ORIGINAL ARTICLE

EVALUATION OF THE ANDROGENIC/ANTIANDROGENIC ACTIVITY OF SELECTIVE SEROTONIN REUPTAKE INHIBITORS

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) are being increasingly prescribed to treat depression, even during pregnancy and *postpartum*, although recent studies suggest that SSRIs may interfere with sexual steroid signalling, potentially causing adverse effects in exposed children. This study aimed to assess potential SSRIs interactions with the androgen receptors (AR) *in vitro*, using a firefly luciferase reporter construct in the MDA-kb2 cell line. The tested compounds were fluoxetine and its metabolite, norfluoxetine, sertraline and paroxetine. None of the compounds induced AR-transcriptional activity during individual testing. In mixtures with dihydrotestosterone (DHT), all the compounds induced a significant increase in luciferase expression (compared to the positive control) at low concentrations. This study shows for the first time that SSRIs may potentiate the DHT-induced androgenic activity, and depending on the mechanism underlying this effect, these results could raise concern regarding the endocrine toxicity.

Rezumat

Compuşii din clasa inhibitori selectivi ai recaptării serotoninei (ISRS) sunt prescrişi frecvent pentru tratamentul depresiei, chiar şi în timpul sarcinii şi alăptării, cu toate că studiile recente sugerează faptul că aceşti compuşi ar putea interfera cu activitatea steroizilor sexuali, cauzând efecte adverse la copiii expuşi. Acest studiu a avut ca scop evaluarea unor potenţiale interacţiuni *in vitro* între ISRS şi receptorii androgenici, utilizând linia celulară MDA-kb2 care exprimă un sistem raportor cu luciferază. Compuşii testaţi au fost fluoxetina şi metabolitul său norfluoxetina, sertralina şi paroxetina. În cazul testării individuale, niciunul dintre compuşi nu a reuşit activarea receptorilor androgenici. În amestecuri cu dihidrotestosteronul (DHT), toţi compuşii au indus o creştere semnificativă a expresiei luciferazei (comparativ cu controlul pozitiv) la concentraţii mici. Acest studiu arată, pentru prima oară, faptul că inhibitorii selectivi ai recaptării serotoninei ar putea potenţa activitatea DHT. În funcţie de mecanismul acestui efect, aceste rezultate ar putea semnala o toxicitate la nivel endocrin.

Keywords: fluoxetine, norfluoxetine, sertraline, paroxetine, luciferase assay

Introduction

Depression is a highly prevalent mood disorder [15] that is often diagnosed in adults between 35 and 49 years of age [16]. Nonetheless, it can also manifest during childhood or adolescence [24], during pregnancy and/or after birth [42, 28, 29], at menopause [10], old-age [16], in the case of various chronic diseases and certain pharmacologic treatments etc. [22]. 7.6% of USA population aged 12 and over experienced moderate or severe depressive symptoms during 2009-2012 [7], while major depression alone has been reported to affect over 30 million people in the EU in 2011 [41].

Depression affects more women than men [33] and even during pregnancy and lactation depression is being increasingly treated with antidepressant medication [5]. For this purpose, selective serotonin reuptake inhibitors (SSRIs) are usually prescribed [5], but these medications cross the placental barrier and are also excreted in breast milk, thus passing to the developing *foetus* and child to different extents [11, 30]. Consequently, there are concerns about some adverse effects of SSRIs in children exposed *in utero* or through lactation [23].

Studies on rodents show that fluoxetine can affect the sexual differentiation of the brain and alter sexual behaviour in both male and female rat offspring exposed through lactation [31, 32]. Continuous exposure to fluoxetine during gestation and lactation can also negatively affect the testicular development and sperm production in rat offspring [6, 26, 40]. These results suggest that SSRIs may interfere with sexual steroid signaling during development. Moreover, an array of studies on aquatic animals and rodents, but also case-reports in humans, point out to adverse endocrine and reproductive effects related to SSRI exposure [27], further supporting the idea of SSRIs interference with sexual steroid signalling. Although fluoxetine has already been shown to stimulate nuclear estrogenic receptors both *in vitro* and *in vivo*, the same study found no interactions

with the androgen receptor *in vivo* [27]. There have been no studies to evaluate interactions of other SSRI compounds with the androgen receptors (AR).

This study aimed to assess potential SSRI interactions with the AR *in vitro*, using the MDA-kb2 breast cancer cell line. The cells express androgen receptors and have been stably transfected with a luciferase reporter construct. The tested compounds were fluoxetine and its active metabolite norfluoxetine, sertraline and paroxetine. These compounds have different rates of transfer through the placenta and in breast milk [11, 30]. All compounds were tested individually and in mixtures with dihydrotestosterone to detect potential additive, synergistic or antagonistic effects.

