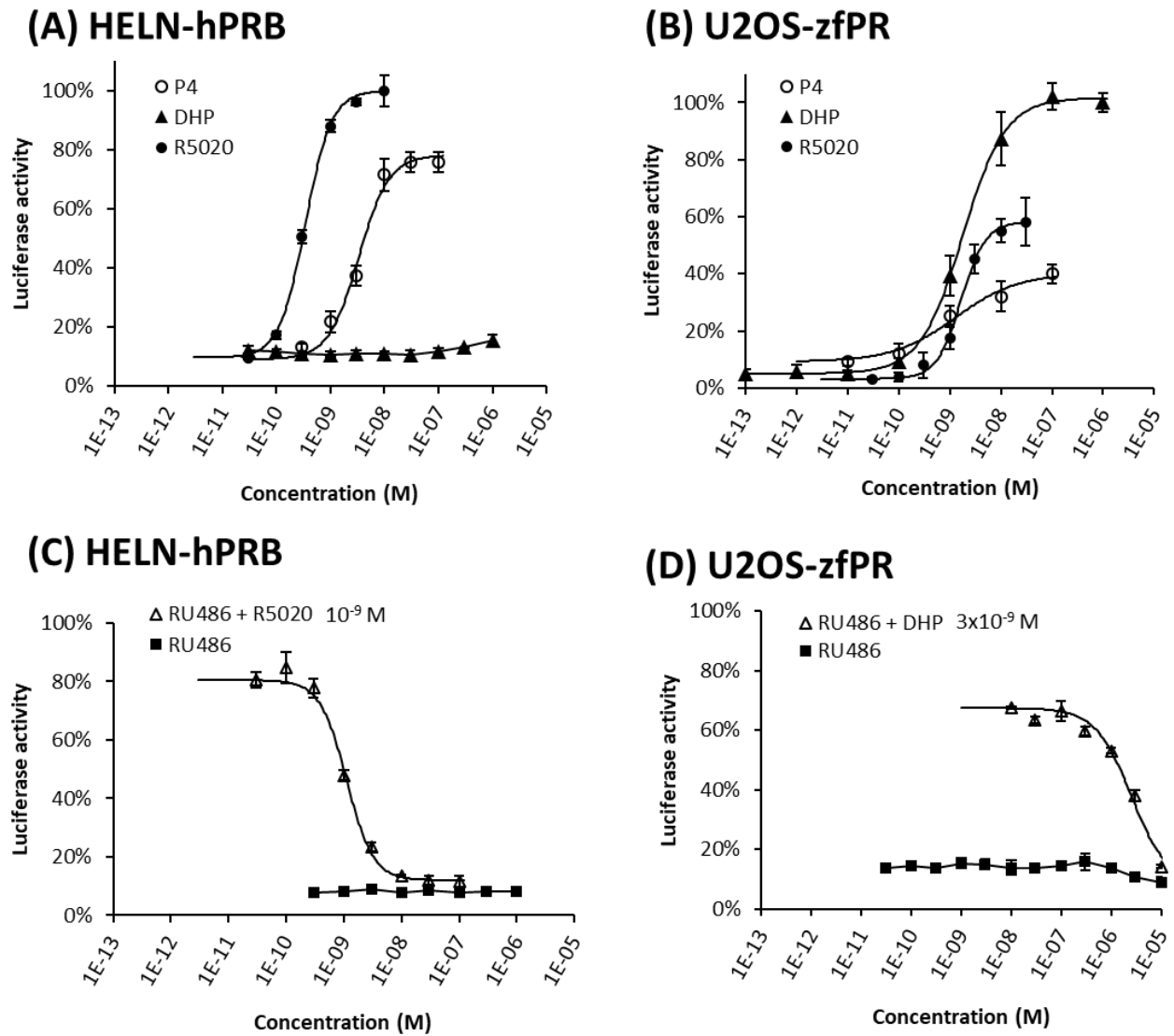
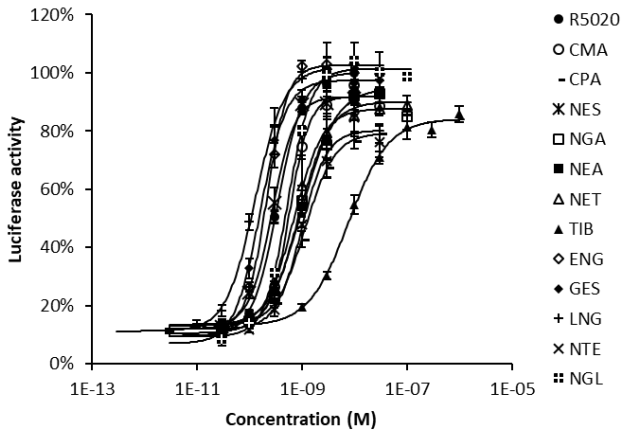
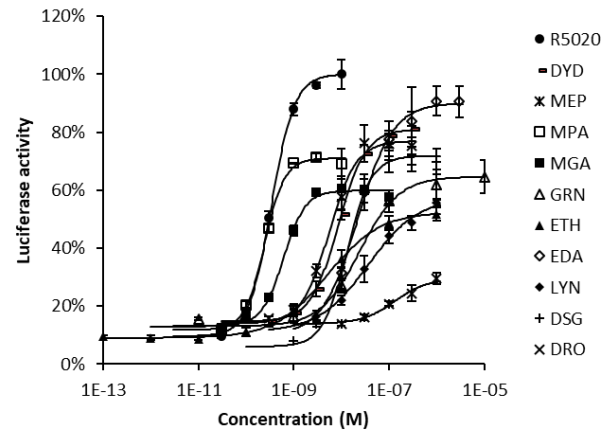


