The six most widely used selective serotonin reuptake inhibitors decrease androgens and increase estrogens in the H295R cell line

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) used as first line of treatment in major depressive disorder (MDD) are known to exert negative effects on the endocrine system and fertility. The aim of the present study was to investigate the possible endocrine disrupting effect of six SSRIs, fluoxetine, paroxetine, citalopram and its active enantiomer escitalopram, sertraline and fluvoxamine using the OECD standardized and validated human *in vitro* adrenocortical H295R cell assay. All the major steroids, including progestagens, corticoids, androgens and estrogens were analysed using a fully validated LC-MS/MS method. All 6 SSRIs were found to exert endocrine disrupting effects on steroid hormone synthesis at concentrations just around C_{max}. Although the mechanisms of disruption were all different, they all resulted in decreased testosterone levels, some due to effects on CYP17, some earlier in the pathway. Furthermore, all SSRIs relatively increased the estrogen/androgen ratio, indicating stimulating effects on the aromatase. Our study demonstrates the potential of SSRIs to interfere with steroid production in the H295R cells around C_{max} levels and indicates that these drugs should be investigated further to determine any hazards for the users.

1. Introduction

Several pharmaceuticals have been developed to attenuate the symptoms of depression. Selective serotonin reuptake inhibitors (SSRIs) are the first line treatment of major depressive disorder (MDD) and they are far more prevalent than other antidepressants such as tricyclic antidepressants (TCA) and selective noradrenalin reuptake inhibitors (SNRIs) (Nierenberg et al., 2007; Werner et al., 2010). MDD is a mental disorder characterized by a persistent low mood and low self-esteem. MDD may also influence sleeping and eating habits (Nestler et al., 2002). SSRIs are speculated to exert their effect by blocking the serotonin reuptake transporter, hence decreasing the reuptake of serotonin to the presynaptic neuron by which the concentration of serotonin in the synaptic cleft is increased. Elevated serotonin levels in the cleft are assumed to attenuate depression (Lattimore et al., 2005; Lindqvist et al., 2015).

Fluoxetine was the first SSRI sold and later other SSRIs have been introduced. Initially, the SSRIs were considered free of adverse effects, but after marketing some side effects have been uncovered. Some of these effects are nausea, dry mouth, appetite changes and sexual dysfunction (Ferguson, 2001). The most sold SSRIs are fluoxetine, sertraline, paroxetine and citalopram. Citalopram is a racemic mixture of R- and S-citalopram, but only S-citalopram is therapeutically active. Consequently, the S-enantiomer is also marketed, as escitalopram. The Institute for Rational Pharmacotherapy (IRF) recommend citalopram/escitalopram and sertraline as first choice for treating moderate and severe depressions, because their effect and adverse profile are assumed to be better than fluoxetine and paroxetine (IRF, 2010).

Altered sex hormone production in humans may be caused by a combination of lifestyle factors, anatomical conditions, autoimmune conditions and environmental factors, including endocrine disruptors (Hauser et al., 2015; Skakkebaek et al., 2001). In recent years, the focus on drugs as endocrine disruptors has increased (Winther et al. 2013; Guldvang et al 2015; Holm et al 2015, 2016; Kristensen et al 2016; Sørensen et al 2016) and studies indicate that adverse effects of SSRIs may be associated with the endocrine system since sexual disorders are common in SSRI users. Between 30 and 60% of all SSRI users have been reported to suffer from sexual dysfunction (Gregorian et al., 2002). In men, this includes low levels of testosterone (TS), luteinizing hormone (LH), and follicle stimulating hormone (FSH), and elevated level of prolactin (Safarinejad, 2008). In women, SSRI use has been associated with breast enlargement and changes in the length of the menstrual cycle (Amsterdam et al., 1997; Steiner et al., 1997). It has also been suggested that binding of serotonin to the brain serotonin ($5\text{-}HT_2$) receptor directly cause decreased libido (Ferguson, 2001). Another explanation may be that increased peripheral serotonin inhibits nitric oxide (NO) levels,

which normally relaxes the smooth muscles of the vasculature. Inhibition of NO may thus cause decreased blood supply to the sexual organs (Kennedy et al., 2000).

Few studies have investigated the relation between SSRIs and steroid production. A previous study observed a decrease in testosterone (TS) in the H295R cell line during exposure to sertraline, fluoxetine, paroxetine, citalopram and fluvoxamine (Jacobsen et al., 2015) but the effects of these SSRIs on the complete steroidogenesis is not known. In the present study we investigated six SSRIs (paroxetine, fluoxetine, citalopram, escitalopram, sertraline and fluvoxamine) in the H295R cell line to clarify how these drugs alter the steroid production. This cell line is capable of *de novo* synthesis of all the major steroids in the entire steroid pathway including progestagens, corticosteroids, androgens and estrogens. Consequently, analysing the complete steroid profile in this cell line during SSRI exposure will provide a detailed overview of the effects of these drugs on the native steroidogenic pathway. In total, we analysed 16 steroids (4 progestagens, 6 corticosteroids, 4 androgens and 2 estrogens), covering all major steroids in the human steroidogenesis. An overview of the mammalian steroidogenesis and the involved enzymes is shown in Figure 1. The chemical structures and selected physicochemical properties of sertraline, citalopram/escitalopram, fluoxetine, paroxetine and fluvoxamine are shown in Table 1.

2 Materials and methods

2.1 H295R steroidogenesis assay

The H295R steroidogenesis assay was conducted in accordance with the OECD (2011) guideline and Nielsen et al (2012) with minor modifications. The cells were grown at 37°C with 5% CO_2 in 75 cm^3 flasks with DMEM/F12 growth media with 1% ITS+premix and 2.5% Nu-serum. The media was changed every second day and the culture was trypsinated and sub-cultured when it reached a confluency of 75-90%. The cells used for experimentation were in passage 4-12 (OECD, 2011).

The culture was trypsinized for plating when an acceptable confluency of approximately 90% was reached. The cells were then counted on a bürker-türk cell counter and diluted with growth media to a concentration of $3 \cdot 10^5$ cells/mL. 1 mL cell culture was seeded in each of the 24 wells. The cells were allowed to attach to the wells for 24 hour incubation at 37°C under 5% CO_2. A serial dilution of the six SSRIs with dimethyl sulfoxide (DMSO), which is the preferred solvent in the H295R assay (OECD, 2011) was prepared to achieve different experimental concentrations: Fluoxetine: 0.001, 0.002, 0.03, 0.1, 0.3, 1, 3.75, 7.5, 10 and 20 µM; paroxetine: 0.03, 0.1, 0.2, 0.3, 1, 2, 3, 10 and 15, 20 µM; citalopram: 0.03, 0.1, 0.3, 1, 3.1, 10, 31.4 and 50 µM; escitalopram: 0.0002, 0.002, 0.02, 0.2, 2, 20, 50 µM; sertraline: 0.01, 0.03, 0.1, 0.3, 1.0, 3.1

and 7.5 µM and fluvoxamine: 0.1, 0.3, 1, 3, 10, 31 and 43. The dilutions were prepared so that the final DMSO concentration in the wells were maximum 0.1%, which is recommended in the guideline (OECD, 2011).

