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## Human and Zebrafish Nuclear Progesterone Receptors Are Differently Activated by Manifold Progestins

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

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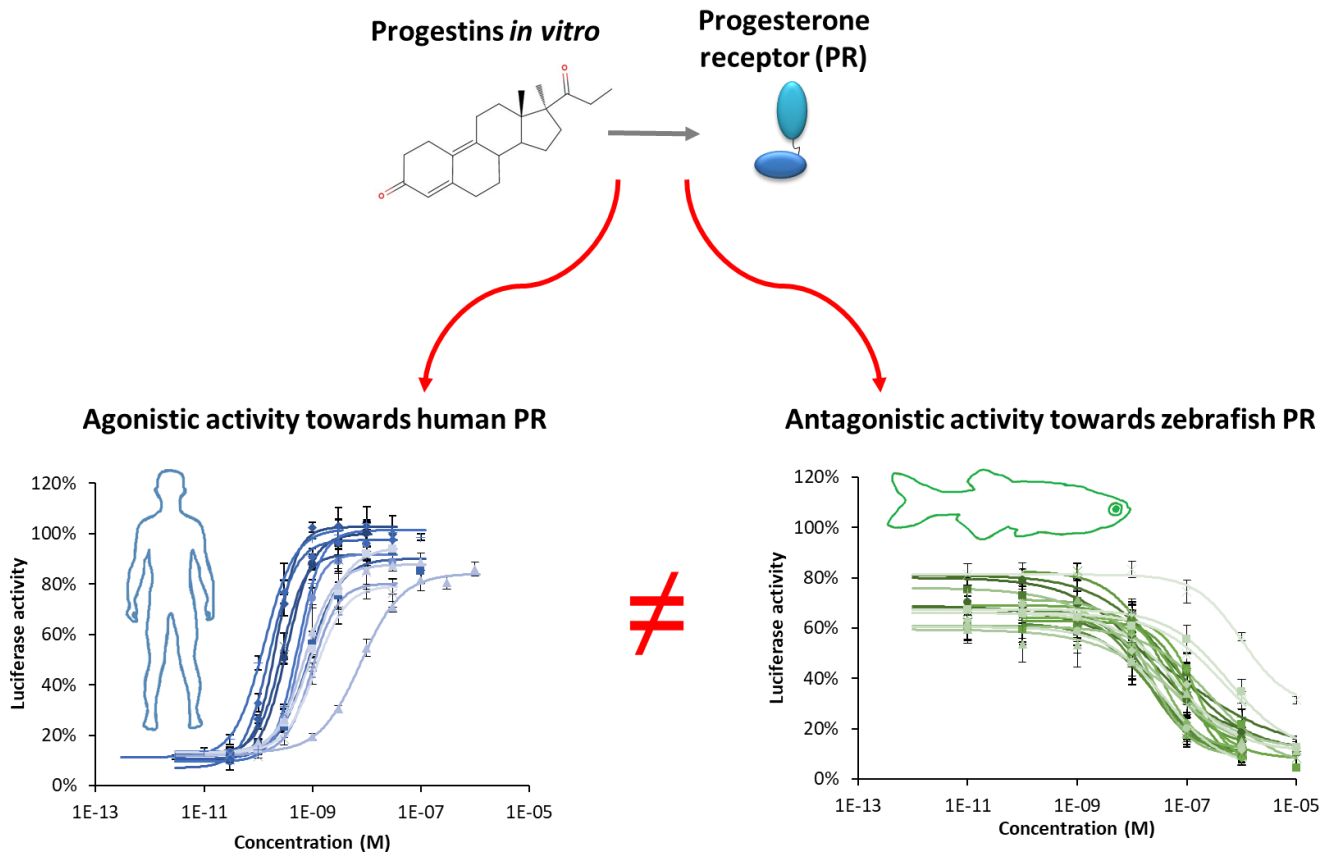
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- 21 *Keywords: synthetic progestins; 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one; human and zebrafish*
- 22 *nuclear progesterone receptor; luciferase reporter cell lines.*

23 **Graphical abstract**



24  
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 ( $20\beta$ -S) <sup>1</sup> 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 <sup>2</sup>. 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 <sup>3</sup> and more recently  
59 PRKO zebrafish <sup>4,5</sup>.