Figure 1: Dose-response curves of the activity of reference ligands in HELN-hPRB and U2OS-zfPR cells expressed as the percentage of the maximum luciferase activity induced by 3×10^{-8} M R5020 (A,C) or 3×10^{-8} M DHP (B,D). P4: progesterone; DHP: $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; R5020: promegestone, RU486: mifepristone. Error bars represent standard deviation.

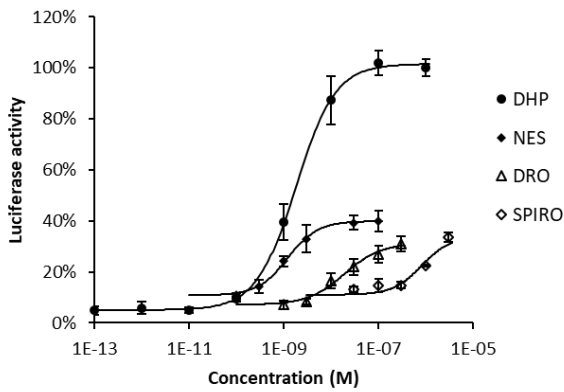
(A) Full agonists on HELN-hPRB



(B) Partial agonists on HELN-hPRB



(C) Agonists on U2OS-zfPR



(D) Antagonists on U2OS-zfPR

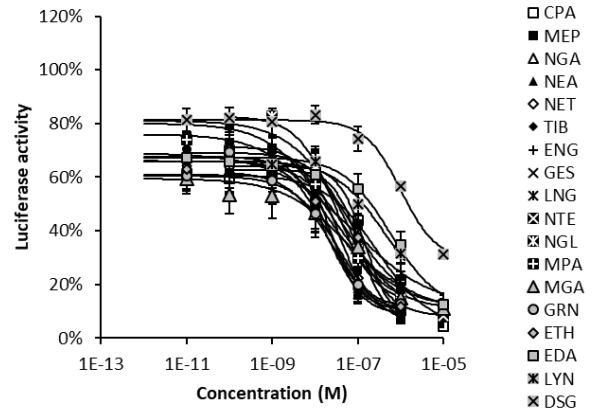


Figure 2: Dose-response curves of the activity of progestins in HELN-hPRB and U2OS-zfPR cells expressed as the percentage of the maximum luciferase activity induced by 3×10^{-8} M R5020 (A, B) or 3×10^{-8} M DHP (C, D). P4: progesterone; DHP: $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; R5020 : promegestone, P5: pregnenolone; DYD: dydrogesterone; MEP: medroxyprogesterone; MPA: medroxyprogesterone acetate; MGA: megestrol acetate; CMA: chlormadinone acetate; CPA: cyproterone acetate; NES: nesterone; NGA: nomegestrol acetate; ETH: ethisterone; EDA: ethynodiol diacetate; LYN: lynestrenol; NEA: norethindrone acetate; NET: norethindrone; TIB: tibolone; DSG: desogestrel; ENG: etonogestrel; GES: gestodene; LNG: levonorgestrel; NTE: norgestimate; NGL: norgestrel; DRO: drospirenone; GRN: gestonorone. Error bars represent standard deviation (SD, n=3 replicates per concentration). All curves for individual compounds are presented separately in Supplementary Materials, Table S1.

