Effects of antiandrogenic progestins, chlormadinone and cyproterone acetate, and the estrogen 17 α ethinylestradiol (EE2), and their mixtures: Transactivation with human and rainbowfish hormone receptors and transcriptional effects in zebrafish (*Danio rerio*) eleuthero-embryos

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Abstract

Synthetic progestins act as endocrine disruptors in fish but their risk to the environment is not sufficiently known. Here, we focused on an unexplored antiandrogenic progestin, chlormadinone acetate (CMA), and the antiandrogenic progestin cyproterone acetate (CPA). The aim was to evaluate whether their *in vitro* interaction with human and rainbowfish (*Melanotaenia fluviatilis*) sex hormone receptors is similar. Furthermore, we investigated their activity in zebrafish (*Danio rerio*) eleuthero-embryos.

First, we studied agonistic and antagonistic activities of CMA, CPA, and 17 α -ethinylestradiol (EE2), in recombinant yeast expressing either the human progesterone (PGR), androgen (AR), or estrogen receptor. The same compounds were also investigated *in vitro* in a stable transfection cell system expressing rainbowfish nuclear steroid receptors. For human receptors, both progestins exhibited progestogenic, androgenic and antiestrogenic activity with no antiandrogenic or estrogenic activity. In contrast, interactions with rainbowfish receptors showed no progestogenic, but antiandrogenic, antiglucocorticoid, and some antiestrogenic activity. Thus, interaction with and transactivation of human and rainbowfish PGR and AR were distinctly different.

Second, we analyzed transcriptional alterations in zebrafish eleuthero-embryos at 96 and 144 hours post fertilization after exposure to CPA, CMA, EE2, and binary mixtures of CMA and CPA with EE2, mimicking the use in oral contraceptives. CMA led to slight down-regulation of the *ar* transcript, while CPA down-regulated *ar* and *pgr* transcripts. EE2 exposure resulted in significant transcriptional alterations of several genes, including *esr1*, *pgr*, *vtg1*, *cyp19b*, and gonadotropins (*fshb*, *lhb*). The mixture activity of CMA and EE2 followed the independent action model, while CPA and EE2 mixtures showed additive action in transcriptional alterations.

Third, we analyzed the interactions of binary mixtures of CMA and CPA, and of CMA and EE2 to analyze for their joint activity *in vitro* and in eleuthero-embryos. Both mixtures behaved according to the concentration addition model in their interaction with human and rainbowfish receptors often showing antagonism. In zebrafish eleuthero-embryos, binary mixtures of CMA and EE2 showed the same expression patterns as EE2 alone, indicating an independent action *in vivo*.

Our study demonstrates that CMA and CPA interact distinctly with human and rainbowfish receptors, suggesting that activities of these and possibly additional environmental steroids determined with yeast expressing human receptors cannot simply be translated to fish. The lack of agonistic activities of both progestins to rainbowfish PGR and AR is the probable reason for the low activity found in zebrafish eleuthero-embryos.

1.Introduction

Endocrine disrupting compounds (EDCs) can act by different modes of action. They can bind as agonists, partial agonists, or antagonists to steroid hormone receptors. They can also interfere on different levels of the hormone signaling pathway, including synthesis, transport, or metabolism. The hypothalamic-pituitary-gonadal axis seems to be the main target of EDCs, affecting reproduction of mammals and fish.

A very potent class of EDCs are synthetic and natural hormones (Sumpter, 2005). While many EDCs act as estrogens, others may exhibit different steroid activities. Antiandrogens, for instance, may reduce plasma androgen levels in organisms or act by blocking of the androgen activity. Androgen receptor (AR) antagonists bind to the AR but elicit no biological effect, probably because the antiandrogen-AR complex does not bind to androgen response elements on the DNA due to conformational differences compared to the androgen-AR complex (Kelce et al., 1998). Most of the antiandrogens are partial AR agonists (Singh et al., 2000).

An important but not yet well studied class of EDCs are progestins (also called progestogens or gestagens) that bind and activate human progesterone receptors (PGR). Among them are natural hormones and synthetic progestins, which are part of oral contraceptive pills (alone and in combination with an estrogen) and hormone replacement therapeutics (Africander et al., 2011). In some countries, they are used as growth promoters in animal farming (Fent, 2015). Progesterone (P4) has mainly progestogenic activity but antiestrogenic, antimineralocorticoid, and antiandrogenic activities are also observed (Schindler et al., 2008). In mammals, progestins mainly bind to nuclear progesterone receptors (PGR α and PGR β) and mediate effects through transcriptional changes, although they also mediate their effects through the membrane PGR. Most synthetic progestins show a higher binding affinity to nuclear PGR than P4 (Kloosterboer et al., 1988). Although designed to bind specifically to the human PGR, progestins show some cross-reactivity with other nuclear steroid receptors (Sitruk-Ware and Nath, 2010).

The nuclear PGRs of several teleost fish, including zebrafish, have been sequenced. They show high homology to other vertebrates. Contrary to mammals and humans, the most predominant and active progestins in fish are 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) and 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S) (Nagahama, 1997). P4 binds to the fish nuclear PGR but with a lower affinity. The ligand-binding domain of fish nuclear PGR differs from that of mammals, and therefore its binding affinities to progestins are also influenced. In transactivation assays, many progestins did not bind to the fish nuclear PGR or bound with very low affinity. They also showed cross-reactivity with other nuclear sex steroid receptors (Bain et al., 2015a; Ellestad et al., 2014; Pinter and Thomas, 1997). Similarly, progestins

may have different interactions with human and fish AR (Bain et al., 2015a, 2015b; Ellestad et al., 2014).

Some progestins were found to have high endocrine disruption potency in fish (Fent, 2015; Kumar et al., 2015). Exposure to low ng/L levels of P4, levonorgestrel, norethisterone, and mifepristone (an antiprogesterone) as found in surface water led to transcriptional changes in genes involved in hormone signaling and steroidogenesis in zebrafish eleuthero-embryos at 48 and 144 hours post fertilization (hpf) (Zucchi et al., 2012). P4 (Blüthgen et al., 2013a; Liang et al., 2015b), levonorgestrel (Overturf et al., 2014), norgestrel (Liang et al., 2015a, 2015b), dydrogesterone (Zhao et al., 2015a), medroxyprogesterone acetate (Zhao et al., 2015a), and drospirenone (Rossier et al., 2016) altered gene expression profiles in different fish species. Transcriptomics revealed that additional genes are altered by progestins, including genes of the circadian rhythm and cell cycle regulation (Zhao et al., 2015b; Zhao and Fent, 2016; Zucchi et al., 2014, 2013). Embryos exposed to levonorgestrel, norgestrel, or P4 for a longer period (1 to 2 months) showed reduced growth and survival (Overturf et al., 2014) and altered sex ratios (Liang et al., 2015a, 2015b). Currently, the effects of CMA and CPA in fish early life stages are unknown.

Chlormadinone acetate (CMA) and cyproterone acetate (CPA) are synthetic progestins structurally related to progesterone (17 α -hydroxy-derivatives). CMA is used in hormone replacement therapy, and in combination with EE2, in oral contraceptive pills. CPA is used for the treatment of androgenization in women (in combination with EE2 in oral contraception) (Schneider, 2003), chemical castration in men (Bradford, 1999), and certain prostate cancers (Mahler et al., 1998). Both of them are potent PGR agonists in humans but also exhibit antiandrogenic effects by acting as AR antagonistst (Térouanne et al., 2002). The *in vitro* antiandrogenic activity of both compounds is in the same range (at about 1 $\times 10^{-8}$ M), and at higher concentrations, CMA exhibited partial agonistic activity on the AR (Térouanne et al., 2002). CMA has antiestrogenic and glucocorticoid activity in mammals (Schindler et al. 2008). They also act by competitive inhibition of the 5 α -reductase, which converts testosterone to 5 α -dihydrosterosterone (DHT), and by suppressing gonadotropin secretion via negative feedback loops (antigonadotropic activity), hence inhibiting the androgen synthesis in gonads and adrenal glands (Raudrant & Rabe 2003), which results in decreased levels of circulating androgens. Furthermore, CPA has antiestrogenic and glucocorticoid activity (Schindler et al. 2008).