Materials and Methods

Chemicals and Reagents

The test compounds, namely fluoxetine (FLX), norfluoxetine (NFLX), sertraline (SRT) and paroxetine (PRX) were purchased from LGC Standards (Germany). 5-alpha-dihydrotestosterone (DHT), resazurin, tricine, Ethylenediaminetetra-acetic acid (EDTA), Dithiothreitol (DTT), and adenozin triphosfat (ATP) were purchased from Sigma Aldrich (Steinheim, Germany). $(MgCO_3)_4Mg(OH)_2.5H2O$ was obtained from Acros Organics (Geel, Belgium), 1,2-diamino-cyclohexane-N,N,N',N'-tetraacetic acid (CDTA) and Tris were from Fluka (Buchs, Switzerland) and luciferin (≥ 99%) was from Roth (Karlsruhe, Germany). All solvents and reagents were of analytical grade. L-15 culture medium was purchased from ATCC (USA) and trypsin, Leibovitz's phenol-red free medium and charcoal stripped foetal bovine serum (FBS) were purchased from Gibco (Paisley, UK). Foetal Bovine Serum (FBS) was obtained from Sigma (Steinheim, Germany) and Dulbecco's Phosphate Buffered Saline (PBS) was obtained from Invitrogen.

Stock solutions

All compounds were prepared as 1000 µL stock solutions in dimethyl sulfoxide (DMSO ≥ 99.5%, Riedel-de Haën, Seelze, Germany) at a concentration of 30 µM. Solutions of 0.005, 0.015, 0.05, 0.15, 0.5, 1.5, 5, 7.5, 10 and 15 µM for all four test chemicals were obtained from their respective stock solutions that were subsequently diluted with DMSO. These serial dilutions were then used to obtain the desired test concentration range for individual compounds. For FLX and NFLX equimolar mixtures of 0.005, 0.015, 0.05, 0.15, 0.5 and 1.5 µM were prepared.

Cell culture

MDA-kb2 human breast cancer cells (ductal adenocarcinoma positive for androgen receptors) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in L-15 medium supplemented with 10% FBS, at 37°C, without additional CO_2, under saturating humidity and passaged every 2 to 3 days. Dulbecco's Phosphate Buffered Saline was used to rinse the cells and trypsin was used to detach cells from culture flasks. During the experiments, the cells were cultured in phenol red-free Leibovitz's medium containing 10% charcoal stripped FBS.

Viability assay

For the viability assay, cells were cultured in 96 well plates. Cells were allowed to attach for 24 h, then rinsed with 200 µL PBS and subsequently exposed to individual test compounds at increasing concentrations and binary mixtures compound-DHT (300 pM DHT) for 24 h. Cell viability was evaluated by measuring the capacity of the cells to reduce resazurin (plate concentration 100 nM), a non-fluorescent reagent, to resorufin, a fluorescent product. This reaction takes place in the mitochondria of metabolically active cells and is therefore an indicator of cell viability [3]. The fluorescence was measured at $\lambda_{excitation}$ = 530/25; $\lambda_{emission}$ = 590/35, using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek).

Luciferase assay

For luciferase induction, the cells ($8.5x10^6$ cells/mL) were seeded in 96-well plates and incubated 24 h at 37˚C. After this period, the medium was removed and the cells were rinsed with PBS. 100 µL fresh medium was added together with another 100 µL medium that contained the tested compounds at increasing concentrations and binary mixtures compound-DHT. DHT at a concentration of 300 pM served as a positive control and was used in combination with the test chemicals in order to screen for anti-androgenic effect. Each chemical was tested in three independent experiments in the presence or absence of DHT. The assay medium contained DMSO at a concentration of 0.2%. After a 24 h exposure to test chemicals or mixtures (compound-DHT), the cells were lysed using a low salt buffer containing 10 µM Tris, 1.99 µM DTT and 2 µM CDTA. Following 15 minutes incubation at -20°C, the plates were frozen at -80°C for a minimum of 30 minutes. Plates were then thawed on ice and shaken for 30 min. at room temperature. Luciferase activity was measured using a luminometer (Synergy 2 Multi-Mode Microplate Reader, BioTek) with automatic injection of 100 µL luciferin FlashMix [0.47 µM luciferin, 20 µM tricine, 1.07 µM $(MgCO_3)_4Mg(OH)_2$ x $5H_2O$, 0.1 µM EDTA, 2 µM DTT and 5 µM ATP, pH 7.8] in each well. Light emission was extinguished with 50 µL NaOH 0.2 M.