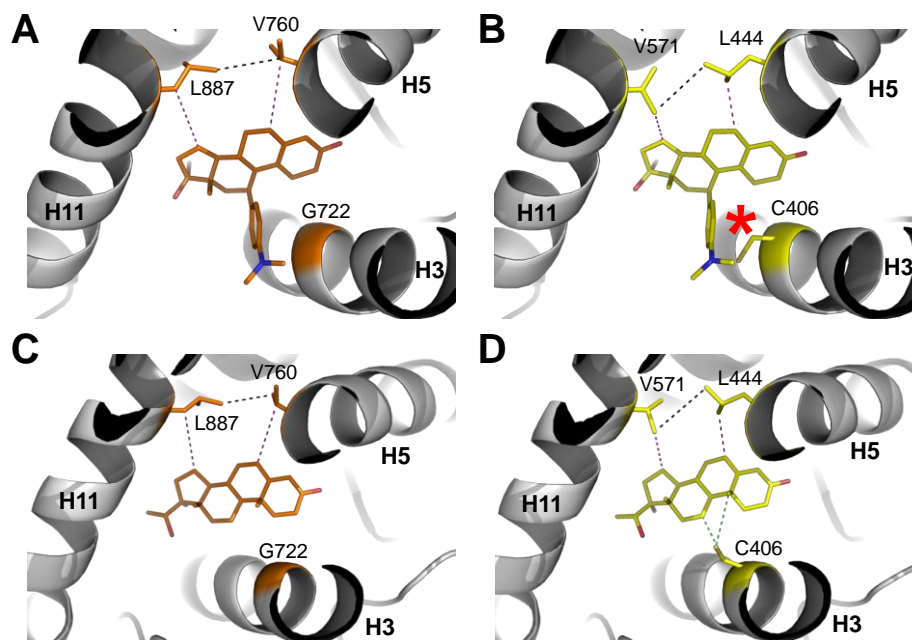


Figure 3: Structural analysis of species-specificity for RU486 and DHP. Structure (A), PDB code 2w8y, and docking model (B) of RU486 bound to human and zebrafish PR, respectively. (C, D) Docking models of DHP bound to human and zebrafish PR, respectively. The ligands and residues discussed in the main text are shown as sticks. The structural elements of PR LBD are shown in grey and labelled. The dashed lines denote the interactions described in the text. The red asterisk denotes a steric clash between C406 and RU486. Docking models were generated using the server EDMon (<http://edmon.cbs.cnrs.fr>) and the default parameters. Figures were prepared with PyMOL (<http://pymol.org/>).

Supporting information

Human and zebrafish nuclear progesterone receptors are differently activated by progestins

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Figure S1: Constructions of the cell lines HELN-hPRB (A) and U2OS-zfPR (B).

Table S1: Typical concentration-response curves of the activity of progestins in HELN-hPRB and U2OS-zfPR cells.

Figure S2: HELN-hPRB cells (A) and U2OS-zfPR (B) cells were treated with indicated concentrations of 17β -estradiol (E2), testosterone (T) and 11-Ketotestosterone (11-KT).

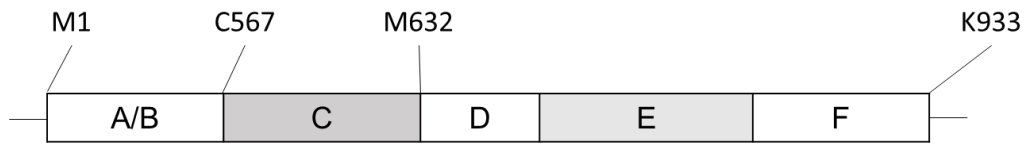
Figure S3: Concentration-response curves of the activity of progestins in U2OS cells expressing hPR (A) and HeLa cells expressing zfPR (B).

Figure S4: Activity of spironolactone and canrenone towards h and zfPR.

Figure S5: Activity of progestins in ZFL-zfPR cells.

Figure S6: Amino acids sequence alignment of the human (hPR-B) and zebrafish (zfPR) progesterone receptor.