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 <sup>6-10</sup>. 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 <sup>11,12</sup>. 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*<sup>13-18</sup>. 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<sup>19,20</sup> and subsequently alter nPR-regulated physiological processes<sup>2</sup>.  
73 Because cross-species variations in nuclear receptor (NR) sequences can significantly  
74 influence their structure, functioning and response to chemical ligands<sup>21</sup>, 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 hPRB<sup>22</sup> 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 $\beta$ -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 $\alpha$ ,20 $\beta$ -  
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 described<sup>22</sup>. Briefly, HELN-hPR cells were obtained  
108        by stably transfection of HELN (HeLa ERE-luciferase-neomycin) cells<sup>23</sup> by a plasmid  
109        expressing hPR where the DNA binding domain was replaced by the hER $\alpha$  DNA binding  
110        domain (pSG5-hPR(ER $\alpha$  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 $\alpha$  DNA binding domain ((pSG5-hPR(ER $\alpha$  DBD)-puromycin, supporting Information,  
113 Figure S1). Selection of HELN-hPR cells was made with 0.5  $\mu$ g/mL puromycin and 1 mg/L  
114 G418 and selection of USO2-zfPR cells was made with 0.5  $\mu$ 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  $\mu$ g/mL  
117 of streptomycin. Culture medium was supplemented with 0.5  $\mu$ g/mL puromycin and 1 mg/mL  
118 geneticin for HELN-hPR and 0.5  $\mu$ g/mL puromycin for U20S-zfPR cells. Cells were cultured  
119 in a 5% CO<sub>2</sub> 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  $\mu$ 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 ARE<sub>6</sub>-  
124 collagenase-Luciferase-hygromycine) and the zfPR expressing plasmid pSG5-zfPR-  
125 puromycin and grown in presence of 0.5  $\mu$ 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 CO<sub>2</sub> 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  $\mu$ 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  $7 \times 10^4$  cells per well in 100  $\mu$ 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  $3 \times 10^{-9}$  M for U2OS-  
144 zfPR. After 16h, medium was removed and replaced with 50  $\mu$ 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  $3 \times 10^{-8}$  M for HELN-hPRB cells and DHP  
148 at  $3 \times 10^{-8}$  M for U2OS-zfPR cells. Concentration–response curves were obtained with the Hill  
149 equation model using the Regtox 7.0.6 Microsoft Excel <sup>TM</sup> macro  
150 ([http://www.normalesup.org/~vindimian/fr\\_index.html](http://www.normalesup.org/~vindimian/fr_index.html)), from which effective (EC) and  
151 inhibitory concentrations (IC) were derived. For a given chemical, EC<sub>50</sub> is defined as the  
152 concentration inducing 50% of its maximal effect and IC<sub>50</sub> represents the concentration  
153 required for 50% inhibition. Relative Effect Potencies (REPs) were calculated by dividing the  
154 EC<sub>50</sub> 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  $3 \times 10^{-8}$  M R5020 or  $3 \times 10^{-8}$  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 EC<sub>50</sub> (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 <sup>2</sup>, the rainbow fish <sup>19</sup> and the  
173 fathead minnow <sup>20</sup>. 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.* IC<sub>50</sub> 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 $\beta$ -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 <sup>24</sup>, 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 EC<sub>50</sub> 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 (EC<sub>50</sub> 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 <sup>10</sup> with EC<sub>50</sub> 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 IC<sub>50</sub> values ranged  
215 from 4.70 nM for MGA to 156 nM for EDA.

216 The progestagenic activities reported herein agree with EC<sub>50</sub> values derived from *in vitro* cell-  
217 based transactivation assays for 8 pharmaceuticals common to our study using either HELN-  
218 hPRB<sup>22</sup> or U2OS-hPR cells<sup>19</sup> albeit the EC<sub>50</sub> reported for tibolone in HG5LN-Gal4-hPR  
219 cells was ten times higher than in our study<sup>25</sup>.

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 EC<sub>50</sub> 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 rainbowfish<sup>19</sup> and  
239 the fathead minnow<sup>20</sup>. 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 IC<sub>50</sub> 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<sup>26,27</sup> and is a  
253 relevant *in vitro* zebrafish model to study the interactions of EDCs with NR-regulated  
254 signaling pathways such as AhR<sup>27</sup> or ERs<sup>28-30</sup>. 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<sup>17,31</sup>. 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<sup>28,32,33</sup>. Among them, the pregnane X receptor (PXR) showed the most significant  
280 differences in ligand specificity across species<sup>34,35</sup>. 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 <sup>35</sup>. 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 <sup>36</sup>  
286 while in zebrafish, one *prg* gene coding for one PR have been cloned and characterized <sup>2</sup>. 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 <sup>2,35,37</sup>. 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 <sup>38</sup>. 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/>) <sup>39</sup>, 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 $\beta$ ) 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 <sup>11</sup>. 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 <sup>14</sup>. 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 <sup>4,5</sup>. 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 <sup>40</sup>. 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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357

358

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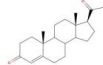
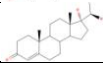
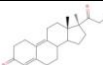
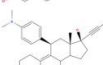



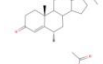
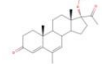
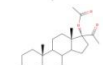

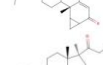


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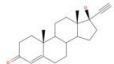
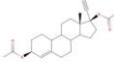
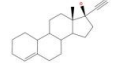
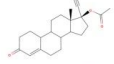
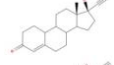

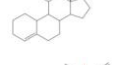
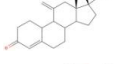
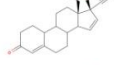
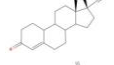


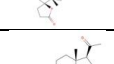
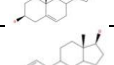
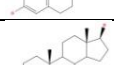
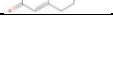
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518



**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 $\alpha$ ,20 $\beta$ -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 $\alpha$ -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 $\alpha$ -hydroxy-19-norprogesterone	Gestonorone	GRN	Clinical use of gestonorone caproate	316.43	2137-18-0	

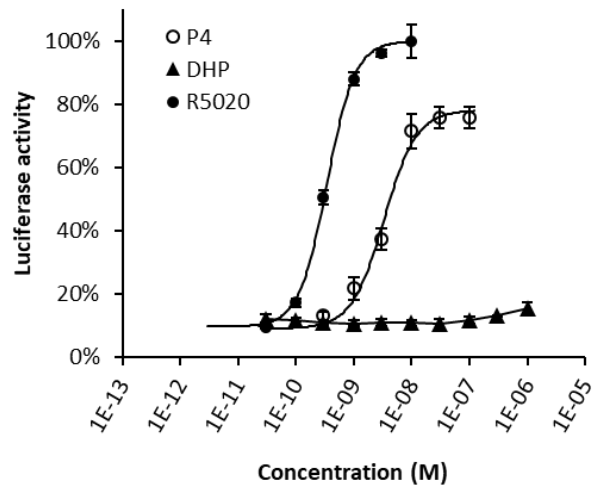
<b>Progestins structurally related to testosterone: derived from 19-nortestosterone</b>	<b>Estranes</b>	Ethisterone	ETH	Stopped	312.45	434-03-7	
		Ethinodiol diacetate	EDA	Clinical use	384.51	297-76-7	
		Lynestrenol	LYN	Clinical use	284.44	52-76-6	
		Norethindrone acetate	NEA	Clinical use	340.46	51-98-9	
		Norethindrone	NET	Clinical use	298.42	68-22-4	
		Tibolone	TIB	Clinical use	312.4	5630-53-5	
	<b>Gonanes</b>	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	
<b>Progestin structurally related to spironolactone</b>	Drospirenone	DRO	Clinical use	366.49	67392-87-4		
<b>Natural hormones and precursors</b>	Pregnenolone (progesterone precursor)	P5	Clinical use	316.48	145-13-1		
	17β-estradiol	E2	Clinical use	272.38	50-28-2		
	Testosterone	T	Clinical use	288,42	58-22-0		