Effects of CMA in fish are unknown. CPA binds to the AR in goldfish but not in rainbow trout (Martinović et al., 2008; Wells and Van Der Kraak, 2000; Wilson et al., 2007). At μ g/L concentrations, CPA led to intersex and gonadal abnormalities, changes in plasma concentrations of sex steroids, and alterations of male parental behavior (Collette et al., 2010; Dey et al., 2010; Kiparissis et al., 2003; Linderoth et al., 2006). Even at lower

1 concentrations (ng/L), plasma sex steroid concentrations were altered (Sharpe et al., 2004).
2 CPA altered oocyte maturation in rainbow trout (Rime et al., 2015), and vitellogenin levels
3 were reduced in female fathead minnows at 200 µg/L CPA, possibly mediated by its
4 antagonistic effects and associated lowered plasma sex steroids (Ankley et al., 2010).
5

6 The sales figures of active ingredients of CPA and CMA are among the highest of all
7 progestins in Switzerland; the annually sold and consumed amount in 2010 was 50 kg and
8 10 kg, respectively (Fent, 2015). CMA consumption was mainly related to contraceptive use,
9 while CPA was also applied for its antiandrogenic activity for human therapeutic use. In
10 France, CPA showed highest relative consumption (Besse and Garric, 2009), and in the
11 United Kingdom, the consumption was 278 kg CPA in 2009 (Runnalls et al., 2010). Currently,
12 there are no data on CMA levels in the environment (Fent, 2015), and CPA has been
13 determined in animal farm waste in China only (Liu et al., 2014). In Switzerland, progestins
14 including CMA and CPA are not used in farm animals.
15

16 The aim of this work was to assess and compare the activity of CPA and CMA *in vitro*
17 in transactivation assays in humans and fish. To test their similar or dissimilar interaction with
18 receptors, we compared their transactivation with human or rainbowfish nuclear steroid
19 receptors in recombinant cell systems. Recently, we analyzed effects of mixtures of the
20 progestin drospirenone and EE2 in zebrafish embryos (Rossier et al., 2016). Transcriptional
21 effects of these binary mixtures were mainly dominated by EE2 indicating an independent
22 action. Thus, an additional aim of our present work was to assess the activity of binary
23 mixtures of the progestins CMA and CPA, as well as CMA and CPA with EE2, which mimic
24 their use in contraceptive pills, both in the different *in vitro* systems and *in vivo* in zebrafish
25 eleuthero-embryos. Thereby, we tested whether the different steroids act according to the
26 concentration addition or independent action model and compared the *in vitro* with *in vivo*
27 reactions.
28

29 A further aim was to compare the *in vitro* with the *in vivo* activity in zebrafish eleuthero-
30 embryos. We examined transcriptional effects of single compounds and binary mixtures of
31 CMA and EE2. Based on our previous findings for binary mixtures of progestins (Rossier et
32 al., 2016; Zhao et al., 2015a; Zucchi et al., 2014), we focused on genes involved in hormonal
33 pathways, such as those encoding nuclear receptors, and enzymes of the steroid hormone
34 biosynthesis. We hypothesized that CMA and CPA alter gene expression levels of genes
35 involved in progestogenic and antiandrogenic action. For binary mixtures of CMA and CPA
36 with EE2, we hypothesized an independent action due to their different action on signaling
37 pathways.
38

2. Materials and methods

Chemicals and materials used are described in the Supplementary Material (SM).

2.1 Yeast expressing human receptors

To assess the agonistic and antagonistic activities of CPA, CMA, and EE2 as single compounds and mixtures, we used three different recombinant yeast (*Saccharomyces cerevisiae*) systems expressing human nuclear steroid hormone receptors, including estrogen and androgen receptor, kindly provided by J. Sumpter, Brunel University (U.K.), and progesterone receptor, kindly provided by Z. Wang (Chinese Academy of Science, Peking). We assessed the estrogenic and antiestrogenic activity (YES) according to Routledge and Sumpter (1996), androgenic and antiandrogenic activity (YAS) according to Sohoni and Sumpter (1998), and progestogenic activity (YPS) according to Gaido et al. (1997) and Lorenzen et al. (2004).

Following the assessment of concentration-response curves of individual substances, we calculated the equieffective concentrations EC50, EC25, and EC10 using the inverse function of the corresponding Hill equation. We then combined the compounds at equieffective concentrations to generate the EC50, EC25, and EC10 of binary mixtures. Equieffective mixtures contain substances at concentrations leading to the same effect but concentrations may differ depending on their potency. All mixture assays were carried out identically to the single compound assays. Details on the experimental setting and mixture calculations are provided in the SM.

We serially diluted stock solutions and chemical mixtures in ethanol and transferred 10 μ L per well to 96-well optically flat-bottomed cell culture plates (ethanol concentration was 4 % but was evaporated within 30 minutes). For single compounds, each plate contained a row of blanks (medium only) and solvent control (ethanol) followed by serial dilutions of positive control and single compounds in duplicates. For mixture experiments, we changed the plate design a little, to have half a row for both blank and ethanol control, one row of positive control, and serial dilutions of mixtures (EC50, EC25, EC10) in duplicates. In the end, we measured absorbance at 540 and 620 nm (YES and YAS) and at 420 and 600 nm (YPS), respectively. We independently repeated the experiments at least three times.

To assess the antagonistic activity in YES and YAS, we used the procedure of Sohoni and Sumpter (1998) with slight modifications. We added the corresponding agonist concentrations (between 1×10^{-8} to 4.88×10^{-12} M 17 β -estradiol [E2] and 5×10^{-8} to 2.44×10^{-11} M DHT, respectively) to the final assay medium (except to the row of blank medium) to produce a submaximal response. This response was set to 65% of maximal transactivation response (which was set to 100%). This was made before seeding the plates.

2.2 Analysis of mixture activity

We calculated mixture effects on the basis of the Hill regression models for each compound using the concept of **concentration addition (CA)** (Loewe and Muischnek, 1926). The concentration addition model assumes that the compounds have the same mode of action and can be replaced by each other. Each compound adds individually to the mixture in proportion to its concentration. The CA model is used for evaluation of mixture activity of compounds of the same class having a similar mechanism of action (Loewe and Muischnek, 1926). To calculate mixture effect estimations of toxicity, mixture ratios (relative abundance of chemicals in mixture) and dose/concentration-response curves of single substances are used. The following Equation 1 applies:

Equation 1. Concentration addition model

$$\sum_{i=1}^n \frac{c_i}{EC_i} = 1$$

Key to equation 1: c_i : concentration of the compound i in a mixture leading to an effect E ; EC_i : the concentration of i needed to get the same effect on its own.

According to Equation 2, we used the EC_{50} values and Hill slopes of the individual substances to calculate additive activity (Olmstead and LeBlanc, 2005).

Equation 2. Calculation according to the CA model

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^n \frac{c_i}{EC_{50_i}}\right)^{hs}}}$$

Key to equation 2: R : response to mixture; c_i : concentration of the compound i in a mixture leading to 50% effect; EC_{50_i} : concentration of a compound i needed to get 50% effect on its own; hs : Hill slope.

In addition, we analyzed the mixture behavior with the method of isoboles (Loewe and Muischnek, 1926) and the toxic unit approach (Kortenkamp and Altenburger, 1998; Sprague and Ramsay, 1965). The **method of isoboles** is a graphical method to test interactions of substances in mixtures for additivity, synergism, or antagonism. We constructed graphs, so-called isobolograms, by plotting equieffective doses/concentrations of two substances in a coordinate system. The axes represent concentrations of the two substances. We connected the equieffective concentrations with a line forming the isobole (additivity line). Additionally, we included the 95%-confidence interval to get an additivity band. We then plotted the activity from our mixture assays in the coordinate system. When the dot fell within the additivity band, the binary mixture was assumed to behave additively. When it fell below the lower confidence interval line, synergism was assumed, and when it fell above the upper confidence interval line, antagonism was assumed. The isobole method assumes that the compounds show analogous dose/concentration-response curves, i.e., similar Hill slope and maximal response (Tallarida, 2006). When these requirements are not fulfilled, the **toxic unit**

approach can be used instead. For this method, additivity in a mixture is given, if Equation 1 equals 1. If compounds in a mixture influence each other, the result of Equation 1 deviates from 1, where a value < 1 indicates synergism and a value > 1 indicates antagonism. To correct for variations in our data, we added a margin of error of ± 0.5 resulting in an additive behavior if $0.5 \leq \text{toxic unit value} \leq 1.5$.