(A) HELN-hPRB



(B) U2OS-zfPR

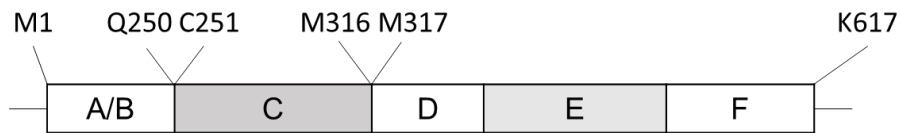


Figure S1: Constructions of the cell lines HELN-hPRB (A) and U2OS-zfPR (B). Each contains six domains. Domain A/B: activation factor; domain C: human estrogen receptor hER α DNA binding domain (C251-M316); domain D: hinge; domain E: hPR-B (A) or zfPR (B) ligand binding domain.

Table S1: Typical concentration-response curves of the activity of progestins in HELN-hPRB and U2OS-zfPR cells. Results are expressed as percentage of the maximum luciferase activity induced by 3×10^{-8} M R5020 in HELN-hPRB or 3×10^{-8} M DHP in U2OS-zfPR. Cells were treated with progestins alone (black circles) or in co-exposure with R5020 or DHP at concentrations inducing 80% of the maximum luciferase activity (10^{-8} M R5020 and 3×10^{-9} M respectively) (white circles). Error bars represent standard deviation.