For each plate, 7 concentrations were added to the wells in triplicates. Furthermore, 0.1% DMSO (n = 3) was added as a solvent control (SC) to measure the background steroid hormone production. Samples were then incubated. After 48 hours, 950 µL cell medium was collected from each sample and 5 ng (50 µL of a 0.1 µg/ml) internal standard (IS) was added. The IS contained the following deuterated standards: d_7-androstenedione (ANd_7), d_4-estrone (E1d_4), d_5-17β-estradiol (βE2d_5), d_8-corticosterone (COSd_8), d_8-11-deoxycorticosterone (11-deoxyCOSd_8), d_9-progesterone (PROGd_9), d_3-testosterone (TSd_3), d_3-dihydrotestosterone (DHTd_3), d_7-aldosterone (ALDOd_7), d_4-cortisol (CORd_4), d_5-11-deoxycortisol (11-deoxyCORd_5) and d_6-dehydroepiandrosterone (DHEAd_6).

Experiments were conducted two to four times on different days. For every experimental trial, a quality control (QC) plate was included to verify the cell growth and hormone expression. The QC plate included blanks (medium, n = 6), and 0.1% DMSO as SC (n = 6). Furthermore, a known inhibitor, prochloraz, (1 and 10 µM, n = 3 for each concentration) and inducer forskolin (0.1 and 1 µM, n = 3 for each concentration) were included (OECD, 2011).

2.2 Viability assay

Potential cytotoxicity was tested with the Alamar blue viability test. Resazurin was added to the wells after 48 hours of exposure to the SSRI. The plates were then incubated for 3 hours at 37°C and 5% CO_2. The fluorescence of Resorufin (formed from Resazurin) was measured using 560 nm excitation/590 nm emission filter set. The fluorescence of Resorufin is proportional to the number of living cells (O'Brien et al., 2000). Cells with viability below 80% compared to the SC were excluded from the data analyses. Cytotoxicity was detected in paroxetine: 15 and 20 µM and in fluoxetine: 20 µM and data were therefor excluded.

2.3 Sample preparation

Precipitation of proteins from the cell culture medium was performed prior to LC-MS/MS analysis, first by adding 900 µL acetonitrile to the 950 µL culture medium. The samples were left on ice to precipitate for 10 min and centrifuged for another 10 minutes at 10.000 RCF. The supernatant was evaporated to app. 1 mL

and a second precipitation was conducted by adding 500 µL methanol. The samples were left on ice for 10 min and centrifuged for 10 minutes at 10.000 RCF. The supernatant was evaporated to dryness by a steam of nitrogen at 60°C and re-dissolved in 1 mL 10% MeOH.

2.4 Quantification by HPLC-MS/MS

Samples were analysed with a newly developed LC-MS/MS method (Weisser et al., 2016). A binary 1290 Agilent infinity series system combined with a binary 1100 Agilent HPLC series pump were used both for on-line cleanup and separation of steroids. The LC setup included a thermostated autosampler set at 8°C, a thermostated column oven set at 40°C with a two position 6 port switching valve connected to an enrichment column followed by an analytical column with a guard column in front. A constant flow of 1 mL/min 10% MeOH (0.1% formic acid) was generated by the 1100 pump. Steroids were retained on the column while salt and proteins were washed into waste. The Agilent 1290 binary pump performed a gradient elution with a flow rate of 0.3 mL/min. Mobile phase A was composed of water with 0.1% formic acid and mobile phase B contained pure MeOH. Total runtime was 16 minutes. To separate the steroids completely an elution gradient was performed. The elution gradient was maintained at 10% B for the first 2 min, 10.0–30.0 % B from 2.0 to 2.2 min, 30.0–60.0 % B from 2.2 to 8.0 min, maintained at 60.0 % B from 8.0 to 10.0 min, 60.0–85.0 % from 10.0 to 12.30 min, 85.0-99.5 % B from 12.3 to 12.5 min and held at 99.5 % B from 12.5 to 14.8 min, before re-equilibrating the column unto 16 minutes. Detailed method describtion and validation is found in Weisser et al (2016).

2.5 Data processing and statistical analyzes

Integration of the peak areas obtained by the LC-MS/MS was performed in MultiQuant™ 3.0 software. The slopes obtained from linear regression on standard curves were used to calculate the levels of steroid in each sample by calculation of ratio between areas of analyte and IS. Cell samples were normalized to a SC conducted in each run. The raw data was processed in Microsoft Excel. GraphPad Prism version 6 (GraphPad Prism Software, La Jolla, USA) was used to create graphs and perform statistical analyzes such as one way analysis of variance (ANOVA) followed by a two sided Dunnetts multiple comparisons test to investigate whether the analytes were significantly different from SC. All results were expressed as mean, with 95% confidence interval and range.

3. Results

Fluoxetine

Four trials with fluoxetine in concentrations ranging from 0.001-10 µM were conducted (n = 6-12). The effects of the H295R steroid production are shown in Figure 2. For concentrations of 3.75 µM and above, a significant decrease was observed for all corticosteroids (except for COR), androgens and progestagens (p-values from 0.01-0.0001), typically in the range 50-70%. This is approximately a factor of 4 higher than the C_{max} for fluoxetine, which is around 0.97 µM (DeVane 1999). The decrease in progestagens indicates that the steroidogenesis is affected at the cholesterol side-chain cleavage enzyme or before this step. However, a significant 50-70% increase in E1 and β-E2 was observed at 3.75 µM and above (p values from 0.05-0.0001) and higher. In general, fluoxetine seems to inhibit the production of steroids in the early part of the pathway, but may enhance CYP19 activity by stimulating estrogen production.