**Table 2:** Calculated EC<sub>50</sub> and IC<sub>50</sub> (nM) in HELN-hPRB and U2OS-zfPR cells.

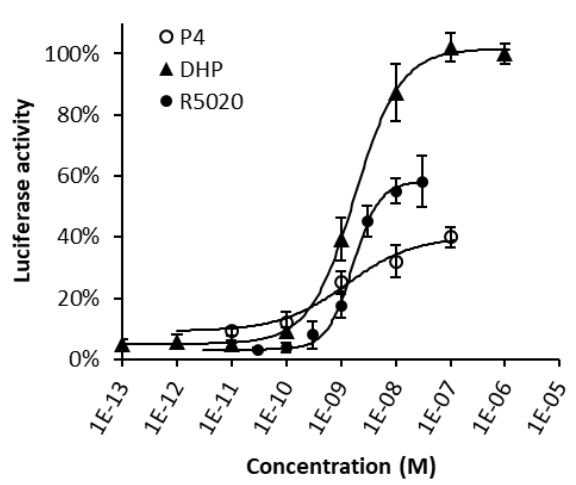
Classification	Abb	HELN-PRB			U2OS-zfPR				
		Agonism		Antagonism	Agonism			Antagonism	
		EC <sub>50</sub> ± sd (nM)	max ± sd (%)	REP	IC <sub>50</sub> ± sd (nM)	EC <sub>50</sub> ± sd (nM)	max ± sd (%)	REP	IC <sub>50</sub> ± sd (nM)
Reference compounds	P4	13.5 ± 7.14	70 ± 9	0.02	17.1 ± 15.7	1.97 ± 0.57	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 (EC<sub>50</sub>(R5020)/EC<sub>50</sub>(chemical)) and U2OS-zfPR cells (EC<sub>50</sub>(DHP)/EC<sub>50</sub>(chemical)). Standard deviations (sd); ne: no effect.