HELN-PRB

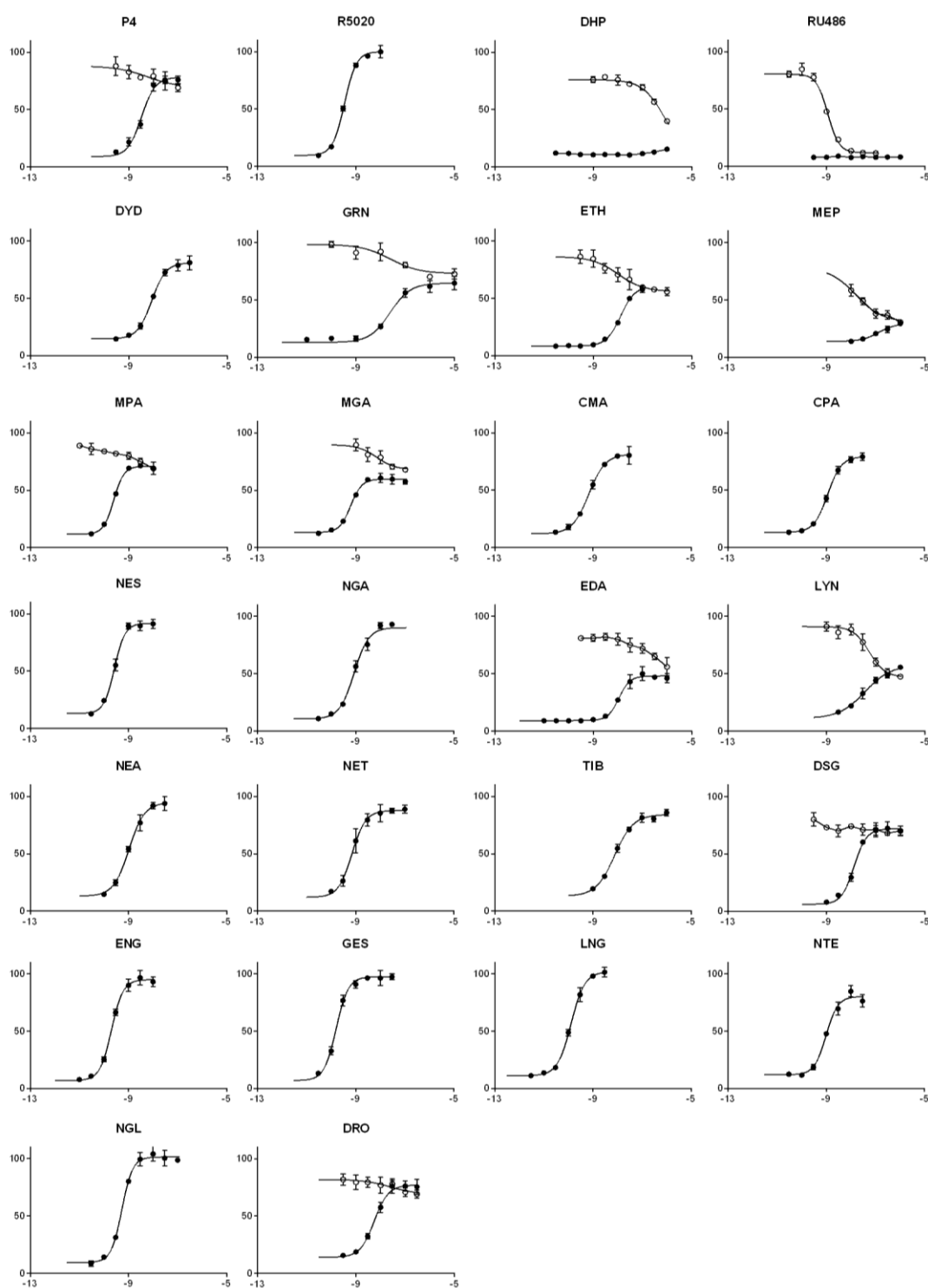
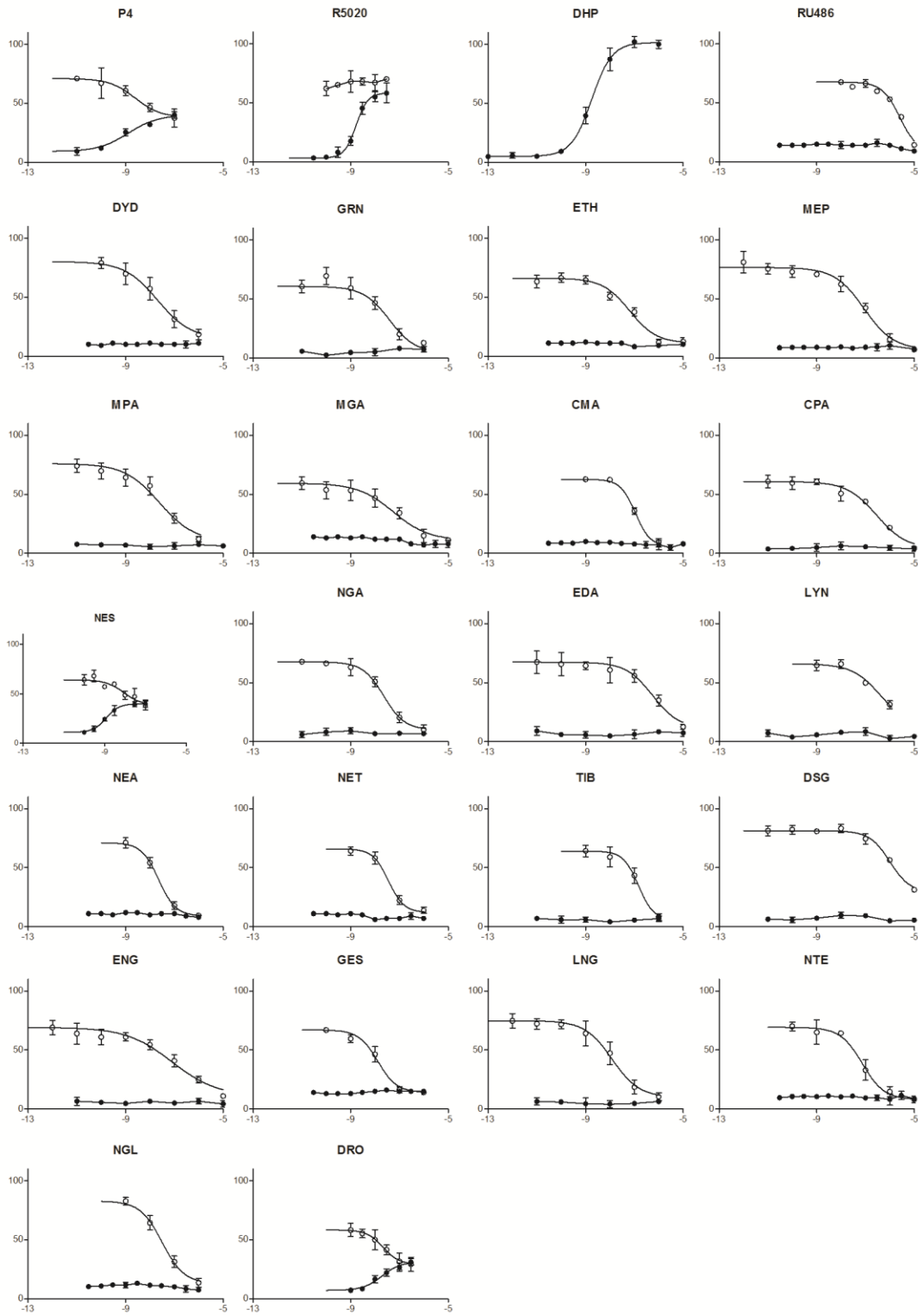