Paroxetine

The H295R cell line was exposed to paroxetine at three different days (n = 3-9) in concentrations ranging from 0.03-10 µM. An overview of the effects in the steroidogenesis is shown in Figure 3. Significant increases were observed for all 4 progestagens at a concentration of 2 µM for PREG and 17-OH-PREG and at 3 µM for PROG and 17-OH-PROG and higher (p-values: 0.05-0.0001). These effects are close to the C_{max} value of 1.6 µM (DeVane 1999). Furthermore increased levels of all corticosteroids, were observed, with the exception of CORNE, the last steroid on the lyase axis. Furthermore, all three androgens (DHEA, AN and TS) significantly decreased at 10 µM (p value from 0.01-0.0001) for all runs. Altogether, this indicates an inhibiting effect of paroxetine on the CYP17-lyase reaction, converting progestagens into androgens. No significant effect was observed on β-E2, but a significant increase was observed for E1 from concentration of 1µM and above ($p \leq 0.0001$). This indicates a weak but nevertheless significant stimulation of the aromatase as also observed for fluoxetine.

Citalopram and escitalopram

Citalopram and escitalopram were both tested in 2 trials (n=6), which reduced all the steroids in the pathway, in the concentration range of 3.14-50 µM (Figure 4). For the progestagens, significant decreases were observed at 3.14 (citalopram) and 2.0 µM (escitalopram) and higher concentrations (p values from 0.01-0.0001), resulting in a 60% and 80% decrease. Similar decreases were observed for the corticosteroids,

although the decrease in COR during citalopram exposure was less pronounced (15%) and not significant. The androgens also decreased during citalopram/escitalopram exposure. However, the decreases in estrogens were lower than that of the androgens and it was only significant at the highest test concentration (50 µM) for E1, whereas no significant change was observed for βE2 indicating that citalopram and escitalopram both have stimulating effects on the aromatase. The C_{max} is in the range 0.25-0.92 µM for citalopram (DeVane 1999) and 0.05-0.25µM for escitalopram (Schulz et al., 2012). The effective inhibitory concentrations are less than an order of magnitude above the Cmax values.

Sertraline

Figure 5 shows the steroid production in the H295R cell line as a function of increasing sertraline exposure ranging from 0.01-7.5 µM from four different experiments (n = 12). For all progestagens and corticosteroids, except for COS, we observed a decrease of around 30-50%, in the concentration range 1.0-7.5 µM (p-values from 0.05 – 0.0001). The C_{max} value for SER is around 0.65 µM, which is similar to the NOAELs for several steroids in Figure 5. Defining a margin of safety (MoS) as NOAEL/Cmax, the MoS will be less than 2.

In this experiment, COS levels were close to detection limits and thus larger standard deviations were observed. The androgens also decreased significantly, corresponding to 40% for DHEA and approximately 50% for AN and TS (p-values from 0.05 – 0.0001). However, a clear increase in estrogens within the range 3.14-7.5 µM was observed, corresponding to approximately 200% for E1 and 300% for β-E2. This is in accordance with the results from the other SSRIs and indicates the ability of sertraline to stimulate the aromatase at supra-therapeutic concentrations.

Fluvoxamine

Finally, we investigated the disrupting properties of fluvoxamine on the steroid production in 2 trials (n = 6). Exposing H295R cells to this drug resulted in a response different from the other SSRIs (Figure 6). All steroids on the CYP17-hydroxylase axis increased significantly with 200-300% (p-values from 0.001–0.0001). In contrast, all steroids on the lyase axis decreased, clearly indicating that fluvoxamine is a CYP17-hydroxylate inhibitor. This is further indicated by the observed decrease in all androgens and estrogens. Effects were observed already at concentrations around 0.1 µM and above (p values from 0.05-0.0001), which is actually lower than the C_{max} value of around 1.6 µM. Therefore there appear to be limited or no margin of safety.

Changes in CYP enzymes product to substrate ratios

The ratios between products and substrates for the three major CYP enzymes in the H295R steroidogenesis are shown in Figure 7. For CYP17, both the hydroxylase and the lyase reactions are shown. In this plot, a ratio significantly higher that 1 indicates a stimulatory effect on the steroid production of the CYPs whereas a significant decrease indicates an inhibition. The aromatase (CYP19) was the most affected enzyme, with increased estrogen/androgen ratio for all six drugs. This was most pronounced for sertraline, fluoxetine and fluvoxamine, but significant effects were also observed for escitalopram, citalopram and paroxetine. The CYP17 was also affected by the drugs, in particular the lyase reaction, whereas the CYP21 was the least affected enzyme.

It is clear from these plots that the six SSRIs affect the CYP enzyme activities of the steroidogenic pathway, however in six different ways. Inspecting Figure 4, one may get the impression that citalopram/escitalopram inhibits the steroidogenesis. However, a closer look reveals that the pathway is actually inhibited prior to PREG formation, and in fact, all three major CYPs in the pathway were stimulated by citalopram and escitalopram, clearly illustrated in Figure 7. It is worth noticing, however, that escitalopram is a stronger CYP17 stimulator than citalopram. Consequently, the stimulatory effect of escitalopram is already observed on the hydroxylase reaction whereas the main effect of citalopram is on the lyase reaction.

Sertraline and fluoxetine also stimulated the aromatase and the CYP21, but exerted no or weak effect on either of the CYP17 reactions. In contrast to the other investigated drugs, fluvoxamine appears to be a true CYP17 inhibitor as pronounced effects were observed both on the hydroxylase and the lyase reactions. Paroxetine was the SSRI exerting the least effects on the H295R steroidogenesis. However, in contrast to the other SSRIs, this drug appears to have a significant inhibitory effect on the CYP17 lyase reaction.

4 Discussion

The overall findings from the experiments are summed up in Table 2 along with the main suggested mechanisms of the six SSRIs. Based on the present studies, fluoxetine appears to be a CYP19 stimulator, but may also inhibit important processes prior to steroid formation such as the StAR and the CYP11A1. Paroxetine appears to be mainly a CYP17-lyase inhibitor, citalopram and escitalopram are general CYP stimulators, sertraline a CYP19 stimulator and fluvoxamine a CYP17-hydroxylase inhibitor. All 6 SSRIs are to some extent aromatase stimulators.