2.3 *In vitro* reporter gene assays expressing rainbowfish nuclear receptors

The agonistic and antagonistic activities of CPA, CMA, and EE2 were further assessed using cell-based luciferase reporter assays based on nuclear hormone receptors from Murray-Darling rainbowfish (*Melanotaenia fluviatilis*). These assays were generated using the human-derived osteosarcoma cell line U-2 OS, which expresses low levels of endogenous steroid hormone receptors and is therefore well suited to the development of reporter gene assays with low background signal (Quaedackers et al., 2001; Sonneveld et al., 2005). Stable reporter gene assays were developed using dual antibiotics selection to ensure the maintenance of the transfected receptor expression vector and along with a selective luciferase reporter construct. Construction of the rainbowfish androgen receptor β (rfAR β) and progestogen receptor (rfPGR) stable reporter gene assays has been previously described (Bain et al., 2015b). Similar assays for rainbowfish estrogen receptor α (rfER α) and glucocorticoid receptor 2 (rfGR2) were developed using pcDNA3.1-based expression constructs and pGL4.26 luciferase reporter vectors containing suitable response elements (Bain et al., manuscripts in preparation).

The agonistic activity of the selected compounds in the rainbowfish reporter gene assays was determined essentially according to the method of Bain et al. (2015b). Briefly, cells were seeded into 96-well plates at a density of 10,000 cells/well in 100 μ L exposure media (phenol red-free DMEM-F12 medium supplemented with 5% dextran-coated charcoal-stripped fetal bovine serum [DCC-FBS], Eagles non-essential amino acids, 20 U/mL penicillin and 20 μ g/mL streptomycin) and incubated overnight in a humidified atmosphere at 37 °C and 5% CO₂. Stock solutions of test compounds were prepared in dimethylsulfoxide (DMSO) and serially diluted in DMSO before adding to exposure medium at a dilution rate of 1 in 1,000 (0.1%). Solvent controls contained 0.1% DMSO. Overnight growth media were removed prior to adding 100 μ L/well exposure media containing various concentrations of test compound. Exposures were performed in triplicate wells for each concentration of test compound, and each plate contained four sets of triplicate solvent control wells (a total of 12 solvent control wells per plate). Each experiment was repeated at least three times on separate occasions.

Antagonistic activity was assessed in a similar way, but the dilutions of test compound were prepared in exposure media containing a suitable agonist at a concentration corresponding to 75% of the maximal response (EC75;E2 for rER α , 6.16×10^{-8} M 11-ketotestosterone (11-KT) for rAR β , 1.55×10^{-8} M DHP for rPGR, and 1.3×10^{-9} M dexamethasone for rGR2). Additional controls comprising of exposure media lacking the agonist were included on each plate (12 wells) to establish background levels for each assay.

Concentration-response curves (Hill equation) were fit to background-subtracted, percent-normalized data using nonlinear regression with the aid of Prism ver. 6.07 software (GraphPad Software, La Jolla, CA, U.S.A., www.graphpad.com).

2.4 Zebrafish culture and embryo collection

Adult zebrafish maintenance was conducted as described previously (Blüthgen et al., 2013a). In brief, we obtained adult zebrafish (*D. rerio*) from Harlan Laboratories, Inc. (Itingen, Switzerland) and kept them in big fish culture tanks (300 L water tanks with 240 L water) containing reconstituted de-ionized water (CaCl $_2$ *2H $_2$ O [147.0 g/L], KCl [2.9 g/L], MgSO $_4$ *7H $_2$ O [61.6 g/L], NaHCO $_3$ [32.4 g/L]) with a conductivity of 470 to 480 μ S/cm and a temperature of 27 $\pm$ 1 °C. We exchanged the water and checked the water parameters (nitrite, nitrate, pH, oxygen concentration) once a week. We fed the fish twice daily with TetraMin XL flakes (Tetra GmbH, Melle, Germany) and twice per week with frozen white mosquito larvae and brine shrimps (*Artemia salina*), and living *Daphnia magna*. The light-dark cycle was 14:10 h. In the evening before starting the embryo experiments, we placed stainless steel spawning trays into the big fish tank. Spawning trays measured 265 x 185 x 30 mm and were covered by a stainless steel grid (mesh size: 2.5 mm). We collected the eggs about 1.5 h after onset of light and rinsed them in reconstituted fish water. We inspected the eggs under a stereomicroscope (Stemi DV4, Carl Zeiss AG, Feldbach, Switzerland) and selected healthy fertilized eggs for the experiment. At the age of about 2-4 hpf (blastula-stage), embryos were placed in exposure water.

2.5 Exposure

Zebrafish embryos were placed either in glass beakers containing 100 mL of water or in plastic Petri dishes containing 25 mL of water. The containers were incubated at 27 $\pm$ 1 °C and 70% humidity for up to 144 hpf. Every 24 h, we exchanged and renewed the exposure water, inspected the embryos for appearance and mortality, and removed dead embryos.

We conducted two separate experiments for the single compounds, one with CMA and one with CPA. For both compounds we performed a second experiment in order to cover a considerable concentration range. Both experiments consisted of eight treatment groups, including six concentrations (nominal concentrations of 1, 10, 100, 1,000, 10,000,

100,000 ng/L) of appropriate progestin, water control, and solvent control (0.01% DMSO), with five replicates each. For experiments with CMA and CPA, we selected 25 embryos at 2-4 hpf and placed them into covered plastic Petri dishes containing 25 mL reconstituted fish water and the corresponding amount of progestin. At 144 hpf, we collected 15 embryos per replicate, pooled them in RNAlater and stored them at -80 °C for later molecular analysis.

For our second experiment with CMA alone, we selected 40 embryos at 2-4 hpf and placed them in covered 150 mL glass beakers containing 100 mL reconstituted fish water and corresponding amount of steroid. This experiment consisted of four CMA concentrations of CMA (nominal concentrations of 10, 100, 1,000, 10,000 ng/L), and a solvent control (0.01% DMSO). Four replicates per treatment were used. At 96 hpf and 144 hpf, we collected 10 to 15 embryos per replicate, pooled them in RNAlater and stored them at -80 °C for later molecular analysis.

For our experiments with binary mixtures of CMA and EE2, we selected 50 embryos, and for the mixtures of CPA and EE2, 100 embryos, at 2-4 hpf and placed them in covered 150 mL glass beakers containing 100 mL reconstituted fish water and corresponding amount of steroid. This experiment consisted of four concentrations of EE2 alone (nominal concentrations of 10, 100, 500, 1,000 ng/L), four binary mixtures of EE2 and CMA (nominal concentrations of 10 + 100, 100 + 1,000, 1,000 + 10,000, 100 + 100 ng/L), and a solvent control (0.01% DMSO). For the binary mixtures of CPA and EE2, the experiment consisted of four binary mixtures of EE2 and CPA (nominal concentrations of 10 + 100, 100 + 100, 100 + 1,000, 1,000 + 10,000 ng/L), and a solvent control (0.01% DMSO). Exposure concentrations were determined by chemical analysis (Table 2, supplementary material). Each treatment consisted of four replicates per treatment. At 96 hpf and 144 hpf, we collected 10 to 15 embryos per replicate, pooled them in RNAlater and stored them at -80 °C for later molecular analysis. Each treatment consisted of four replicates per treatment. At 96 hpf and 144 hpf, we collected a pool of 10 to 30 embryos per replicate, pooled them in RNAlater or froze them in liquid nitrogen (in case of embryos of mixture of CPA and EE2), and stored them at -80 °C for later molecular analysis.

We selected the exposure concentrations of CMA and CPA based on concentrations of previous progestin experiments that showed reproductive and transcriptional effects (Rossier et al., 2016; Zhao et al., 2015a; Zucchi et al., 2014, 2013, 2012). EE2 concentrations were chosen based on our previous study showing transcriptional effects (Rossier et al., 2016). We chose the lowest concentrations of progestins and EE2 based on expected environmental levels, and higher concentrations based on pharmacologically active values (Schindler et al., 2008).

2.6 Gene expression analysis

RNA extraction. At each sampling time point, 10 to 15 embryos per replicate (n = 4-5) were pooled, submerged in RNAlater, and stored at -80 °C for later gene expression analysis. Total RNA was extracted from larvae using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). To remove DNA contaminations, we included a step with the RNase-free DNase set (Qiagen, Basel, Switzerland). RNA concentrations and quality were spectrophotometrically determined using a NanoDrop 1000 spectrophotometer at 260 nm (ND-1000 spectrophotometer, NanoDrop Technologies Inc., Wilmington, DE, USA). Purified RNA was stored at -80 °C.

RT-qPCR analysis. For cDNA synthesis, 1 µg of RNA and RNase-free water (total volume of 14 µL) were incubated with 1 µL of random hexamers at 70 °C for 5 min. Then, 10 µL of a mastermix solution, consisting of 5 µL M-MLV 5x reaction buffer, 3.35 µL RNase-free water, 1.25 µL dNTPs, and 0.4 µL M-MLV reverse transcriptase, were added to each sample. The samples were incubated at 37 °C for 50 min (reverse transcription) and subsequently for 5 min at 95 °C to stop the reaction. The cDNA samples were stored at -20 °C.