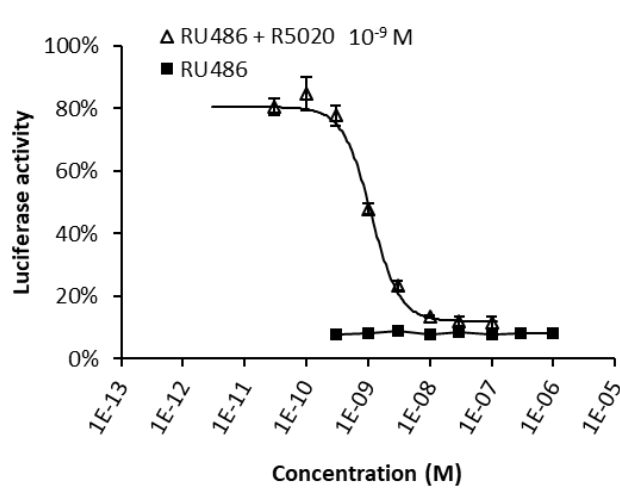
### (A) HELN-hPRB



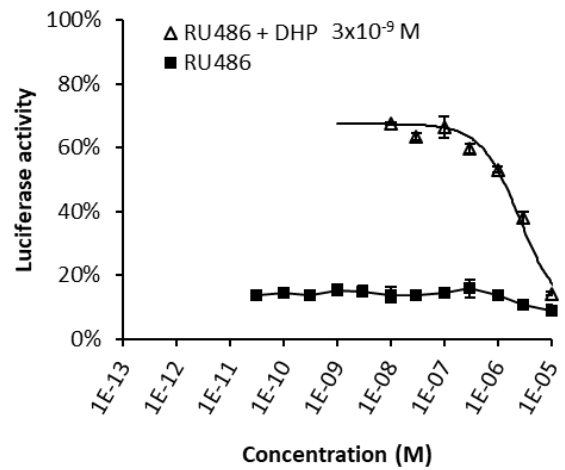
### (B) U2OS-zfPR



### (C) HELN-hPRB

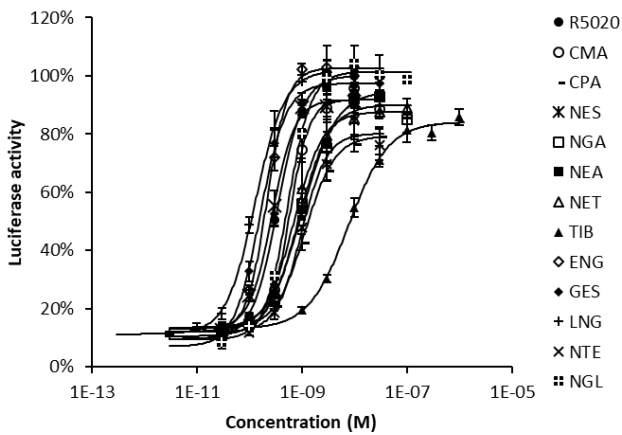


### (D) U2OS-zfPR

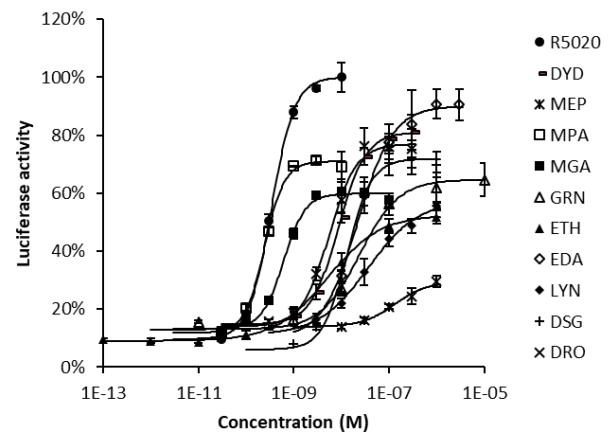


**Figure 1:** Dose-response curves of the activity of reference ligands in HELN-hPRB and U2OS-zfPR cells expressed as the percentage of the maximum luciferase activity induced by  $3 \times 10^{-8}$  M R5020 (A,C) or  $3 \times 10^{-8}$  M DHP (B,D). P4: progesterone; DHP:  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one;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

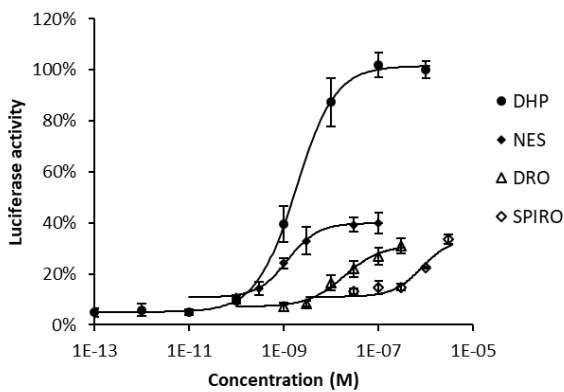
### (A) Full agonists on HELN-hPRB



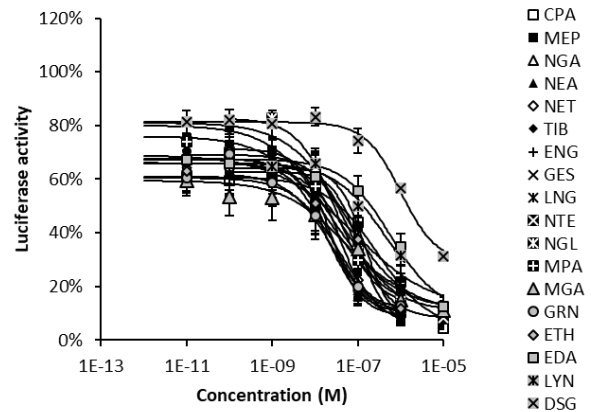
### (B) Partial agonists on HELN-hPRB



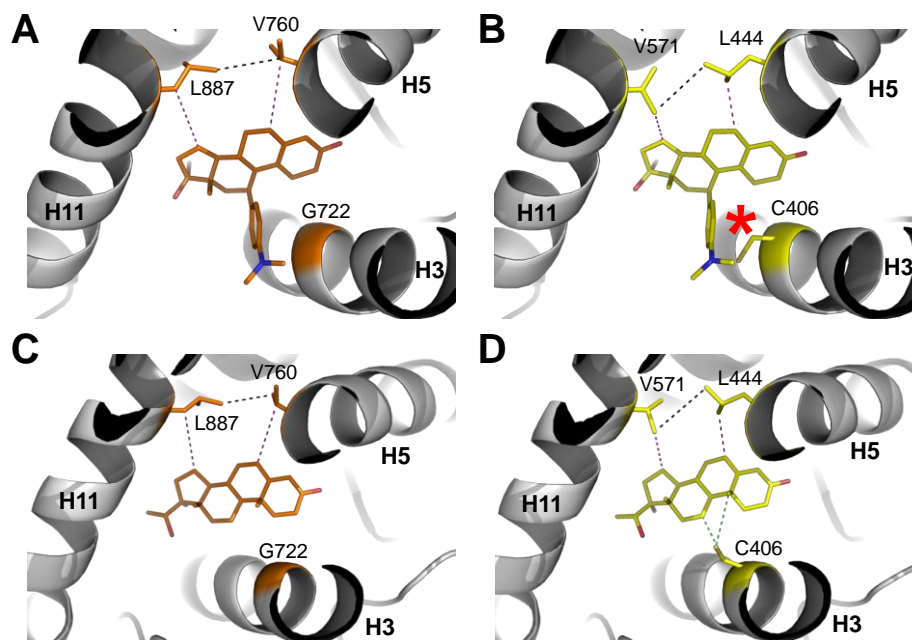
### (C) Agonists on U2OS-zfPR



### (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  $3 \times 10^{-8}$  M R5020 (A, B) or  $3 \times 10^{-8}$  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: nestorone; 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

Clémentine Garoche, Selim Aït-Aïssa, Abdelhay Boulahtouf, Nicolas Creusot, William Bourget, Patrick Balaguer, François Brion

#### Table of content

**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  $17\beta$ -estradiol (E2), testosterone (T) and 11-Ketotestosterone (11-KT).