Results from the H295R assay showed decreased levels of progestagens, corticosteroids and androgens with increasing concentration of fluoxetine starting from 3.75 µM, indicating a reduced activity of CYP11a1

or StAR. Furthermore, fluoxetine may be able to activate the estrogenic pathway (via CYP19 induction) as significant increases in both E1 and E2β were observed at 3.75 µM fluoxetine. Increased estrogenic activity of E2β during fluoxetine exposure has previously been reported in an *in vitro* study using MCF-7 human mammary tumor cells (Müller et al., 2012). Concentrations of 11 and 17 µM fluoxetine reported in the above study elicited the estrogenic response, which is in agreement with the present study. The *in vitro* effect concentrations are approximately an order of magnitude above the clinical C_{max} (0.92 µM) indicating that such effects could potentially also be observed *in vivo.* Several *in vivo* studies have been carried out to investigate the effects of fluoxetine on estrogen levels. Conflicting results have been reported (Mennigen et al., 2008; Mennigen et al., 2010; Müller et al, 2012)**.** A 24 hour uterotrophic assay with 21 female Wistar rats receiving 0.4, 1.7 and 17 mg fluoxetine/kg BW was conducted by Müller et al (2012). The therapeutic doses are 0.33-1 mg/kg/day for a standard 60 kg woman, so the doses used by Müller et al (2012) are therefore comparable to suggested human doses, although direct comparison between plasma levels is preferable. The two highest doses caused a significant increase in uterine weights when compared to the control group. This was ascribed to an increased activity of CYP19, and thereby an increased estrogenic effect (Müller et al., 2012). This increased activity of CYP19 in female rats is in accordance with the present *in vitro* study. However, another study by Mennigen et al. (2008), injecting female goldfish (*Carrasius auratus*) with 5 µg fluoxetine/g BW five times over 17 days found decreased E2β levels. The endocrine disrupting effects of fluoxetine in male goldfish were also investigated by Mennigen et al. (2010), by introduction of fluoxetine in the water tank with a concentration of 0.54 µg/L and 54 µg/L. A decrease in TS and an increase in E2β were observed at both concentrations. Since the goldfish were exposed through water, the actual uptake by the fish is unknown. Nevertheless, the results are in accordance with the results in the present study i.e. a decrease in TS and an increase in E2β at elevated levels of fluoxetine. Furthermore the drug label for tablets, containing fluoxetine (FDA, 2016) state that both male and female rats administered with 3, 10, or 30 mg/kg fluoxetine from the weaning period to adulthood has delay in sexual development. Also, reproductive functional impairment (decreased mating at all doses and impaired fertility at the high dose) were seen. This indicates the endocrine potential of fluoxetine during therapy, which is a cause for concern. Clearly, more clinical and non-clinical studies on the effects of fluoxetine on sex steroids are needed.

The present study indicates that paroxetine is a CYP17-lyase inhibitor. A previous study performed in the H295R cell line with similar concentrations of paroxetine showed a significant increase in PROG and E2β and a decrease in TS (Jacobsen et al, 2015). This supports the hypothesis that paroxetine is a potential CYP17-lyase inhibitor, although further studies are needed.

Only a few *in vivo* studies have investigated the endocrine disrupting effect of paroxetine. A study by El-Gaafarawi et al. (2005) showed a significant reduction in the serum LH, FSH and TS levels after 20 days when male albino rats were treated with 0.36 mg paroxetine/100 g BW/day in a 30-days experiment. Furthermore, an increase was observed in E2β, similar to the *in vitro* experiments by Jacobsen et al. (2015). For female rats given same dosages for 10 days, a decrease in LH, FSH and E2β was observed as well as an increase in TS (EL-Gaafarawi et al, 2005). The decrease in E2β and LH may cause impaired reproduction in the females. Reproductive function was investigated by Gaukler et al. (2015) who found that female mice exposed to 22.5 mg/kg/day paroxetine took 2.3 times longer to produce their first litter compared to the control group. This could be caused by altered hormone levels and/or altered libido. These findings indicate that the decrease found in both TS and estrogens in the current *in vitro* study could potentially also occur *in vivo*, even though the effect concentrations found *in vitro* were around 10 times greater than the C_{max} values.

In a prospective study including 35 healthy men that were treated with paroxetine, an escalating dosing schedule starting from 10 to 20 mg daily was used (Tanrikut et al, 2010) and the serum hormones TS, E2β, LH and FSH were analysed by enzyme assays. Testosterone and E2β levels decreased significantly but remained within the normal range for adult men, whereas no change in LH and FSH levels were observed. These observations, and the decrease in TS levels observed in the present *in vitro* study, indicate the potential of paroxetine to decrease TS levels *in vivo*. Since TS is involved in sperm production in the seminiferous tubules (Walker, 2011), this could potentially exert negative effects on male fertility.

With the exception of COR during citalopram exposure, a decrease was observed for all steroids downstream from CHOL when the H295R cells were treated with citalopram or escitalopram. Because the decrease was observed in the beginning of the steroidogenesis, citalopram and escitalopram may cause alterations prior to the steroidogenetic pathway perhaps at StAR or CYP11A1 (OECD, 2011). When CYP11A1 is affected, it may lead to adrenal insufficiency with low adrenal and gonadal steroid production, smaller size of adrenals and gonads and premature maturation (Hauffa et al., 2011; Miller and Auchus, 2011; Rubtsov et al., 2009). Changes in blood pressure (hypotension), glucose levels (hypoglycemia) and water/salt balance may be seen in StAR deficiency in humans (King et al., 2011; Miller and Auchus, 2011). The present results are in agreement with an earlier study also performed in the H295R cell line with similar concentrations of citalopram (Jacobsen et al., 2015) showing decreased levels of PRO and TS but less effects on E2β. The less pronounced effects on the estrogens suggest a compensatory increase in the aromatase activity as also observed in the other investigated SSRIs. Interestingly, an aromatase inhibition assay performed by Jacobsen et al. (2015) showed an inhibition of recombinant CYP19 with increasing

amounts of citalopram, which indicates a decrease in the estrogens although the concentrations needed were 18-194 times higher than concentrations normalizing estrogen production in the present study.

We have been unable to identify any experiments exploring the endocrine disrupting effects of escitalopram. However, based on the present studies, escitalopram seems to be at least as potent an endocrine disruptor as citalopram. Since citalopram is marketed as a racemic drug containing both the R and S enantiomers, patients are exposed to both. Also, the studies refered to above were conducted with both enantiomers. Based on this and the present results, it may be speculated that the endocrine disrupting effects of citalopram is mainly due to effects exerted by S-citalopram, whereas the R-citalopram may have lower endocrine dirrupting potential. Further studies should be conducted to clarify this important aspect.

An earlier investigation of sertraline in the H295R cell line, using similar concentrations as those used in the present study, showed a significant decrease in TS and PROG levels and a significant increase in the levels of E2β (Jacobsen et al, 2015). These results are similar to those of the present study and underline the potential of sertraline to decrease androgens and increase estrogens.