For RT-qPCR, each sample contained 1 µL of cDNA, 9.5 µL nuclease free water, 2 µL of appropriate primer mix (forward and reverse primer), and 12.5 µL FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland), as recommended by the manufacturer's guidelines. The program we used consisted of 10 min at 95 °C for enzyme activation, followed by 40 cycles of transcript amplification (15 s at 95 °C, 60 s at 60 °C, plate read), and then the temperature was increased gradually in 0.5 °C steps every 5 s with plate read for melting curve analysis. All qPCR analyses were performed on BIORAD CFX96™ real-time PCR detection system (BIO-RAD, Switzerland).

We selected several genes including hormone receptors, steroidogenic enzymes, circadian rhythm genes and metabolizing enzymes based on mechanistic considerations and previous data, and the reference gene *β-actin* for normalization (Rossier et al., 2016; Zhao et al., 2015a). We evaluated melting curves to ensure the specificity of the qPCR products. For analysis of the qPCR data, we performed relative quantification using the $\Delta\Delta C_t$ -method (Livak and Schmittgen, 2001). The mRNA expression levels of target genes were expressed as fold changes (log2) according to the formula $2^{-\Delta\Delta C_t}$, with $\Delta\Delta C_t = (C_t(\text{target gene, treated}) - C_t(\text{reference gene, treated})) - (C_t(\text{target gene, control}) - C_t(\text{reference gene, control}))$. Details on the used primers are in the SM (Table S1).

2.7 Chemical analysis

The analysis was performed by solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) modifying an analytical method described previously (Zucchi et al., 2014). Mass spectrometric analysis was done using an

API5500 triple quadrupole (AB-Sciex, Thornhill, Ontario, Canada) equipped with a turbo ion spray source (Applied Biosystems – Sciex, Thornhill, Ontario, Canada). Analyses were performed both in negative ion mode for EE2, using estradiol-D3 as internal standard (IS), and in positive mode for the other analytes, using medroxyprogesterone (MPA) as IS. Recoveries in the investigated matrix were 91.3 ± 3.8 for chlormadinone, 69.7 ± 1.3 for CMA, 76.5 ± 0.5 and 90.6 ± 5.1 for CPA and EE2, respectively. The recovery of the IS was 101.5 ± 5.1 as previously reported (Zhao et al., 2015b). The limits of quantification of the method were calculated directly from samples as the value giving a signal to noise ratio (S/N) of 10 and were 0.25 ng/L for chlormadinone and CMA, 0.38 ng/L for CPA, and 2.16 ng/L for EE2. Detailed information about the analytical procedures used for chemical analysis are provided in the SM.

2.8 Statistical analysis and graphical illustration

For statistical analysis and graphical illustration of our data, we used Prism ver. 6.00 (GraphPad Software, La Jolla, CA, U.S.A., www.graphpad.com). We designed heat maps using the MultiExperimental Viewer v4.9 (<http://www.tm4.org/mev.html>; Dana-Farber Cancer Institute, Boston, MA, USA). We analyzed RT-qPCR data using one-way analysis of variance (one-way ANOVA) with subsequent post-hoc Bonferroni test for multiple comparison of differences between treatment and respective control. Results are shown as mean $\pm$ standard deviation (SD) of mean, unless otherwise indicated. We set the significance level α to 0.05 (95% confidence interval).

3. Results

3.1 *In vitro* activities of single compounds in human receptor transactivation systems

CMA and CPA exhibited complete concentration-response curves, indicating progestogenic, androgenic, and antiestrogenic activity, but no estrogenic and antiandrogenic activity (Figure 1). CMA was more potent than CPA in its androgenic and progestogenic activity. When analyzed for antiandrogenic activity, both progestins led to an increase in activity to about 140% (SM Figure S1). Details on the curve parameters and relative potency of the yeast screens are listed in the SM (Table S9). EE2 showed estrogenic, androgenic, and antiandrogenic activity with full concentration-response curve in the YES, but only partial agonism in the YAS (up to 25%). The order of decreasing potencies for the progestogenic activity was P4 > CMA > CPA > EE2 (3-, 19-, and 54-times less potent than P4). The decreasing order for androgenic activity was DHT > CMA > CPA > EE2 (80-, 257-, and 271-times less potent than DHT).

3.2 *In vitro* activities of single compounds in rainbowfish receptor transactivation systems

Reporter gene assays driven by rainbowfish nuclear hormone receptors (Figure 2) revealed that CPA and CMA exhibited minor antiestrogenic and antiprogestogenic activity (at 20 μ M), moderate antiandrogenic activity (at or above 4 μ M), and marked antiglucocorticoid activity (at or above 0.16 μ M). Estrogenic, androgenic, progestogenic or glucocorticoid activity was negligible for both CPA and CMA. Full concentration-response curves were obtained for CPA and CMA in the antiandrogenic assay, allowing the derivation of accurate median effect values (EC50s). The antiandrogenic potency of the compounds tested was EE2 > Bicalutamide (BCL) > CMA ~ CPA (97-, 1-, 0.28- and 0.24-times more potent than BCL, respectively). In the antiglucocorticoid assay, both CPA and CMA were antagonists at lower concentrations (in the range centered around 4 μ M) while a partial return towards the control effect level was observed at high concentrations (20 μ M). EE2 exhibited potent estrogenic and antiandrogenic activity, moderate glucocorticoid activity, and partial androgenic and antiprogestogenic activity (at 10 μ M). Like CPA and CMA, EE2 elicited a biphasic response in the antiglucocorticoid assay, with intermediate concentrations (0.08 to 0.4 μ M) reducing DXM-induced activity to approximately 50% of maximum, and higher concentrations (2 to 10 μ M) returning to above maximum activity. Interestingly, while EE2, CPA and CMA all exhibited partial antagonism of the rfPGR, RU-486 appeared to be at least a partial agonist of this receptor, enhancing the background DHP signal.

3.3 *In vitro* activities of binary mixtures in human and rainbowfish receptor transactivation systems

In human receptor systems, we first analyzed binary mixtures of CMA and CPA, and second, of CMA and EE2 at equieffective concentrations of EC50, EC25, and EC10 for progestogenic activity, based on their individual concentration-response curves. We used the mixture ratios as listed in the SM Table S3. The EC50 mixture of CMA and CPA deviate from the predicted curve assuming CA in low concentrations (but EC50 values and Hill slopes did not differ statistically significantly) (Figure 3). Further analysis by isoboles and toxic unit methods indicated antagonistic interaction (Figures 3B, 3C). On the other hand, the EC25 and EC10 mixtures of CMA and CPA showed an additive behavior in the isoboles but antagonism in the toxic unit approach.

The EC50 mixture of CMA and EE2 slightly deviated from the predicted curve assuming CA towards antagonism, especially at low concentrations (Figure 4), but the differences in EC50 values and Hill slopes were not statistically significant. The isobolograms and toxic unit approach both showed an antagonistic behavior. The EC25 mixture

(CMA+EE2) also showed an antagonistic behavior in the isoboles and toxic unit approach, while the EC10 mixture (CMA+EE2) showed an additive behavior (Figures 4B, 4C). The results of the mixture behavior for progestogenic activity of both binary mixtures are summarized in Table 1.

The mixtures were also analyzed in the rainbowfish androgen receptor systems for antiandrogenic activity. Overall, the results also suggest that the mixtures acted according to the CA model as with human receptors. Figure 5 demonstrates that the EC50 mixtures of CMA and CPA, as well as CMA and CPA with EE2 showed an additive interaction.

3.4 *In vivo* experiments with zebrafish eleuthero-embryos

3.4.1 Chemical Analysis

Analyte concentrations were measured during the exposure experiment immediately after the beginning of the exposure (t_0) and after 24 hours (t_{24}). Different experiments were conducted: single exposures of CMA and CPA at concentrations ranging between 1 ng/L and 100,000 ng/L, and of EE2 at concentrations ranging from 10 to 1,000 ng/L; mixture exposure of EE2 and CMA and EE2 and CPA at concentrations ranging between 10 ng/L and 10,000 ng/L. The mean exposure concentrations were 0.6, 5.4, 54.7, 692, 6,788, 65,650 ng/L for CMA, and 0.9, 8.9, 82.6, 808, 7,828 and 70,525 ng/L for CPA (Table 2, raw data are reported in the SM in Tables S5, S6, S7 and S8). The mean EE2 concentrations were 9.7, 103, 454, and 950 ng/L in the single exposures, and 10.2, 96.1/99.1 and 1,053 ng/L in the mixtures. Mean concentrations of CMA in mixtures were 69/70.9, 729, 5,090 ng/L, and for CPA in mixtures mean concentrations were 88 (mixture with 10 ng/L EE2) and 73 (mixture with 100 ng/L EE2), 889, and 9,472 ng/L.