Data analysis and statistics

The results were expressed as mean ± standard deviation (SD). For each experiment, relative light

units (RLUs) in every well were corrected by subtracting the mean response of control wells. For individual testing, the signals from compound-treated cells were compared to the negative control (solvent control). To compare data for mixtures compound-DHT, the mean induction of luciferase obtained at 300 pM DHT, was set at 100%. Data were expressed as percentages of the signal observed for 300 pM DHT (positive control) and were compared to the DHT signal. Statistical Analysis Origin software (OriginLab, Northampton, USA) was used for graphical analyses. Data were analyzed by one-way analysis of variance (ANOVA). Differences in p values of < 0.05 were considered statistically significant.

Results and Discussion

Viability assay

To assess cell viability for the androgenic assay, the MDA-kb2 cell line was exposed to FLX, NFLX, SRT and PRX in increasing concentrations (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 15, 20 µM) for 24 hours. PRX was the most toxic compound, as concentrations higher than 3 µM produced a significant decrease in the cell response to resazurin. For SRT, concentrations lower than 15 µM did not decrease cell viability in a significant manner, while for NFLX and FLX it was possible to further test for (anti)androgenic activity concentrations up to 15 µM and 20 µM, respectively.

For the antiandrogenic assay, citotoxicity was assessed by incubating the cells for 24 hours to binary mixtures of the studied compounds (increasing concentrations) and 300 pM DHT. A suitable concentration of DHT was chosen based on its dose-response curve which showed that 300 pM corresponds to an effect level in the upper third of the linear range (EC50 for the DHT = 202.45 pM). In a previous study, Ermler *et al* argued that DHT at a high concentration may overcome the effect of weak AR antagonists and also that using lower DHT concentrations may result in a flawed dynamic range of the assay [9]. The range of tested compound concentrations in the binary mixtures were as follows: FLX 0.01-20 µM, NFLX 0.01-15 µM, SRT 0.01-15 µM and PRX 0.01-3 µM.

(Anti)androgenic assay

None of the tested compounds was able to induce a significant luciferase expression, in case of individual exposure.

When tested in the presence of DHT, only SRT showed a statistically significant anti-androgenic activity, but only at 15 µM, the highest concentration tested (and most probably not biologically relevant in case of therapeutic doses) (Figure 1d). At low concentrations, all the compounds revealed an increase in the cellular response (Figure 1a-d), indicating a potential synergistic effect between SSRIs and DHT.

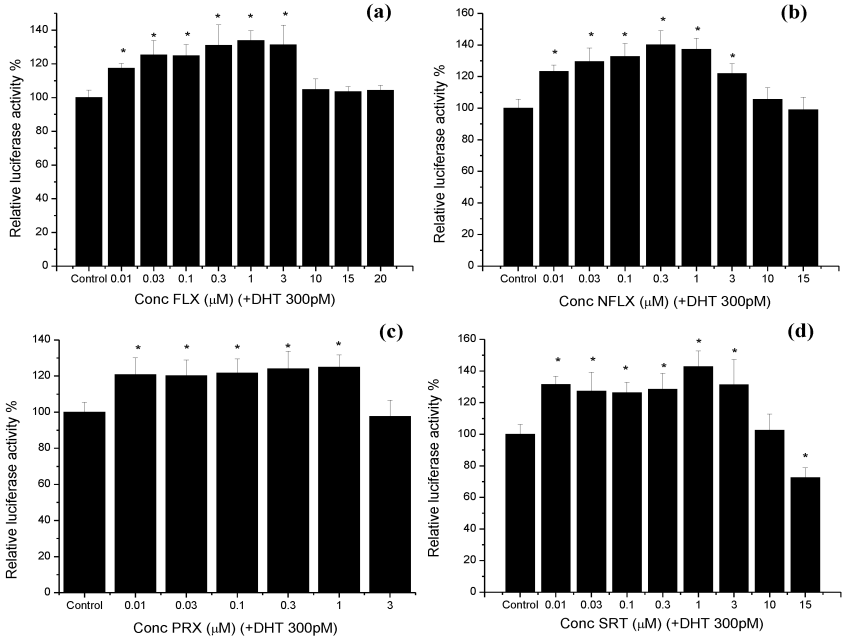

Figure 1.
Effects of mixtures compound (increasing concentrations)-DHT (300pM) on luciferase activity in MDA-kb2 breast cancer cells (a) FLX, (b) NFLX, (c) PRX, (d) SRT. (*) statistical significance compared to positive control (DHT)

According to a recent report of the Organization for Economic Cooperation and Development, the use of antidepressants (AD) has increased considerably in the last decade, SSRIs being the most widely used class of AD [12]. Among SSRIs, the antidepressant efficacy is similar, but their pharmacokinetic profiles are substantially different [4]. Since their metabolism presents a high interindividual variability, the blood concentrations are highly variable between individuals [13], but therapeutic concentrations are in the nanomolar to low micro molar range for all compounds [37]. SSRIs are prescribed as pharmacotherapy even for pregnancy and *postpartum* depression, although they can cross the placenta and pass into breast milk, therefore reaching the foetus and new-born [11, 28, 30]. The long-term effects of gestational and lactational SSRI exposure are unknown, but research into some potential adverse effects is emerging.