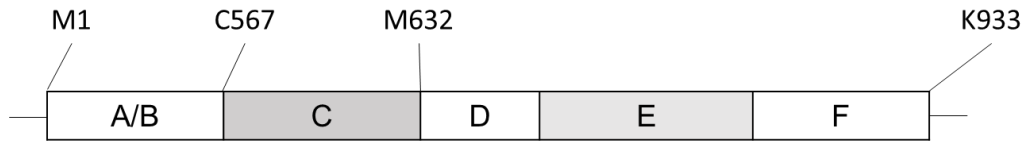
**Figure S3:** Concentration-response curves of the activity of progestins in U2OS cells expressing hPR (A) and HeLa cells expressing zfPR (B).

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

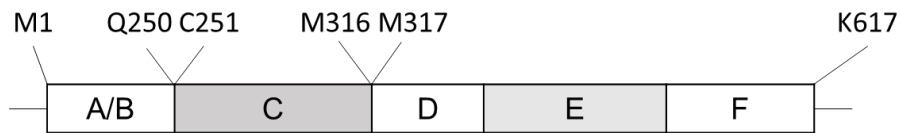
**Figure S5:** Activity of progestins in ZFL-zfPR cells.

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

**(A) HELN-hPRB**



**(B) U2OS-zfPR**

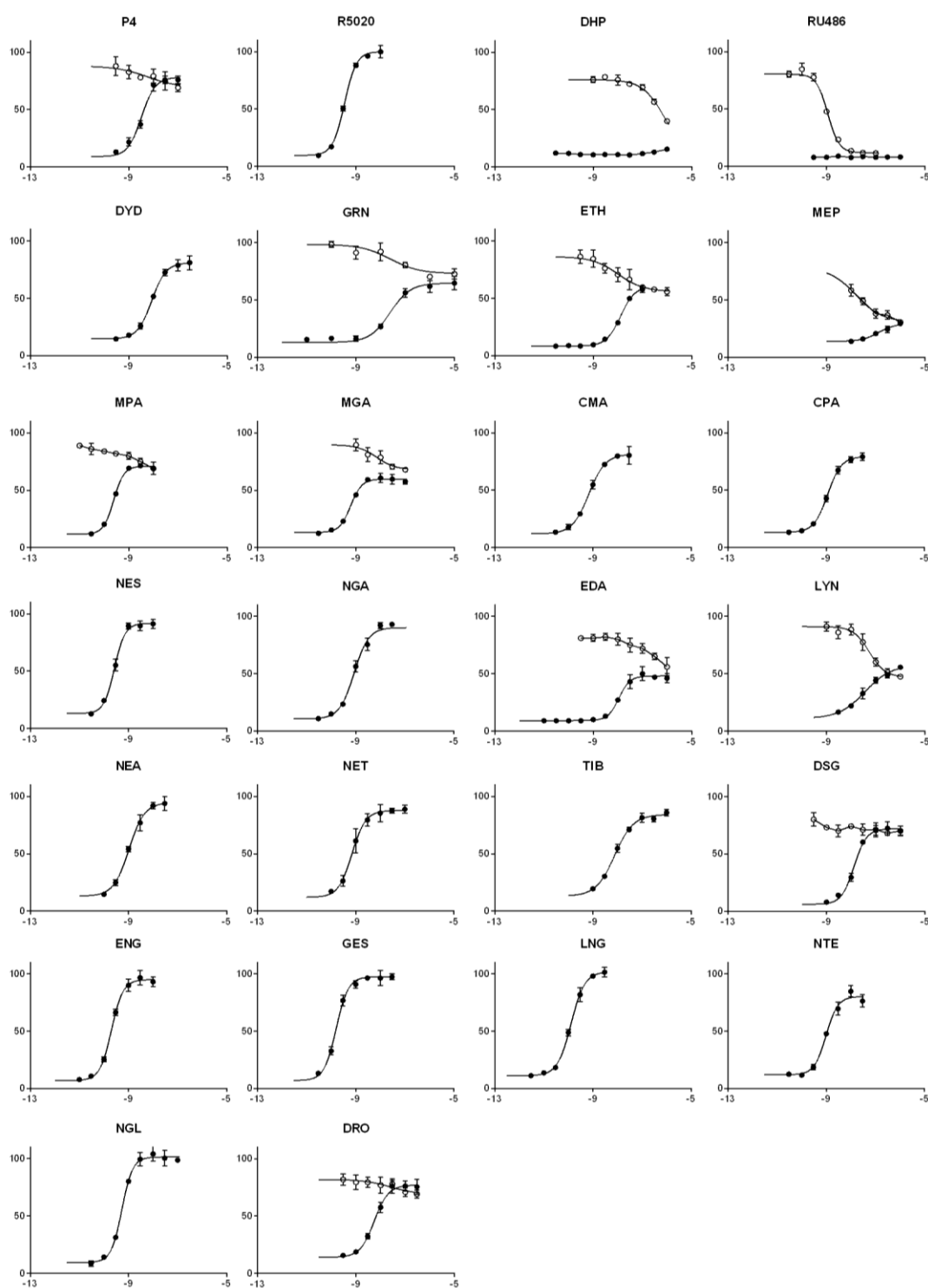


**Figure S1: Constructions of the cell lines HELN-hPRB (A) and U2OS-zfPR (B).** Each contains six domains. Domain A/B: activation factor; domain C: human estrogen receptor hER $\alpha$  DNA binding domain (C251-M316); domain D: hinge; domain E: hPR-B (A) or zfPR (B) ligand binding domain.