Table S1 (continued)

U2OS-zfPR



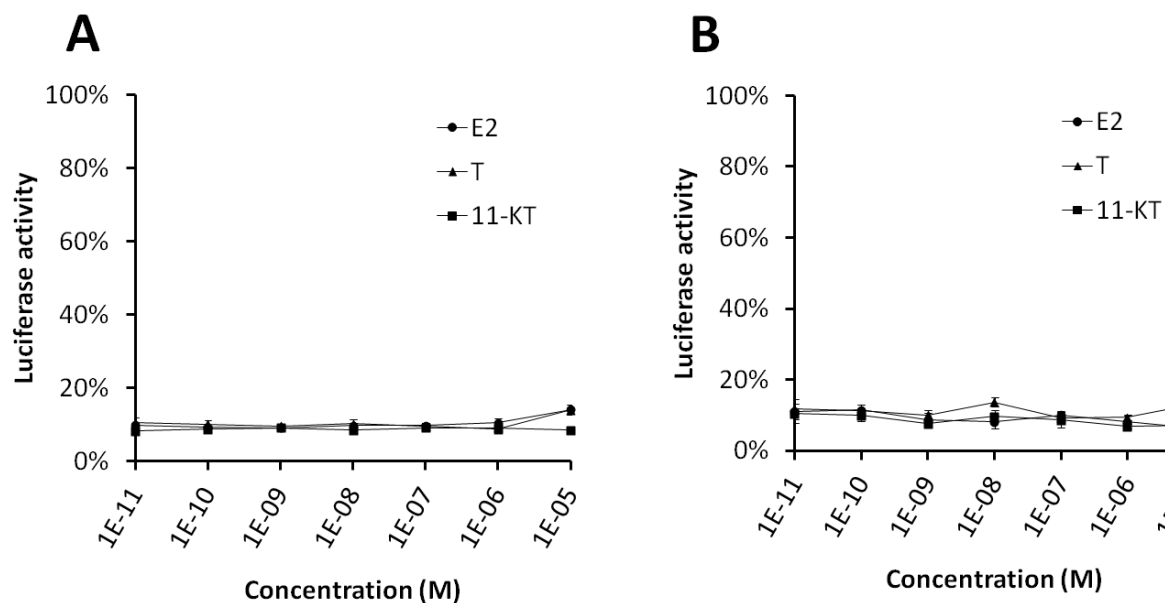


Figure S2: HELN-hPRB cells (A) and U2OS-zfPR (B) cells were treated with indicated concentrations of 17 β -estradiol (E2), testosterone (T) and 11-Ketotestosterone (11-KT). Results are expressed as the percentage of the maximum luciferase activity induced by 3×10^{-8} M R5020 (A) or 3×10^{-8} M DHP (B). Error bars represent standard deviation. The absence of luciferase activity by the steroidal hormones indicates the specificity of response of the cell models.

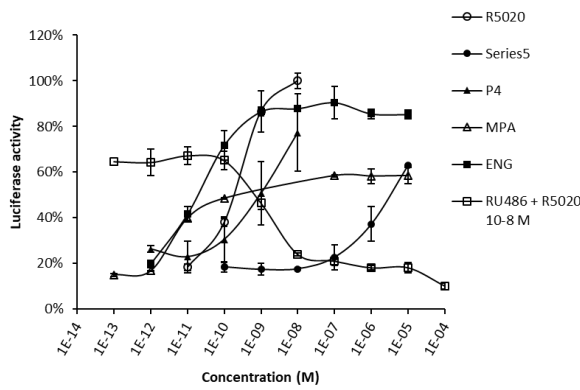
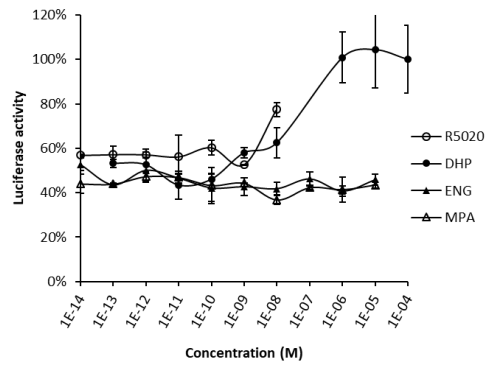
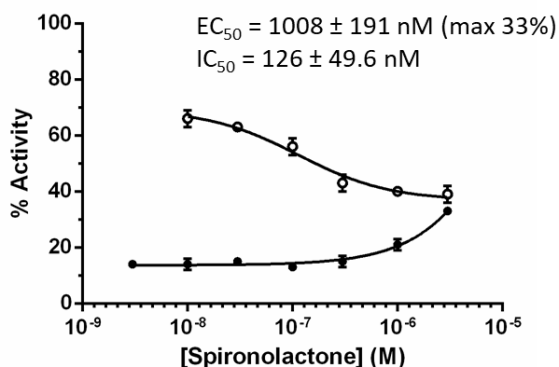
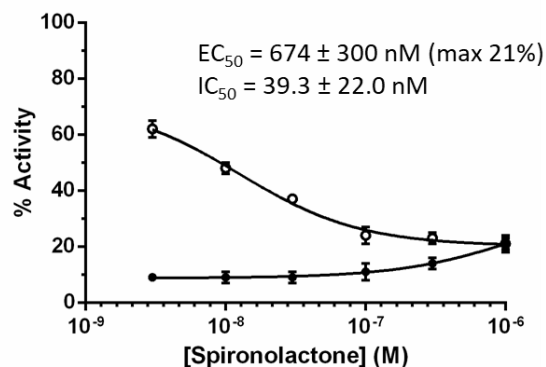
(A) U2OS-hPRB**(B) HeLa-zfPR**