For example, various studies on fish suggest that FLX can act as an anti-reproductive neuroendocrine disruptor [21]. Another study by Mueller et al shows that FLX can induce an uterotrophic response in rats, although the authors have not investigated indirect effects on hormone control [27]. However, taken together with the *in vitro* data for oestrogen receptor activation in MCF-7 cells, Mueller et al suggested that FLX may be a xenoestrogen. In the same study, an increase (up to 33.8% ± 3.8%) of the luminescence, when the cells were incubated with FLX and oestradiol was observed compared to cells exposed to oestradiol, indicating a possible additive effect. FLX was also evaluated by the same group for (anti)androgenic activity *in vivo*, using the Hershberger assay, but no significant effects were found [27]. Montagnini et al evaluated SRT for estrogenic activity using a rat uterotrophic assay, but no positive response was found either [25].

Other studies in rats have shown that gestational, lactational and combined gestational + lactational exposure to some SSRIs may impact the sexual differentiation of the brain, sexual behaviour, testicular development and sperm production in rat offspring [31, 32].

Continuous exposure to fluoxetine during gestation and lactation can also negatively affect testicular development and sperm production in rat offspring [6, 26, 40]. FLX, SRT and PRX were also tested in adult rats for their effects on testicular tissue. Erdemir et al showed that PRX can affect the spermatogenesis, PRX and FLX decreased the follicle-stimulating hormone (FSH) levels, while SRT lowered the levels of testosterone [8]. Safarinejad et al and Kumar et al also discovered that sperm parameters were negatively affected by FLX, SRT, PRX and an additional spermicidal effect was demonstrated [19, 34]. Alteration of the sperm DNA and also the association of SSRI use with low serum free testosterone levels may suggest an endocrine disruptive effect of these compounds on the hypothalamic pituitary gonadal axis testes (HPTA axis), on sexual steroid metabolism or on estrogenic or androgenic receptors.

Regarding the sexual differentiation of the brain, Rayen et al showed that lactational FLX exposure of male rat pups results in a reduced area of the sexually dimorphic nucleus of the preoptic area (SDN-POA) and altered sexual behaviour [31]. Previous studies have shown that androgen receptor function is essential for complete masculinization of the brain [36] and that AR are also present in the medial preoptic area (MPOA) [35], which contains the SDN-POA [14] and is involved in the control of male copulatory behaviour in vertebrates [2, 18].

Conclusions

In our study we show for the first time that SSRIs may potentiate the DHT-induced androgenic activity in human breast cancer cells. This may lead to a down-regulation of the receptors to avoid prolonged overstimulation. A study by Soga et al showed that male mice treated with citalopram (another SSRI) postnatal have decreased numbers of AR-positive cells in the MPOA [38], but the mechanism behind this change remains unclear.

This type of effect of the combined treatment DHT + SSRIs could be explained by a possible facilitation and promotion of AR nuclear translocation, which might result in an increased transcription [17, 20]. In our study, only at lower concentrations (achievable during the SSRIs therapy) the AR-mediated transcriptional activity was observed and these results could raise concern regarding the endocrine toxicity.

Besides the mechanism mentioned above, literature reports the existence of small molecules (MW_{avg} = 325.8 ± 63.3) that act as luciferase inhibitors but display a paradoxical luminescence increase [39] by forming an enzyme-inhibitor complex that is more resistant to degradation than the free enzyme [1]. Even though these types of compounds commonly contain a thiazole, imidazole, oxadiazole or pyridine ring [39] we cannot rule out the possibility that FLX, NFLX, SRT and PRX are, in fact, stabilizers for the DHT-induced luminescence. Therefore it could be "enlightening" to study the *in vitro* androgenic/antiandrogenic effect of the selected compounds by using a cell line transfected with another reporter gene. This could offer the possibility to confirm/exclude the luciferase stabilization as the mechanism responsible for the observed "superinduction" in case of SSRIs + DHT co-treatment.

The existing studies on FLX present it as an endocrine disruptor. Our study provides a new insight into the effects of FLX, its active metabolite, NFLX and two other important SSRIs, SRT, respectively PRX.

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