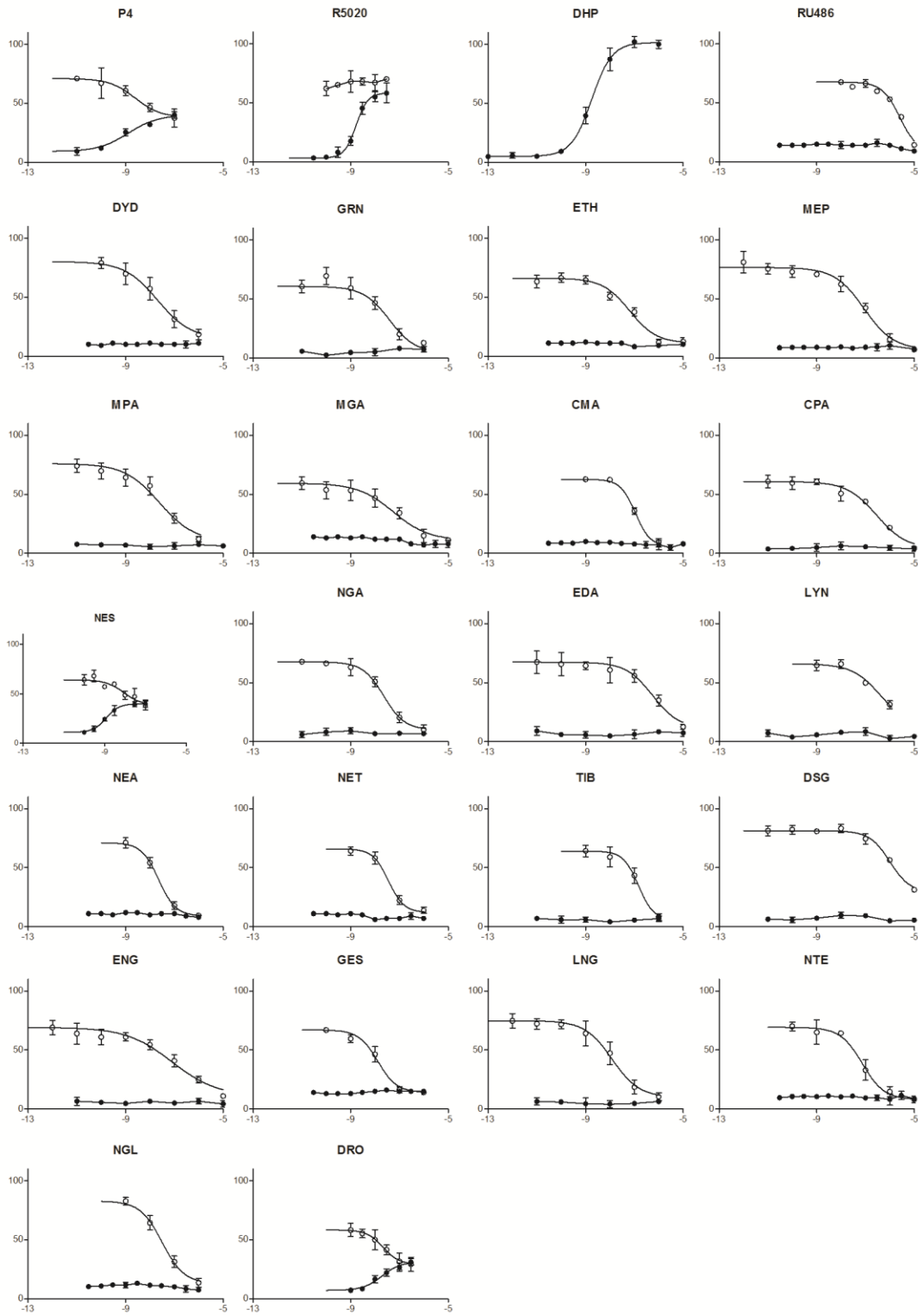
**Table S1: Typical concentration-response curves of the activity of progestins in HELN-hPRB and U2OS-zfPR cells.** Results are expressed as percentage of the maximum luciferase activity induced by  $3 \times 10^{-8}$  M R5020 in HELN-hPRB or  $3 \times 10^{-8}$  M DHP in U2OS-zfPR. Cells were treated 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  $3 \times 10^{-9}$  M respectively) (white circles). Error bars represent standard deviation.