Figure S3: Concentration-response curves of the activity of progestins in U2OS cells expressing hPR (A) and HeLa cells expressing zfPR (B). The results are expressed as the percentage of the maximum luciferase activity induced by 3×10^{-8} M R5020 (A) or 3×10^{-8} M DHP (B). P4: progesterone; DHP: $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; R5020: promegestone, MPA: medroxyprogesterone acetate; ENG: etonogestrel; RU486: mifepristone. Error bars represent standard deviation. U2OS cells were transiently cotransfected by a plasmid expressing ERE-luciferase and a second one expressing the hPR with the EhR α DNA binding domain. HeLa cells were transiently cotransfected by a plasmid expressing ERE-luciferase and a second one expressing the zfPR with the hER α DNA binding domain.

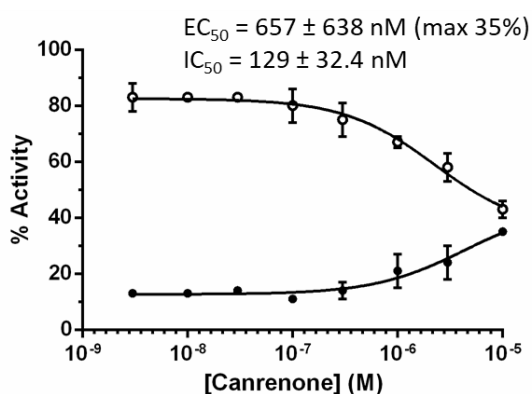
(A) Spironolactone on hPR



(B) Spironolactone on zfPR



(C) Canrenone on hPR



(D) Canrenone on zfPR

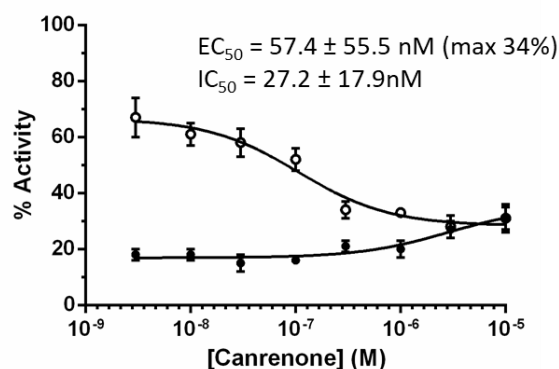


Figure S4: Activity of spironolactone and canrenone towards h and zfPR. Typical concentration-response curves of the activity of spironolactone and canrenone in HELN-hPRB and U2OS-zfPR cells expressed as percentage of the maximum luciferase activity induced by 3×10^{-8} M R5020 in HELN-hPRB or 3×10^{-8} M DHP in U2OS-zfPR. Cells were treated with chemical substances alone (black circles) or in coexposure with R5020 or DHP at concentrations inducing 80% of the maximum luciferase activity (10^{-8} M R5020 and 3×10^{-9} M respectively) (white circles). Error bars represent standard deviation.