Generally, the concentrations were consistent with the nominal for all the hormones, and none of the analytes underwent a decrease of concentration during the exposure experiments. CMA concentrations were slightly lower than the nominal ones (about 30%) and this can be explained by the lower recovery obtained for this compound (70% versus 100% recovery of the IS). The presence of chlormadinone was checked in order to exclude the degradation of the corresponding acetate compounds and traces were detected only at the highest concentration (Table S5), thus a degradation during the experiment can be excluded. The lower than nominal concentrations of CMA and CPA had already been observed in our previous progestin experiments (Rossier et al., 2016; Zhao et al., 2015a; Zucchi et al., 2012). Reasons are sorption of CMA and CPA to surfaces (pipet tips, glass walls, embryos, and debris), and partly due to biotransformation of the acetate into chlormadinone and cyproterone, respectively.

3.4.2 *In vivo* activities of CMA and EE2 as single compounds and binary mixtures

We did not observe any effects on gross morphology or development, and no compound-related mortality. Transcriptional changes upon exposure to single compounds and mixtures were determined at 96 hpf and 144 hpf for 15 selected genes belonging to different signaling pathways based on previous studies (Rossier et al., 2016; Zhao et al., 2015a; Zucchi et al., 2014, 2012). Only minor alterations occurred upon exposure to CMA, and they were almost identical at 96 hpf and 144 hpf. Figures S2 and S3 show data at 96 hpf and Figure S4 shows results of additional genes. A significant down-regulation occurred for *hsdb11b2* at 5.4 and 55 ng/L, for *cyp11a* at 55 ng/L, and for *cyp2k7* and *fshb* at 692 ng/L. In contrast, EE2 strongly induced several genes at all concentrations, including *pgr*, *vtg1*, *cyp19b*, and *lhb*. Alterations also occurred for the *estrogen receptor alpha* (*esr1*) at 103 ng/L, and for *cyp2k7*, *androgen receptor* (*ar*) and *hsd17b3* at 9.7 ng/L EE2 and higher. Minor changes occurred for *cyp11a* and *hsd11b2*. The pattern of expressional changes in embryos exposed to the binary mixtures were very similar to EE2 alone.

Figures 6 and 7 show transcriptional alterations of target genes at 144 hpf with slight down-regulation of *ar*, *pgr*, *hsd17b3*, *hsd11b2* and *lhb* at 0.57 ng/L CMA and higher. In contrast, exposure to EE2 led to significant induction of *pgr*, *cyp19b* and *lhb* at all, and *esr1* and *vtg1* at higher concentrations. Significant alterations also occurred for *cyp2k7*, *cyp11a* and *hsd17b3*. Again, the transcription profile of the binary mixture was very similar to that of EE2 alone (Figures 8, 9). Slight differences occurred only at the lowest mixture concentration in *vtg1* and *pgr* transcripts. This indicates that the interaction in the mixture followed the independent action model.

3.4.3 *In vivo* activity of cyproterone acetate as single compound and binary mixture with EE2

To analyze for effects of CPA, a series of ten selected genes were assessed at 144 hpf in eleuthero-embryos. They belong to different signaling pathways based on previous studies (Rossier et al., 2016; Zhao et al., 2015a; Zucchi et al., 2014, 2012), and were determined in the CMA exposure. Exposure to CPA led to down-regulation of *pgr*, *ar*, *gr* and *hsd17b3* transcripts (Figure 10). The transcription pattern of CPA was very similar to that of CMA, although down-regulation of *pgr* and *ar* seemed more pronounced.

We also analyzed the activity of binary mixtures of CMA and EE2 and measured transcriptional changes of six genes at 144 hpf. Figure 11 shows that the mixture activity was lower than that of EE2 alone. The lowering of transcriptional reaction to EE2 by CPA was particularly visible for *vtg1*, *esr1* and *cyp19b*. As a single compound, CPA alone did not induce these transcripts, and EE2 alone led to higher alterations than in the mixture with CPA. Thus, CPA in the mixture led to a lower transcriptional change. This mixture activity

therefore suggests an additive interaction of both compounds, in that CPA lowered the EE2-induced transcriptional changes. Therefore, the joint action in the mixture can be regarded as an additive interaction of both compounds.

4. Discussion

Our transactivation experiments showed that the interaction of CMA and CPA with human receptors differed from those with rainbowfish. While they exhibited progestogenic, androgenic and antiestrogenic activity with no antiandrogenic or estrogenic activity in human receptor transactivation systems, interactions with rainbowfish receptors showed no progestogenic, but antiandrogenic, antiglucocorticoid and slight antiestrogenic activity. Thus, interaction with human and fish receptors deviates as observed for other progestins (Bain et al., 2015a; Ellestad et al., 2014). We further showed that the *in vitro* interaction of binary mixtures followed the concentration addition model with often antagonistic interactions, while transcriptional effects of CMA and EE2 *in vivo* clearly followed an independent action. This indicates that interactions with a single hormone receptor *in vitro* cannot predict the more complex *in vivo* interaction, where interactions via different signaling pathways occur.

4.1 *In vitro* activities

We found progestogenic, antiestrogenic, and androgenic activity as for other synthetic progestins (Rossier et al., 2016; Runnalls et al., 2013). CMA and CPA were about 70 times less potent than 4-hydroxytamoxifen in their antiestrogenic activity, and they did not show any estrogenic activity. CMA and CPA seemed to antagonistically interact with the estrogen receptor and/or its associated cofactors, which decreased receptor transactivation. Both progestins showed androgenic activity with full concentration-response curves but no antiandrogenic activity; they rather enhanced the androgenic activity, as seen for other progestins (Blüthgen et al., 2013b; Rossier et al., 2016). Their activity was similar to drospirenone (Rossier et al., 2016). Our data confirm the reported androgenic activity of CPA but not its antiandrogenic activity (Térouanne et al., 2000), or that of CMA (Térouanne et al., 2002) found in other cell-based assays. For EE2, our data confirmed previously reported estrogenic, progestogenic, antiandrogenic and partially androgenic activity (Rossier et al., 2016).

The interaction in binary mixtures of CMA and CPA, and CMA and EE2 showed that they interacted according to the concentration addition model in recombinant yeast expressing the human progesterone receptor. The interaction was antagonistic, in particular for the EC50 and EC25 mixtures. This confirms our previously found antagonistic interaction in mixtures of P4 and drospirenone (Rossier et al., 2016). We hypothesize that the

compounds bind differently to the progesterone receptor, leading to inhibition of receptor activation and transactivation.

Recombinant yeast systems are a good model to investigate receptor interaction and transactivation, as they strictly reflect the transactivation with the expressed steroid receptor, but on the other side, this is also a limitation. Yeast assays only represent one small part of complex interactions in the metabolic system of organisms involved in estrogenic, progestogenic, and androgenic activities. Results should be therefore retrospectively confirmed *in vivo*. Considering that our interest lies in prediction of effects in fish, caution should be applied when analyzing progestin interaction with human receptors used for predicting effects in fish. Steroid receptors are highly conserved among vertebrates. However, there are differences in their binding affinities in humans and fish as previously shown (Bain et al., 2015a, 2015b; Ellestad et al., 2014).

To elucidate differences between the responses of human-derived and fish-derived receptors to CMA, CPA and EE2, we employed stable reporter gene assays expressing rainbowfish nuclear steroid hormone receptors. Teleost fish exhibit considerable diversity of genes encoding nuclear receptors, which is thought to be largely attributable to an ancestral whole-genome duplication event (Maglich et al., 2003). The fate of duplicated genes varies among different lineages – two copies of some genes are maintained over the course of evolution, often undergoing functional divergence, while for other genes one copy has been lost. For example, only one AR locus has been identified in the genomes of diverse teleosts including zebrafish, stickleback, takifugu and tetraodon, while several species of the acanthomorpha lineage (spiny-finned fishes, a large clade within the teleostei) express two separate AR genes encoding receptors denoted AR α and AR β . Conversely, cypriniformes such as zebrafish, roach and fathead minnow, express a single AR (Douard et al., 2008; Ogino et al., 2009). In terms of amino acid sequence similarity, acanthomorpha AR β is more closely related to the AR of cypriniformes, while acanthomorpha AR α is more divergent and exhibits novel functionality (Ogino et al., 2016). Because our *in vivo* experiments were performed in zebrafish, we used the rainbowfish AR β -driven reporter gene assay to test for (anti)androgenic activity in the present study since this receptor is more closely related to zebrafish AR.