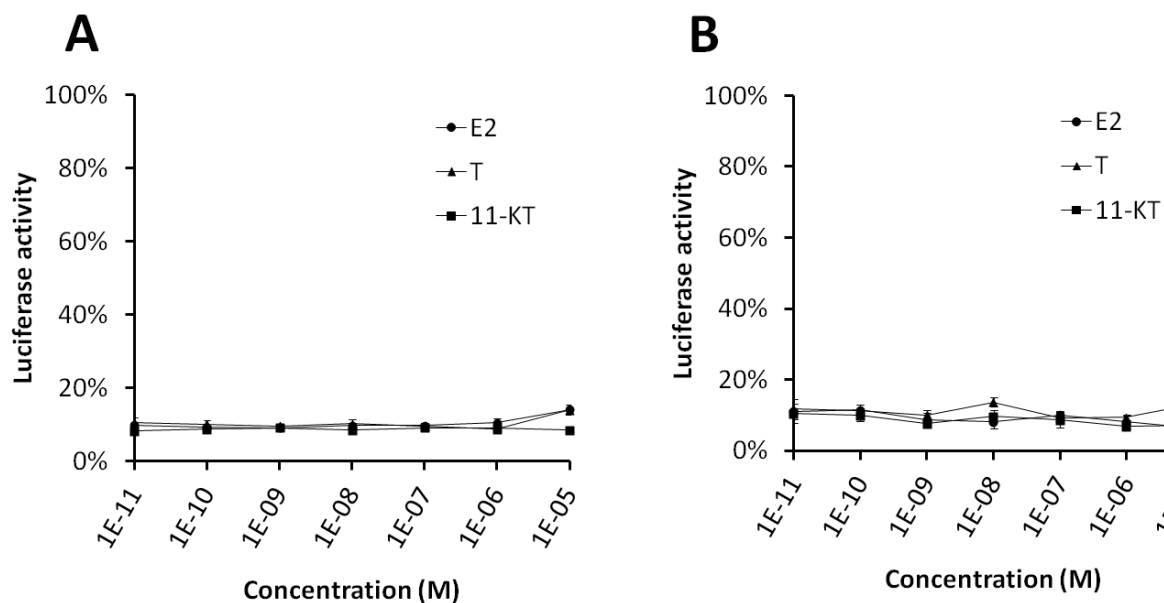
### HELN-PRB



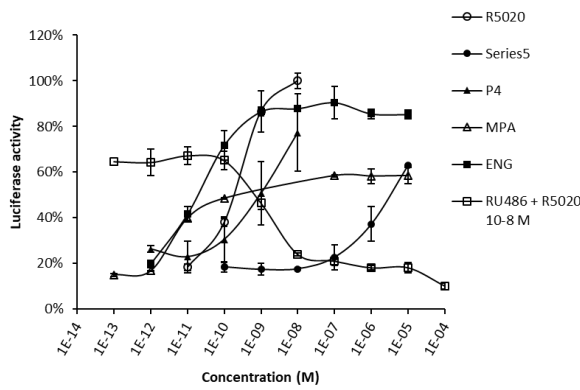
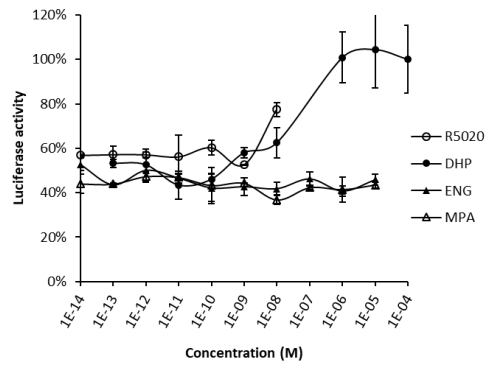
**Table S1 (continued)**

**U2OS-zfPR**

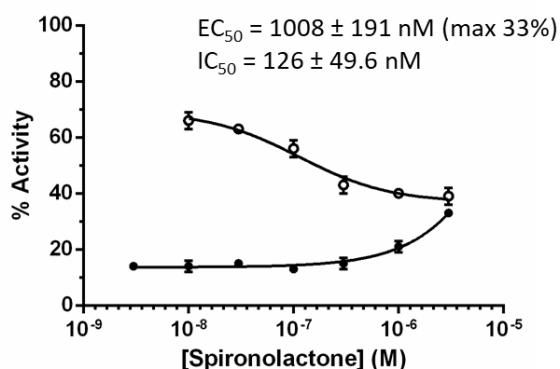
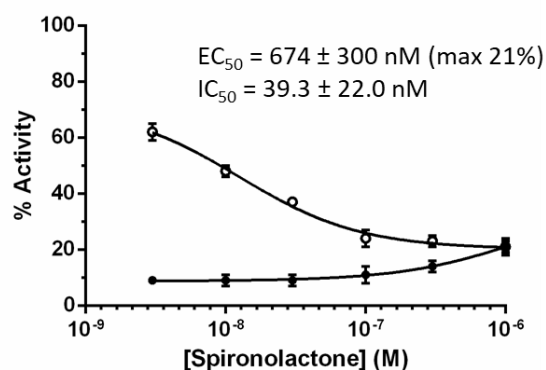
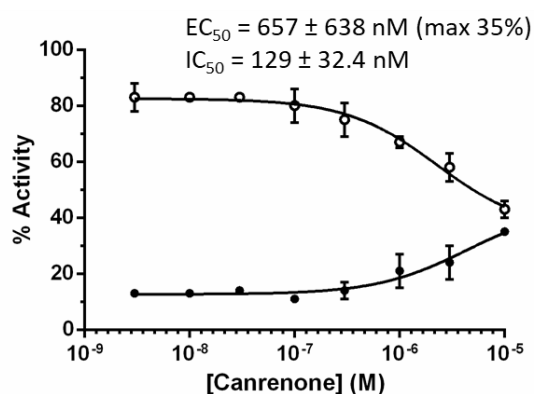
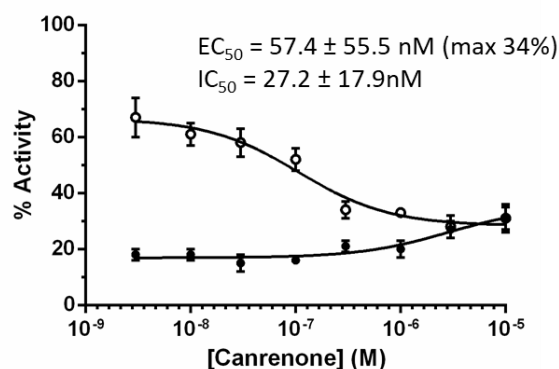