A 3 months clinical trial study with 60 male patients showed a significant reduction in sperm concentration, sperm morphology and an increase in DNA fragmentation when treated with 25 mg sertraline per day (Akasheh et al., 2014). This suggests that sertraline adversely affects fertility controlled by TS, although it must be mentioned that TS was not analysed in the study by Akasheh et al (2014). In men, some cases of infertility may be related to high aromatase activity. In a study, 27 infertile men were treated with the aromatase inhibitor letrozole. The men then exhibited an increased TS/E2 ratio, which increased the sperm motility and sperm count (Gregoriou et al., 2012; Saylam et al., 2011). This suggests a connection between the sperm count and the levels of sex hormones in some patients, which in turn may affect fertility. In women, aromatase activity is also finely tuned. For example, aromatase activity is involved in the E2β surge triggering ovulation. Any interruption of aromatase activity may therefore also exert negative effects on female reproduction. In the present *in vitro* study, a decrease in TS was observed for all SSRIs at margins of one to twenty times over the expected Cmax values, which raises concern regarding a possible adverse effect on fertility in men and women in their reproductive age using SSRIs for extended periods of time.

In the present study, all steroids on the PRE-ALDO axis increased during exposure to increasing levels of fluvoxamine, whereas all other steroids in the pathway decreased. The increase in all steroids on the CYP17-hydroxylase axis and a decrease in all other steroids is charactistic for true CYP17 inhibitors and usually result in significant inhibition at the hydroxylase reaction (Bonomo et al 2016). In accordance with the remaining five SSRIs studied, we also observed an apparent compensatory stimulation of the aromatase, but it should be kept in mind that the total levels of estrogens decreased due to the sequential inhibition of the CYP17.

In a previous study, fluvoxamine has been investigated in both male and female rats. Exposing female rats to 10 mg/kg BW/day significantly reduced circulating E2β and PRO levels, but TS levels in male rats were not affected (Rehavi et al., 2000). Although the decrease in E2β is consistent with our *in vitro* results, results are scarce and there is presently little evidence that fluvoxamine stimulates the aromatase *in vivo*.

Overall, the study confirms that SSRIs have the potential to exert endocrine disruption by interfering with steroidogenesis in a transformed cell line in vitro. Although the investigated SSRIs are very different from a chemical structure point-of-view, the outcome may still be an imbalance in sex steroids and changes in androgen to estrogen ratios, which may partly explain some of the sexual disorders described for SSRI users.

For some drugs, we observed inhibitory effects on some CYP enzyme but stimulatory effects on other enzymes. To the best of our knowledge, there are no good examples in the scientific literature in which a chemical have an inhibiting effect on one CYP enzyme and a stimulatory effect on another closely related enzyme. However, the effects of the SSRIs studied here may be a combination of several effects, including direct effects i.e. direct interactions with the enzymes, and indirect effect i.e. effects on transcription or autocrine feed-back mechanisms (OECD 2011). Our study therefore indicates that the effects of the SSRIs on the steroidogenesis are complex and may affect several processes in the cells. Further studies to understand how stimulatory effects are produced are warranted.

The present study confirms previous findings that different mechanistic effects early in the steroidogenesis may result in similar effects on the sex steroids further down the pathway (Nielsen et al 2012). This further demonstrates the importance of analyzing the entire steroid profile to obtain a better mechanistic understanding as to how chemicals affect this very dynamic pathway.

6. Conclusions

The present *in vitro* study using the H295R cell line showed that six SSRIs, fluoxetine, paroxetine, citalopram, escitalopram, sertraline and fluvoxamine, all exerted endocrine disrupting effects on the mammalian steroidogenesis when used at sufficient concentrations. Although the target enzyme through which the six investigated SSRIs affected steroidogenesis was different, the outcome was the same i.e. a decrease in androgens and a compensatory stimulation of the estrogen production. Since these effects were observed in the range of one to fifty times of the C_{max} values for therapeutic treatment, the study indicates limited margins of safety for these drugs in relation to hormone production. The study demonstrates that the impact on the steroidogenesis may be very different between related compounds. Nevertheless, the effect on the male sex steroid TS was the same, i.e. a decrease. We also conclude that the investigated SSRIs are aromatase stimulators. The mechanism for this is not known. It could be direct stimulation by binding non-competitively to the enzyme or it could be an indirect effect, for example by increasing the *CYP19* gene transcription. Finally, our study indicates that fluvoxamine is a CYP17-hydroxylase inhibitor whereas paroxetine is a CYP17-lyase inhibitor. CYP17 inhibitors are rare and in high demand in drug therapy such as cancer therapy. Presently, abiraterone is the only drug on the market which targets the CYP17-hydroxylase and there are no drugs on the market where the therapeutic effect is via the lyase. This aspect should be investigated further. Overall, we conclude that the endocrine disrupting potential of these drugs should encourage careful use of these drugs in therapy. In particular fluvoxamine and fluoxetine, which seem to have MoS values (defined as NOAEL/Cmax) lower than 1.

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Table legends

Table 1: Chemical structures, physicochemical properties, pharmacokinetic data, and effect on the steroid hormones in vitro for SSRIs. [a] Values obtained using EPI SuiteTM v4.0. [b] DeVane (1999). In DeVane (1999) concentrations were given in ng/ml. Here the values are given as µM for easy comparison with the NOAEL values. [c] IRF (2010). [d] Schultz et al (2012). The molar concentrations have been calculated using the shown molar weights (M_w).

Table 2: Overall effects of the five investigated SSRIs on the three major CYPs in H295R steroidogenesis and the main mechanisms suggested. ↑: increased effect; ↓: decreased effect. -: No major effect. *: $p < 0.05$ **: $p < 0.01$ ***: $p < 0.001$, ****: $p < 0.0001$.

Table 1

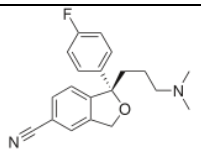
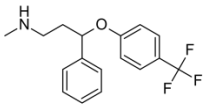
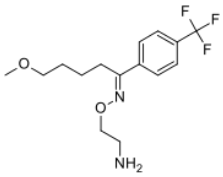
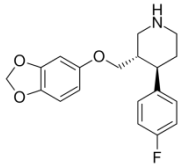
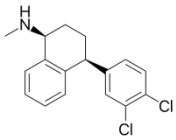
Drug	Chemical structure	Log P[a] pK_a - values[a] M_w (g/mol)	Therapeutic dose (mg/day)	Steady state plasma conc. (µM)[b]
(Es)Citalopram CAS # 59729-33-8 (128196-01-0)		3.7 9.1 324.4	20-60[b] (10-20)[c]	0.12-0.93 (0.05-0.25)[d]
Fluoxetine CAS 54910-89-3		4.1 9.2 309.3	20-60[b]	0.29-0.97
Fluvoxamine CAS # 54739-18-3		3.1 8.2 318.3	50-100[b]	0.063-1.6
Paroxetine CAS # 61869-08-7		2.9 9.6 329.3	20-60[b]	0.027-1.8
Sertraline CAS # 79617-96-2		5.3 8.9 306.2	50-200[b]	0.065-0.65