Thus, disregarding the differences between the reporter assay systems used in the study such as carrier solvent, media, and in the transcriptional co-regulators present in the host cells, the rainbowfish *in vitro* system appears to be a better predictor of *in vivo* effects in fish than the human receptor-driven yeast-based assays. Because positive control chemicals in the two reporter assay systems used behaved as expected, responses are considered to be driven primarily by the activation of the exogenous receptors overexpressed in each reporter assay used, allowing comparison between fish and human receptors despite differences in the nature of the respective reporter assays.

We found that both CPA and CMA were strong antagonists of rfAR β , with potencies similar to that reported for the model non-steroidal antiandrogen, flutamide (Bain et al., 2015b). This is consistent with the well-characterized antagonism of the human AR by CPA and CMA (Raudrant and Rabe, 2003). Our study has confirmed that synthetic progestins exhibit variable off-target effects depending on the parent structure, indicating that structure-activity relationships could be developed that may allow one to predict the likely effects of different classes of progestins in fish. The present study contributes mechanistic evidence that may be useful in such approaches, adding to the pool of data from mechanistic studies of other classes of progestin such as 19-nortestosterone derivatives (Bain et al., 2015a; Ellestad et al., 2014).

The focus of the present study was to examine the *in vitro* (anti)androgenicity, (anti)progestogenicity and (anti)estrogenicity of CPA and CMA based on their known modes of action in mammals. However, because progestins and corticosteroids are both C21 steroids and others have reported activity of progestins in a fish-derived GR reporter assay system (Sato et al., 2013) we also investigated their potential to activate or antagonise a rainbowfish GR. We used a rainbowfish GR2 reporter assay since studies in Japanese medaka showed that GR2 was considerably more sensitive than GR1 to a range of corticosteroids and was therefore likely to be the most active GR *in vivo* (Miyagawa et al., 2014). Although the concentration-response relationships for both CPA and CMA were somewhat non-monotonic, the predominant mode of action was antagonism of rfGR2. Furthermore, the apparent EC₅₀ of the inhibitory phase of the concentration-response curve was considerably lower than that observed for rfAR β , indicating that inhibition of GR2 may be a more relevant mode of action *in vivo* than antiandrogenicity, at least at low exposure concentrations.

4.2 *In vivo* activities of CMA, EE2 and CPA in zebrafish embryos

CMA and CPA induced transcriptional changes in zebrafish eleuthero-embryos. A significant down-regulation of *ar* may be indicative for an antiandrogenic mechanism due to antagonism on the androgen receptor. Similar results have been reported for the antiandrogen flutamide (Filby et al., 2007). The down-regulation of the *lhb* transcript might be explained by an antigonadotropic action of progestins, i.e., CMA might exhibit a negative feedback on luteinizing (LH) and follicle-stimulating (FSH) hormone release like endogenous fish progestins. Down-regulation of *hsd11b2*, *hsd17b3*, *cyp2k7*, and *pgr* were only very weak (and not consistent across different concentrations).

Down-regulation of *pgr* and *ar* has also been observed for the antiprogestin mifepristone, which suggests an antiprogestogenic and antiandrogenic action in zebrafish embryos (Blüthgen et al., 2013b; Zucchi et al., 2012). Down-regulation of *ar* was described

for flutamide (Filby et al., 2007). The weak effects of CPA on gene expression in zebrafish embryos contrasts with data from adult fish of previous studies. As low as 1 ng/L CPA led to decreased estradiol and testosterone biosynthesis in adult mummichogs (*Fundulus heteroclitus*) (Sharpe et al., 2004). Furthermore, antiandrogenic effects were found in adult female fathead minnows (prevention of androgenic tubercle development in females when co-treated with CPA and an androgen) (Ankley et al., 2010). Reasons for these differences might lie in different sensitivities of developmental stages and between species. Interspecies differences in binding affinities to the androgen receptor have been shown for CPA with binding in the brain and gonads of goldfish but not in rainbow trout (Wells and Van Der Kraak, 2000).

In comparison to previously investigated progestins, such as levonorgestrel, norgestrel, medroxyprogesterone acetate, drospirenone, as well as to EE2 in our study, CMA and CPA exposure resulted in only weak expressional changes and for a few genes only (Liang et al., 2015b; Rossier et al., 2016; Zhao et al., 2015a; Zucchi et al., 2012). For both CMA and CPA, transcriptional changes tended to be more pronounced at lower than at higher concentrations. Low-dose effects and non-monotonic dose-response curves have been described for hormones, and on this basis, it might be possible that CMA and CPA exhibited stronger effects at lower doses (Vandenberg et al., 2012). The apparent lack of concentration-response relationship could reflect different time courses of transcriptional responses exhibited at different concentrations. Developmental processes and ontogeny of normal gene expression are time-dependent, and may not be the same at different concentrations, thus non-monotonic responses could result.

The transcription pattern upon exposure to EE2 was almost identical at 96 and 144 hpf. Our data confirm previous data with strong induction of *esr1*, *vtg1*, *cyp19b*, and *pgr* transcripts at about 10 ng/L (Kishida et al., 2001; Rossier et al., 2016). All four genes are mainly regulated by activated estrogen receptors containing estrogen-responsive elements (EREs) in their promotor region (Carroll et al., 2006; Coumailleau et al., 2015). Our study provides data on the EE2-induced modulation of *lhb* and *fshb* in fish embryos. Up-regulation of *lhb* and down-regulation of *fshb* has been demonstrated in pituitary of salmon after exposure to EE2 (Harding et al., 2013). Elevated levels of estrogens in the blood plasma exhibit negative feedback on FSH release. Whether this is leading to the observed down-regulation of *fshb* levels by EE2 at this early developmental stage is not known, however.

4.3 *In vitro* and *In vivo* activities of binary mixtures

The activity in binary mixtures were analyzed in both recombinant yeast expressing the human PGR and with the rainbowfish AR. The mixture components interact from additively to antagonistically in their transactivation of the human PGR. The interaction of all mixtures with

the rainbowfish AR was additive. Overall, the data suggest that the mixtures interacted with the receptor systems according to the CA model and were mostly additive.

The expressional patterns of investigated genes in eleuthero-embryos exposed to binary mixtures of CMA and EE2 basically equaled those of EE2 alone at both time points (96 and 144 hpf). This indicates a mechanism of independent action, where EE2 predominates gene expression, overriding effects of CMA alone. The alternative interpretation that the mixture effect showed an antagonism, in that EE2 antagonized the effects of CMA, is less probable, as CMA induced almost no transcriptional changes, whereas EE2 had strong effects even at lowest concentrations. In contrast, the activity of the CPA and EE2 mixture was additive for most of the gene transcripts. Thus, CPA interacted with EE2 and lowered the sum activity. This cannot be explained by the independent action model, but the concentration addition model. Thus, CMA and CPA differed in their effect on the sum activity with EE2.

Overall, physiological responses of zebrafish embryos exposed to mixtures of CMA or CPA with EE2 are complex, and mixture interactions may differ among genes. This was also indicated for binary mixtures of levonorgestrel and EE2, where effects were additive on reproductive performance of adult zebrafish, but followed an independent action for vitellogenin induction, which was influenced by EE2 only (Runnalls et al., 2015). For responses clearly mediated via estrogens, such as induction of *cyp19a* and *vtg1*, we considered an independent action, because CMA did not have any influence on the expression of these genes. Our present results are in agreement with our previous study on interactions in mixtures of drospirenone and EE2, where gene expression was mainly influenced by EE2 (Rossier et al., 2016). However, in case of binary mixtures of CPA and EE2, the joint activity seemed additive, suggesting that CPA lowered the activity of EE2. Thus, transcriptional changes cannot be explained by independent action alone (as in case of CMA and drospirenone with EE2) but also by additive interaction, as in case of CPA and EE2. Furthermore, our data also suggest that the *in vitro* interaction with receptors were mainly additive, while they were more complex *in vivo*.