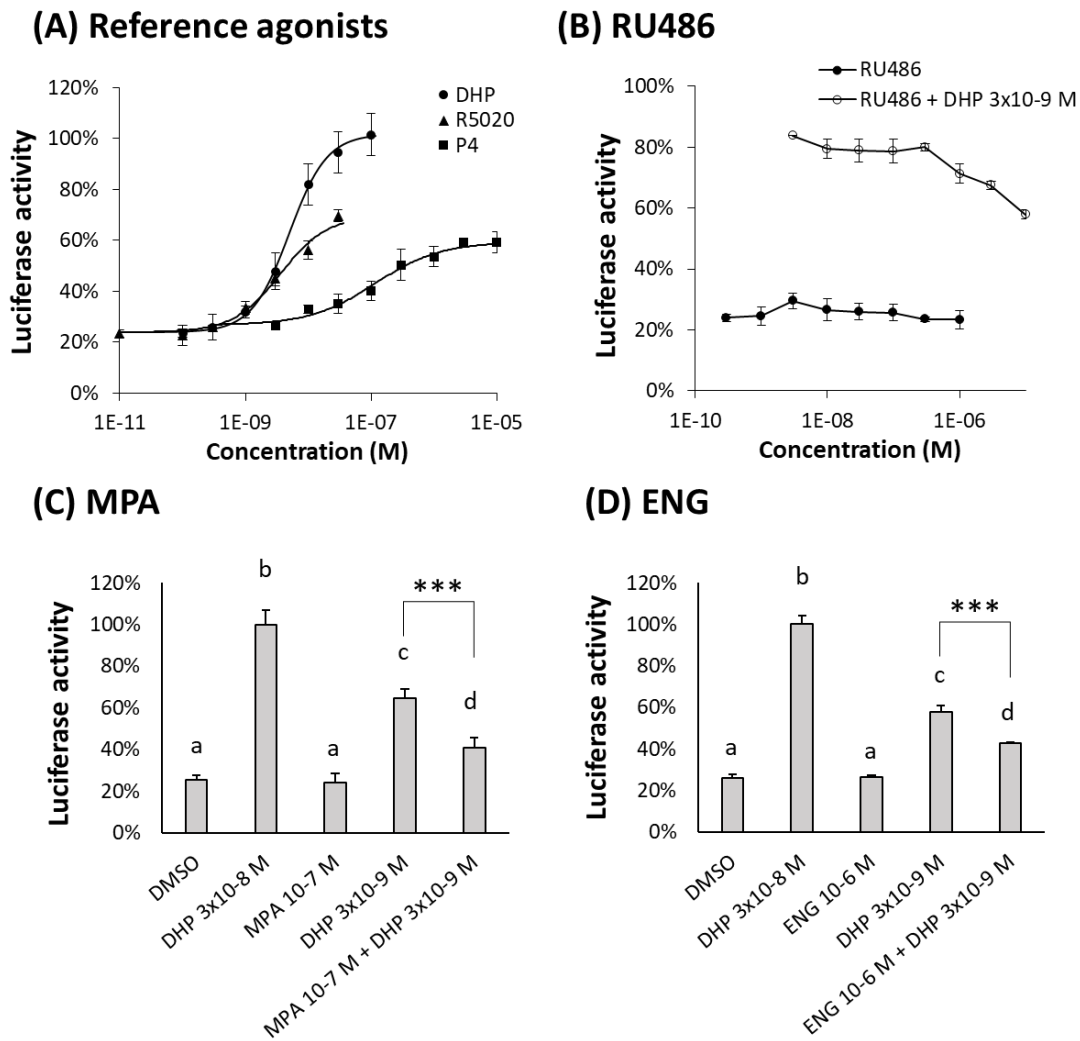


Figure S5: Activity of progestins in ZFL-zfPR cells. Results are expressed as the percentage of the maximum luciferase activity induced by 3×10^{-8} M DHP. P4: progesterone; DHP: $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one; R5020: promegestone, MPA: medroxyprogesterone acetate; ENG: etonogestrel; RU486: mifepristone. Coexposure are made with DHP at a concentration inducing 80% of the maximum luciferase activity (3×10^{-9} M). Error bars represent standard deviation. Letters correspond to significant differences between groups after t-test Student, *** $p < 0.001$.

Figure S6. Amino acids sequence alignment of the human (hPR-B) and zebrafish (zfPR) progesterone receptor ligand binding domain.

The five residue substitutions of amino acids in contact or in proximity to the ligands (G722/C406, V760/L444, S793/P477, F794/I478, L887/V571) are framed.