Table 2:

SSRI	CYP enzyme								Suggested main mechanism
	CYP17 hydroxylase		CYP17 lyase		CYP19		CYP21		
	Effect	Concentration/ Significant level	Effect	Concentration/ Significant level	Effect	Concentration/ Significant level	Effect	Concentration/ Significant level	
Fluoxetine	-	-	-	-	↑	3.75**, 7.5****	-	-	CYP19 stimulator, StAR or CYP11A1 inhibitor
Paroxetine	-	-	↓	10**	↑	10*	-	-	CYP17 lyase inhibitor
Citalopram	-	-	↑	10****	↑	10*, 31.4**, 50****	↑	50***	CYP19 stimulator, general CYP stimulator
Escitalopram	↑	20**	↑	50***	↑	20***, 50****	↑	20**, 50****	CYP19 stimulator, general CYP stimulator
Sertraline	-	-	-	-	↑	3.14****	↑	3.14**, 7.5***	CYP19 stimulator
Fluvoxamine	↓	1**, 3.14****	↓	1****	↑	43**	↓	31.4****	CYP17 hydroxylate inhibitor

Figure legends

Figure 1: Overview of the mammalian steroidogenesis showing the chemical structures of all major steroids. Blue boxes indicate the enzymes involved in the pathway.

Figure 2: Effects on steroid production in the H295R cell line during fluoxetine exposure. Steroid production is shown as the relative concentration (% of SC) as a function of fluoxetine concentration (n = 6-9). *: $p < 0.05$ **: $p < 0.01$ ***: $p < 0.001$, ****: $p < 0.0001$. Cmax for fluoxetine was found to be 0.29-0.97 µM Abbreviations: DHEA: Dehydroepiandrosterone, 3-betaHSD: 3-beta-hydroxysteroid dehydrogenase, 17-beta-HSD: 17-beta-hydroxysteroid dehydrogenase, CYP19: Aromatase, CYP21: 21-alpha-hydroxylase.

Figure 3: Effects of paroxetine exposure on steroid production in the H295R cell line. The y-axis shows the relative concentration (% of SC) as a function of PAR concentration (n = 6). Cmax for paroxetine is 1.8 µM. Symbols and legends otherwise as in Figure 2.

Figure 4: Effects in H295R cell line after citalopram (top) and escitalopram (bottom) exposure. The effect is shown as the relative concentration (% of SC) as a function of CIT concentration (n = 6). Cmax for citalopram and escitalopram are 0.92 µM and 0.25 µM respectively. Symbols and legends otherwise as in Figure 2.

Figure 5: Effects on steroid production in the H295R cell line after sertraline exposure. The effect is shown as the relative concentration (% of SC) as a function of sertraline concentration (n = 12). Cmax for sertraline is 0.65 µM Symbols and legends otherwise as in Figure 2.

Figure 6: Effects on steroid production in the H295R cell line after fluvoxamine exposure. The effect is shown as the relative concentration (% of SC) as a function of fluvoxamine concentration (n = 6). Cmax for fluvoxamine is 1.6 µM Symbols and legends otherwise as in Figure 2.

Figure 7: Product/substrate ratios for the three main CYP enzymes in the H295R steroidogenesis. Increased ratios indicate a stimulatory effect whereas a decrease in the ratio indicates an inhibition. For CYP17, both the hydroxylase reaction and the lyase reaction are shown.