4.4 Comparison of *in vitro* and *in vivo* results

The expected progestogenic, antiestrogenic, and antiandrogenic activity of CMA (Schindler et al., 2008) was confirmed in our yeast transactivation assays. The fact that we did not detect antiandrogenic activity *in vitro* might be based on the recombinant yeast system that lacks specific corepressors and coactivators necessary for antiandrogenic activity *in vivo*. Inhibition of 5 α -reductase ultimately leads to reduced levels of potent DHT and represents part of the mechanism of antiandrogenic activity of CPA and CMA (Raudrant and Rabe, 2003). In zebrafish embryos, CMA induced slight antiandrogenic activity as

1 deduced from the down-regulation of *cyp2k7* and steroidogenesis gene that converts
2 androstenedione to testosterone. The antigonadotropic activity is suggested by down-
3 regulation of *lhb* transcript. Based on transcriptional changes, we observed similar
4 antiestrogenic and slightly antiandrogenic activity, and in males, also progestogenic activity
5 in adult zebrafish (Siegenthaler et al., 2016). Thus, the *in vivo* activity of CMA in embryos
6 and adult fish was similar.
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9 For CPA, we expected progestogenic, antiestrogenic, and antiandrogenic activity in our
10 experiments (Schindler et al., 2008), which was confirmed in our yeast assays. For the lack
11 of antiandrogenic activity in yeast, the same reasons as for CMA apply. In embryos, the
12 magnitude of transcriptional changes was low, and therefore suggest slight
13 antiprogestogenic and antiandrogenic activities. Even at high concentrations transcriptional
14 changes were low with suggests that the *in vivo* activity was rather weak compared to the *in*
15 *vitro* effects, where we found full concentration-response curves. On the other hand, even
16 the lowest concentration of 1 ng/L CPA exhibited highest relative down-regulation of *pgr* and
17 *ar*.
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19 In general, nuclear steroid hormone receptors show high homology among vertebrates.
20 The zebrafish PGR shows the highest homology to the PGR of fathead minnow and goldfish
21 (two cyprinid species) (Chen et al., 2010; Hanna et al., 2010). The DNA binding domain and
22 the ligand binding domain are most conserved between humans and teleost fish, whereas
23 the amino-terminal A/B domain shows the largest differences, which can influence the
24 carboxy-terminal ligand-binding domain that might lead to differences in binding affinities to
25 synthetic progestins between species (Todo et al., 1999; Wells and Van Der Kraak, 2000).
26 As demonstrated for other progestins, it is possible that the fish PGR and AR differ from
27 human PGR and AR in such a way that CMA and CPA cannot bind and activate them (Bain
28 et al., 2015a; Ellestad et al., 2014). This was confirmed by our experiments with rainbowfish
29 receptors where CMA and CPA lacked interaction with fish PGR. No progestogenic, but
30 antiandrogenic, antiglucocorticoid and some antiestrogenic activity occurred. Thus, the
31 rainbowfish *in vitro* system is a better predictor of *in vivo* effects in fish.
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33 In humans, both CMA and CPA are potent antiandrogens (Raudrant & Rabe 2003) that
34 act via several modes of action. First, they are AR antagonists, inhibit receptor activation,
35 and prevent endogenous androgen from binding and activating AR. Second, they
36 competitively inhibit the enzyme 5 α -reductase, which leads to decreased levels of DHT.
37 Third, they inhibit gonadotropin release from the pituitary, consequently leading to decreased
38 androgen synthesis. All these mechanism of action could have differential effects in fish.
39 First, zebrafish AR may differ from human AR leading to lower binding affinity, hence weaker
40 antagonism. Second, DHT is not the most potent androgen in fish and testosterone is, via the
41 enzyme 3 beta-hydroxysteroid dehydrogenase converted into gonadotropin-related
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11-ketotestosterone, which is the most prominent androgen in fish. Therefore, this mechanism might not be relevant in fish. Third, receptor binding to the fish PGR might be lower and, therefore, antigonadotropic activity might be weaker.

Our *in vitro* mixture experiments in recombinant yeast showed that the mixture components interact additively to antagonistically in their transactivation of the human PGR. Furthermore, the *in vitro* interaction with rainbowfish AR was additive. We should also point out that we did not always find identical outcomes by the isobole and toxic unit approach for certain mixtures, similarly as found previously for other progestins (Rossier et al. 2016). This may in part be related to the violated assumption for the isobole method that concentration response curves should have identical slopes, which was not always the case (for EE2 and CMA, as shown in Figure 1). Other reasons are rather methodological. The toxic unit approach does not work with confidence intervals or standard deviations, thus deviations from additivity may not be significant.

For transcriptional effects of the mixtures of CMA and EE2 *in vivo*, the interaction followed the independent action model. In contrast to the *in vitro* experiments, where we analyzed equieffective concentrations, we analyzed binary mixture concentrations in zebrafish embryos in a ratio of 1:10, due to the lack of concentration-response curves in transcriptional responses. Nevertheless, CMA exhibited almost no transcriptional effects. The expression pattern of the binary mixtures was clearly mediated by EE2. In contrast, transcriptional changes in the CPA and EE2 mixtures in eleuthero-embryos were additive, similar as *in vitro*. Although in this case the *in vitro* and *in vivo* data match, one should consider that the *in vitro* results represent a more simple system where only receptor activation is investigated, whereas the *in vivo* reaction is much more complex with many mechanisms, pathways and feedback loops involved in responses to the progestins.

5. Conclusions

CMA and CPA elicited different transactivation in human and fish systems. Our study shows that fish receptor systems seem better predictors of *in vivo* activity in fish. Binary mixtures of both progestins, and of CMA and EE2 showed additive to antagonistic interactions in yeast screens. Human receptors therefore were not predictive for the *in vivo* effects. The transcriptional alterations in eleuthero-embryos followed the independent action model. The effects were mainly related to the activity of EE2, overriding the activity of CMA. Effects elicited by CMA and CPA in zebrafish embryos were less pronounced than those of other progestins. Probable reasons are the lower or lack of binding to zebrafish PGR or AR. Further ecotoxicological studies are needed to unravel the mode of action of these two progestins.

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Tables

Table 1. Summary of the progestogenic activity of binary mixtures in yeast hormonal screens.

Binary mixtures		Predicted curves CA	Toxic unit approach	Isobole method
CMA + CPA	EC50 mix CMA+CPA	Additivity	Antagonism	Antagonism
	EC25 mix CMA+CPA	—	Antagonism	Additivity
	EC10 mix CMA+CPA	—	Antagonism	Additivity
CMA + EE2	EC50 mix CMA+EE2	Additivity	Antagonism	Antagonism
	EC25 mix CMA+EE2	—	Antagonism	Antagonism
	EC10 mix CMA+EE2	—	Additivity	Additivity

Additive activity is shown in yellow, antagonistic activity is shown in red. (—) not analyzed.

Table 2. Concentrations of single compounds and binary mixtures in exposure water of the *in vivo* experiments in zebrafish eleuthero-embryos.

Nominal concentration	Concentration at t0 (mean ¹)	Concentration at t24 (mean ¹)	Mean 24 h (SD) ²	Accuracy t0 – nominal conc. (%)	Residual amount at t24 (%)					
DMSO control										
	< LOQ ³	< LOQ ³	-	-	-					
CMA										
1 ng/L	0.58	0.55	0.568 (0.131)	58	95					
10 ng/L	5.24	5.65	5.44 (0.959)	52	108					
100 ng/L	58.15	51.15	54.88 (11.0)	58	88					
1,000 ng/L	768	616	692 (106)	77	80					
10,000 ng/L	6,995	6,580	6,788 (1049)	70	94					
100,000 ng/L	62,900	68,400	65,650 (7,267)	63	108					
CPA										
1 ng/L	0.93	1.04	0.986 (0.142)	93	111					
10 ng/L	8.66	9.29	8.973 (0.591)	87	107					
100 ng/L	81.8	83.3	82.55 (9.11)	82	102					
1,000 ng/L	814	801.5	808 (55.8)	81	98					
10,000 ng/L	8,010	7,645	7,828 (508)	80	95					
100,000 ng/L	67,500	73,550	70,525 (7,251)	68	109					
EE2										
10 ng/L	9.8	9.6	9.69 (0.300)	98	98					
100 ng/L	99.2	106.7	103 (11.9)	99	108					
500 ng/L	500	408	454 (54.6)	100	82					
1,000 ng/L	954	946	950 (69.3)	95	99					
Mixture EE2+CMA										
	EE2	CMA	EE2	CMA	EE2	CMA	EE2	CMA	EE2	CMA
10+100 ng/L	10.2	75.2	10.2	62.8	10.2 (0.483)	69.0 (7.46)	102	75	100	84
100+1,000 ng/L	95.2	740	97.0	718	96.1 (2.97)	729 (67.2)	95	74	102	97
1,000+10,000 ng/L	1,064	4,930	1,041	5,250	1,052 (85.3)	5,090 (393)	106	49	98	106
100+100 ng/L	99.5	75	98.7	66.8	99.1	70.9	10	75	99	89

					(2.20)	(6.08)	0			
Mixture EE2+CPA										
	EE2	CPA	EE2	CPA	EE2	CPA	EE 2	CPA	EE2	CPA
10+100 ng/L	N. A.	74.7	N. A.	94.1	-	87.7 (23.0)	-	75	-	126
100+1,000 ng/L	N. A.	872	N. A.	905	-	889 (39.3)	-	87	-	104
1,000+10,000 ng/L	N. A.	8,999	N. A.	9,957	-	9,472 (810)	-	90	-	111
100+100 ng/L	N. A.	71.2	N. A.	73.8	-	72.5 (2.36)	-	71	-	104
¹ Mean is referred to two replicates analyzed for all the samples. ² Mean 24 h is calculated as mean of t 0 and t 24 of two replicates including the standard deviation. ³ LOQ = limit of quantification. N.A. = not analyzed.										