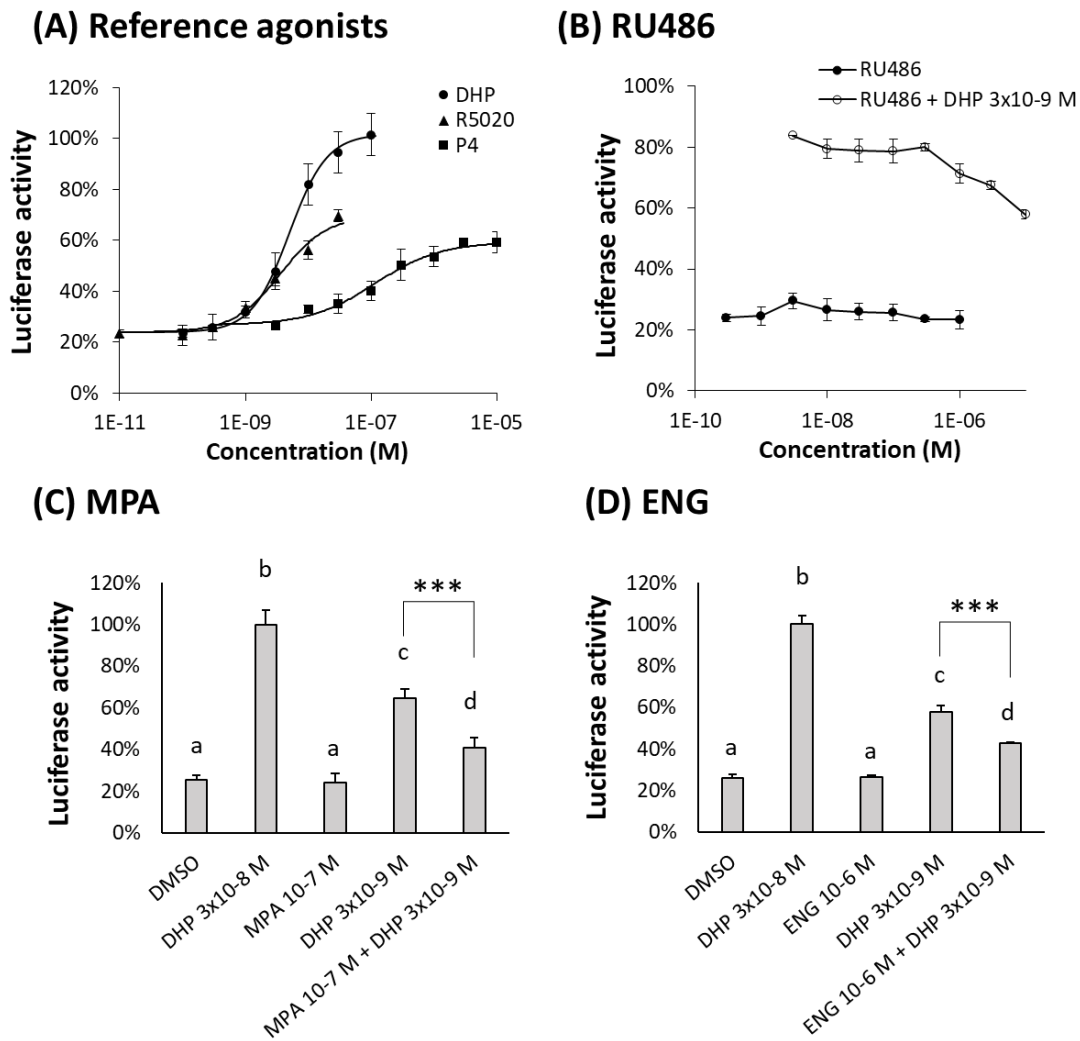
**Figure S2: HELN-hPRB cells (A) and U2OS-zfPR (B) cells were treated with indicated concentrations of 17 $\beta$ -estradiol (E2), testosterone (T) and 11-Ketotestosterone (11-KT). Results are expressed as the percentage of the maximum luciferase activity induced by  $3 \times 10^{-8}$  M R5020 (A) or  $3 \times 10^{-8}$  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  $3 \times 10^{-8}$  M R5020 (A) or  $3 \times 10^{-8}$  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 $\alpha$  DNA binding domain. HeLa cells were transiently cotransfected by a plasmid expressing ERE-luciferase and a second one expressing the zfPR with the hER $\alpha$  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  $3 \times 10^{-8}$  M R5020 in HELN-hPRB or  $3 \times 10^{-8}$  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  $3 \times 10^{-9}$  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  $3 \times 10^{-8}$  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 ( $3 \times 10^{-9}$  M). Error bars represent standard deviation. Letters correspond to significant differences between groups after t-test Student, \*\*\*  $p < 0.001$ .

hPRB (V633-K933)  
zfPR (M317-K617)

VLGGRKFKKFNKVRVVRALDAVALPQPVGVPNESQALSQRFTFSPGQDIQLIPPLINLLMS  
MLGGRKMRFAGLKVMGLSPSLMFQSPLSLLTDGQTLSSLPCMSAMRELQLSPQMISILEN  
\*\*\*\*\*

IEPDVIYAGHDNTKPDTSSSLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYS  
IEPQVVYSGYDNTQPEVPHLLLNSLNRLCERQLLWIVRWSKSLPGFRNLHINDQMTLIQYS  
\*\*\*\*\*

WMSLMVFGLGWRYSYKHVSGQMLYFAPDLILNEQRMKESSFYSLCLTMWQIPQEFVKLQVSQ  
WMGLMFLSLGWRTFQNVTPDYLYFAPDLVLSNDQLRRSPIYDLCLAMQFVPQEFANLQVTK  
\*\*\*\*\*

EEFLCMKVLLLLNTIPLLEGLRSQTQFEEMRSSYIRELIKAIGLRQKGVVSSSQRFYQLTKL  
EEFLCMKALMLLNTVPLEGLKSQTQFDEMRQNYICELSKAIQLKEKGVVASSQRFYHLTKL  
\*\*\*\*\*

LDNLHDLVKQLHLYCLNTFIQSRALSVEFPEMMSEVIAAQLPKILAGMVKPLL FHKK  
MDNMHEIVKKVNLYCLTTFIQADAMKVEFPEMMTEVIASQLPKVLAGMVKPLMFHHK  
\*\*\*\*\*

**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.