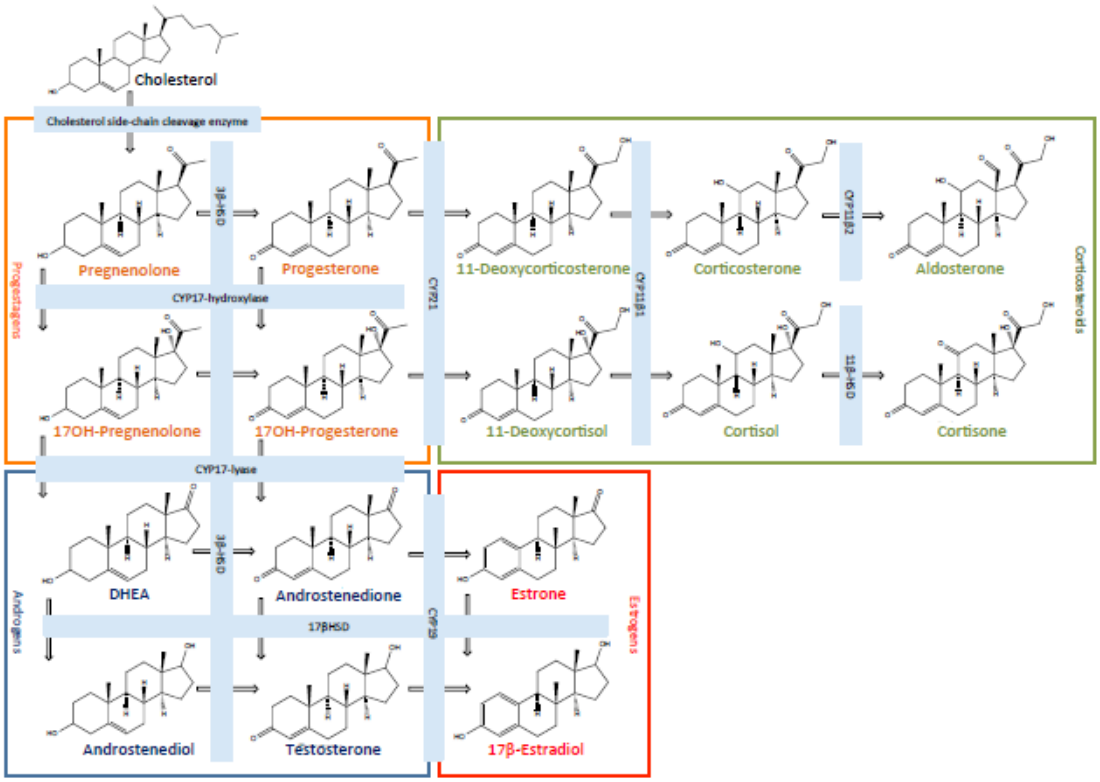


Fig. 1

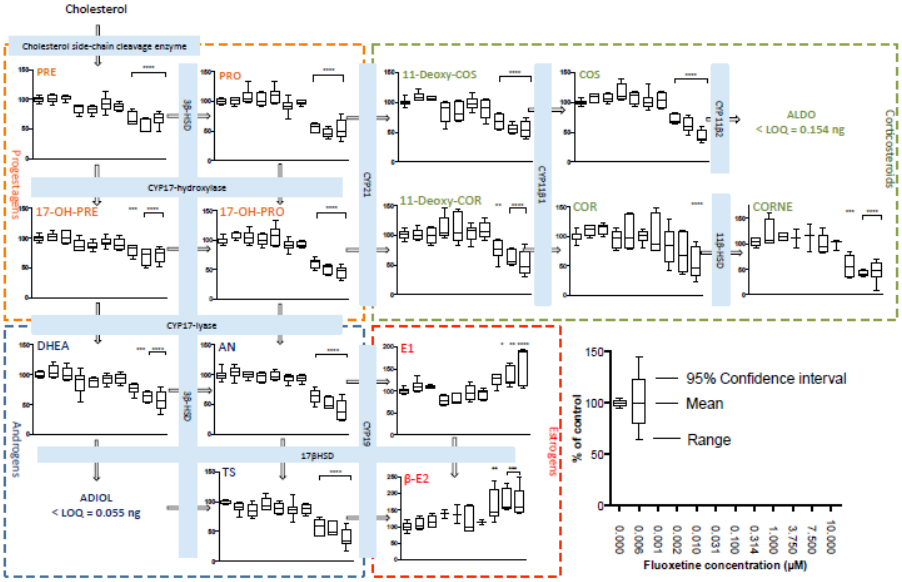


Fig. 2

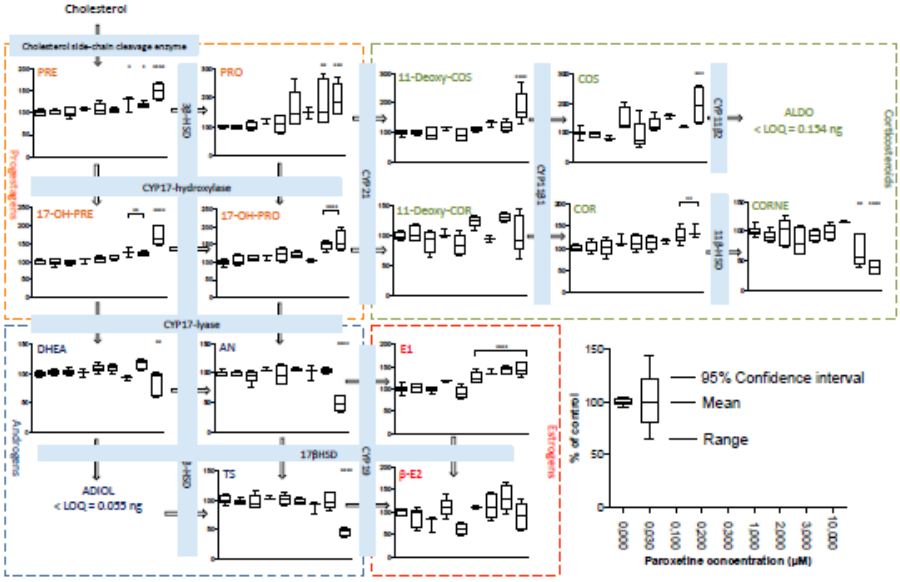


Fig. 3

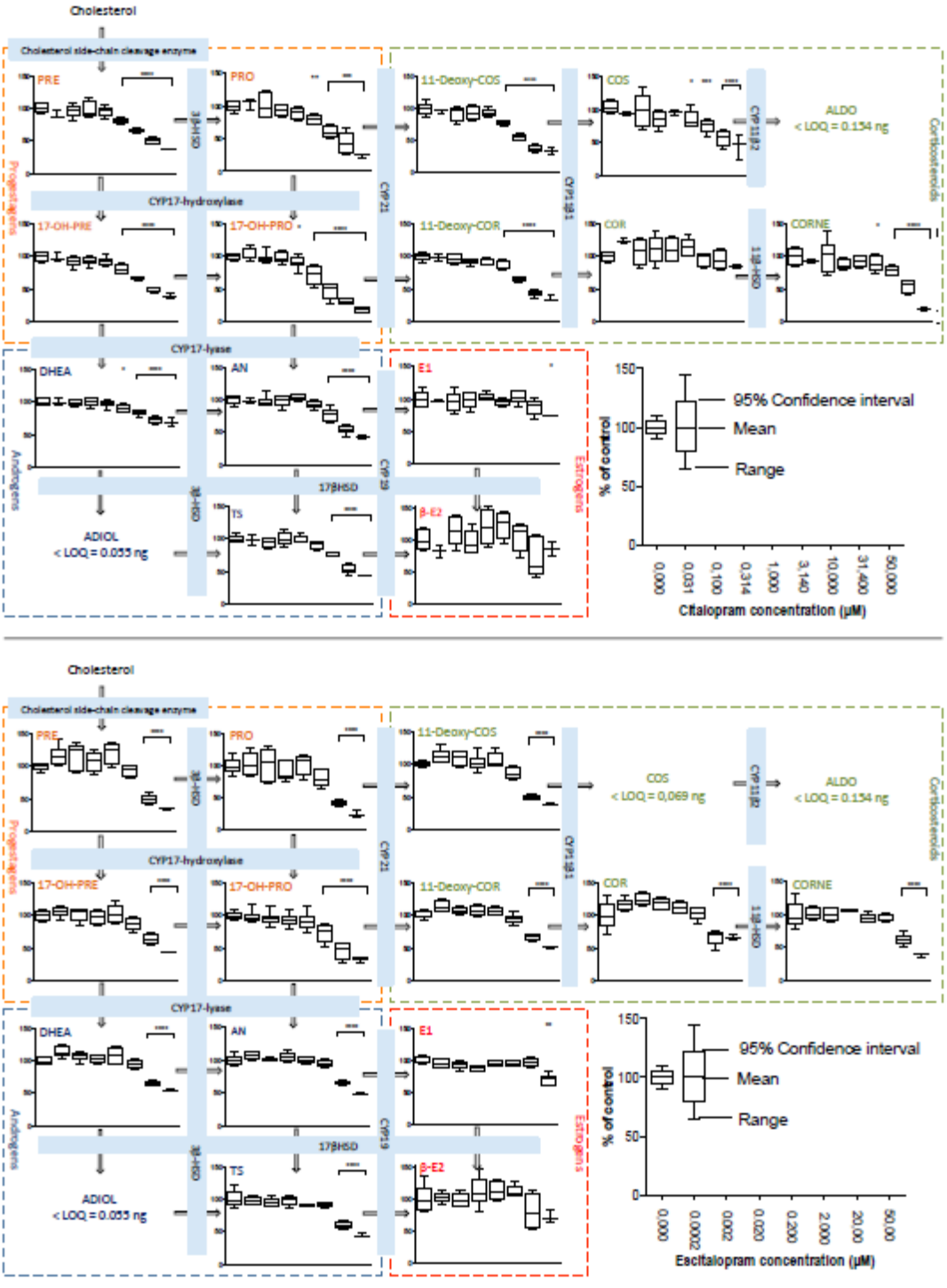


Fig. 4

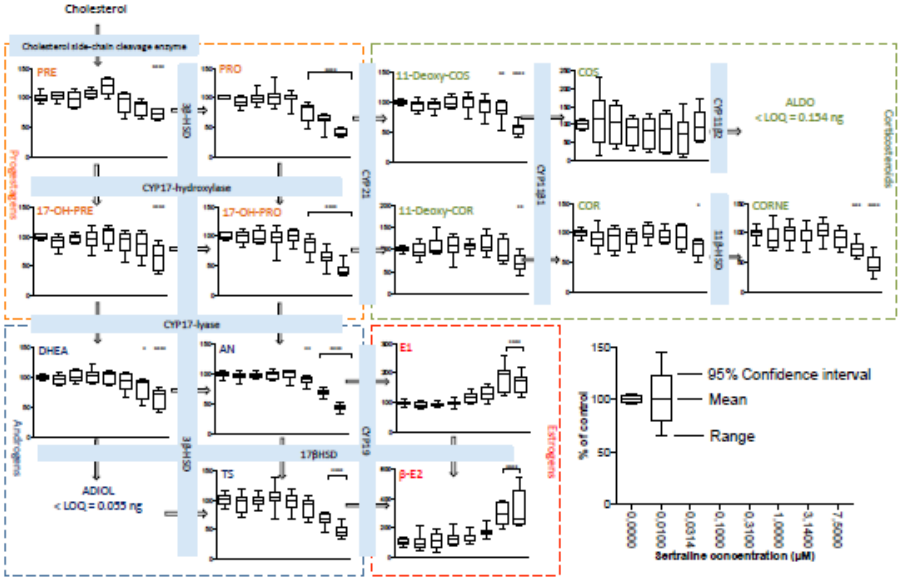


Fig. 5

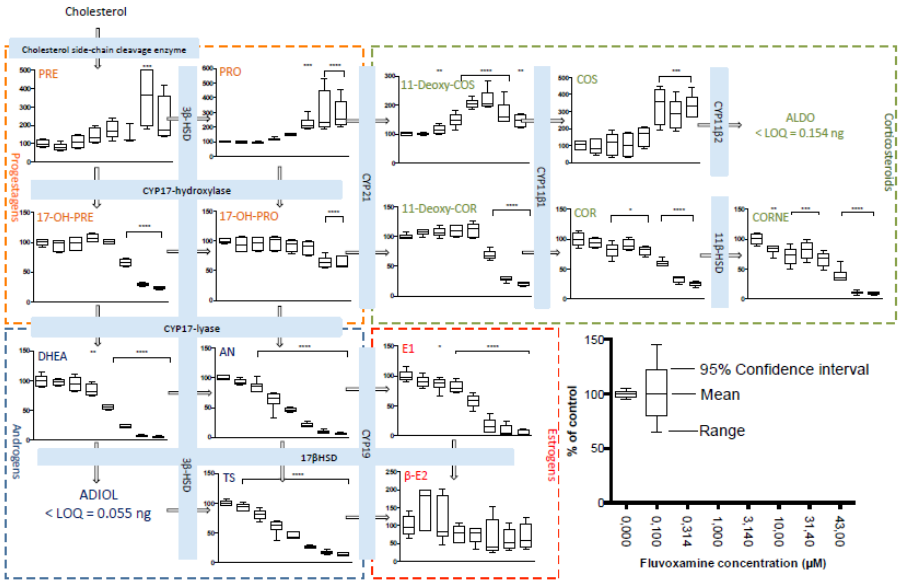


Fig. 6

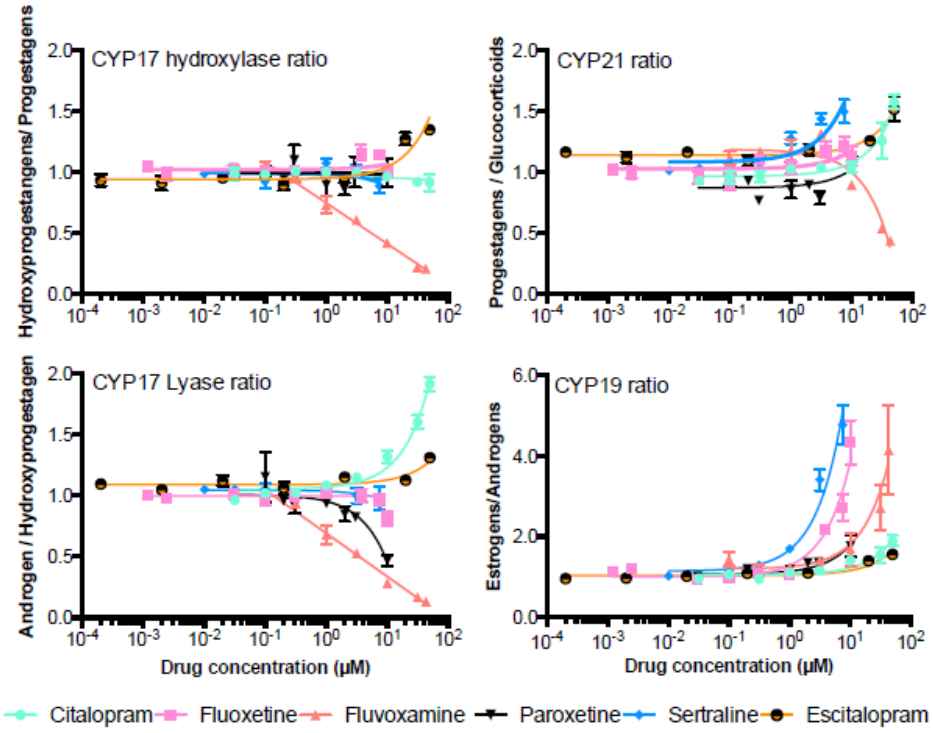


Fig. 7

Highlights

- The effects of 6 SSRIs on steroid production were investigated in H295R cells.
- All 6 SSRIs were shown to inhibit androgen production.
- CYP19 substrate/product ratios increased for all 6 SSRIs.
- All the major CYP enzymes in the steroidogenesis were affected by the SSRIs.
- Based on product/substrate ratio the aromatase was the only enzyme consistently stimulated.