Figure captions

Figure 1. Estrogenic, antiestrogenic, androgenic, antiandrogenic, and progestogenic activities of single compounds in yeast hormonal screens (YES, YAS, YPS).

(A) Estrogenic activity of single compounds with fitted concentration-response curves. 17β -estradiol (E2) is the positive control. (B) Antiestrogenic activity of single compounds with fitted concentration-response curves. Activity is measured by the decrease of E2-induced activity (65% of maximal E2 activity). 4-hydroxytamoxifen (4-HT) is the positive control. (C) Androgenic activity of single compounds with fitted concentration-response curves. 5α -dihydrotestosterone (DHT) is the positive control. (D) Antiandrogenic activity of single compounds with fitted concentration-response curves. Activity is measured by the decrease of DHT-induced activity (65% of maximal DHT activity). Flutamide (FLU) is the positive control. (E) Progestogenic activity of single compounds with fitted concentration-response curves. Progesterone (P4) is the positive control. Data are shown as means $\pm$ SD of at least three independent experiments.

Figure 2. Estrogenic, antiestrogenic, androgenic, antiandrogenic, progestogenic, antiprogestogenic, glucocorticoid and antiglucocorticoid activities of single compounds in stable reporter gene assays developed using nuclear receptors from Murray-Darling rainbowfish (*Melanotaenia fluviatilis*).

(A) Estrogenic activity of single compounds as indicated by the activation of rainbowfish estrogen receptor α (rfER α). 17β estradiol (E2) is the positive control. (B) Antiestrogenic activity of single compounds as indicated by the decrease of E2-induced activity of rfER α (75% of maximal E2 activity). 4 hydroxytamoxifen (4 HT) is the positive control. (C) Androgenic activity of single compounds as indicated by the activation of rainbowfish androgen receptor β (rfAR β). 11-ketotestosterone (11-KT) is the positive control. (D) Antiandrogenic activity of single compounds as indicated by the decrease of 11-KT-induced activity of rfAR β (75% of maximal 11-KT activity). Bicalutamide (BCL) is the positive control. (E) Progestogenic activity of single compounds as indicated by the activation of rainbowfish progesterone receptor (rfPGR). $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) is the positive control. (F) Antiprogestogenic activity of single compounds as indicated by the decrease of DHP-induced activity of rfAR β (75% of maximal DHP activity). Mifepristone (RU-486) is the positive control. (G) Glucocorticoid activity of single compounds as indicated by the activation of rainbowfish glucocorticoid receptor 2 (rfGR2). Dexamethasonse (DXM) is the positive control. (H) Antiglucoctocorticoid activity of single compounds as indicated by the decrease of DXM-induced activity of rfGR2 (75% of maximal DXM activity). Mifepristone (RU-486) is the positive control. Data are shown as means $\pm$ SD of at least three independent experiments.

Figure 3. Progestogenic activity of binary mixtures of CMA and CPA. (A) Concentration-response curves of equieffective mixtures (EC50, EC25, EC10) and the single compound activity. Progestogenic activity of CMA (green) and CPA (orange), and binary mixture of CMA+CPA (blue) at equieffective levels of EC50, EC25, and EC10. For EC50, we included the predicted equieffective mixture curve according to the CA model (black). (B) Equieffective isobole graph for binary mixtures of CMA and EE2 at effect levels of EC50 (green), EC25 (red), EC10 (blue). Solid lines represent the additivity line with the 95%-confidence interval (CI) bands (dotted lines) for single compounds. (C) Toxic unit calculations of binary mixtures of CMA and CPA at effect levels of EC50, EC25, and EC10. For the toxic unit method an additive behavior was assumed when the calculated value (x) was within the following range: $0.5 \leq x \leq 1.5$.

Figure 4. Progestogenic activity of binary mixtures of CMA and EE2 in human progesterone receptor system. (A) Concentration-response curves of equieffective mixtures (EC50, EC25, EC10) and the single compound activity. Progestogenic activity of CMA (green) and EE2 (red), and binary mixture of CMA+EE2 (blue) at equieffective level of EC50, EC25, and EC10. For EC50 we included the predicted equieffective mixture curve according to the CA model (black). (B) Equieffective isobole graph for binary mixtures of CMA and EE2 at effect levels of EC50 (green), EC25 (red), EC10 (blue). Solid lines represent the additivity line with the 95%-confidence interval (CI) bands (dotted lines) for single compounds. (C) Toxic unit calculations of binary mixtures of CMA and EE2 at effect level of EC50, EC25, and EC10. For the toxic unit method an additive behavior was assumed when the calculated value (x) was within the following range: $0.5 \leq x \leq 1.5$.

Figure 5. Antiandrogenic activity of binary mixtures in rainbowfish receptor system. (A) Concentration-response curves of single compounds and equieffective EC50 mixtures of CMA and CPA. The predicted equieffective mixture curve according to the CA model is included in black. (B) Concentration-response curves of single compounds and equieffective EC50 mixtures of CPA and EE2. The predicted equieffective mixture curve according to the CA model is included in black. (C). Concentration-response curves of single compounds and equieffective EC50 mixtures of CMA and EE2. The predicted equieffective mixture curve according to the CA model is also included in black. (D) Toxic unit calculations of the binary mixtures. For the toxic unit method an additive behavior was assumed when the calculated value (x) was within the following range: $0.5 \leq x \leq 1.5$.

Figure 6. Relative transcript levels in zebrafish embryos after exposure to CMA and EE2 as single compounds and their binary mixtures at 144 hpf. Relative transcript levels of *pgr*, *ar*, *esr1*, *gr*, *mr*, *vtg1*, *cyp19a*, and *cyp19b* at 144 hpf. Significant transcriptional changes compared to solvent control are indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Data are shown as mean ± SD of four or five biological replicates.

Figure 7. Relative transcript levels in zebrafish embryos after exposure to CMA and EE2 as single compounds and their binary mixtures at 144 hpf. Relative transcript levels of *lhb*, *fshb*, *hsd11b2*, *hsd17b3*, *cyp2k7*, *cyp11a*, and *cyp17* at 144 hpf. Significant transcriptional changes compared to solvent control are indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Data are shown as mean ± SD of four or five biological replicates.

Figure 8. Heat map of the gene expression changes in zebrafish embryos of 15 investigated transcripts in a color scheme after exposure to CMA and EE2 as single compounds and in binary mixtures at 96 hpf and 144 hpf. Red color indicates significant upregulation, green color indicates significant downregulation, and yellow indicates no significant change. The significance level is indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Empty fields indicate that gene transcripts were not analyzed.

Figure 9. Hierarchical clustering of transcriptional responses of zebrafish eleuthero-embryos exposed to different concentrations of CMA, EE2 or binary mixtures of EE2+CMA at 96 hpf (-96) or 144 hpf (-144). Grey fields indicate that genes were not analyzed.

Figure 10. Relative transcript levels in zebrafish eleuthero-embryos after exposure to CPA. Relative transcript levels of *pgr*, *ar*, *esr1*, *gr*, *hsd17b3*, *vtg1*, *cyp19a*, *cyp19b*, *per1a*, and *nr1d2a* at 144 hpf. Significant transcriptional changes compared to solvent control are indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Data are shown as mean ± SD of five biological replicates.

Figure 11. Relative transcript levels in zebrafish eleuthero-embryos after exposure to CPA and EE2 as single compounds and their binary mixtures. Relative transcript levels

of *ar*, *pgr*, *esr1*, *vgt1*, *cyp19b*, and *cyp2k7* at 144 hpf. Significant transcriptional changes compared to solvent control are indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Data are shown as mean $\pm$ SD of four or five biological replicates. In the mixture, CPA concentrations were measured, while EE2 concentrations were taken as nominal. Transcriptional changes of single compounds were taken from the single exposures and shown here for comparison with the mixture activity.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

EC50 mixture of CMA and CPA

B

EC50 mixture of CPA and EE2

C

EC50 mixture of CMA and EE2

D

Figure 6

Figure 7

Figure 8

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Figure 9

Figure 10

Figure 11