DOI: 10.15386/cjmed-476

IN VITRO MODULATION OF ESTROGEN RECEPTOR ACTIVITY BY NORFLUOXETINE

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Abstract

Background and aims. *Selective serotonin reuptake inhibitors (SSRIs) are antidepressants increasingly prescribed for pregnancy and postpartum depression. However, these compounds can cross the placenta and also pass into breast milk, thus reaching the fetus and infant during critical developmental stages, potentially causing adverse effects. Fluoxetine, a widely used SSRI, has been shown to affect (neuro) endocrine signaling in various organisms, including humans. This compound can also interact with estrogen receptors in vitro and cause an estrogen-dependent uterotrophic response in rodents. Consequently, the aim of the present study was to assess if the active metabolite of fluoxetine, namely norfluoxetine (NFLX), shares the same capacity for estrogen receptor interaction.*

Methods. *The in vitro (anti)estrogenic activity of norfluoxetine was assessed using a firefly luciferase reporter construct in the T47D-Kbluc breast cancer cell line. These cells express nuclear estrogen receptors (ERs) that can activate the transcription of the luciferase reporter gene upon binding of ER agonists. Light emission was monitored in case of cells exposed to norfluoxetine or mixtures of norfluoxetine-estradiol. Cell viability was assessed using a resazurin-based assay.*

Results. *During individual testing, NFLX was able to induce a significant increase in luciferase activity compared to control, but only at the highest concentration tested (10 μM). In binary mixtures with estradiol (30 pM constant concentration) a significant increase in luminescence was observed at low submicromolar norfluoxetine concentrations compared to estradiol alone.*

Conclusion. *Norfluoxetine can induce estrogenic effects in vitro and can potentiate the activity of estradiol. However, further studies are needed to clarify if these observed estrogenic effects may have detrimental consequences for human exposure.*

Keywords: norfluoxetine, estrogen receptors, *in vitro*

Background and aims

Selective serotonin reuptake inhibitors (SSRIs) are antidepressant drugs recommended as first-line pharmacotherapy for unipolar depression [1]. The prevalence of depression is high during pregnancy and postpartum (range 6.5–12.9%), and in the past decade the use of SSRIs to treat depression during pregnancy and breast-feeding has increased considerably [2-4].

However, the use of SSRIs during gestation and postpartum may carry risks for the offspring, as these psychoactive drugs can cross the placenta and also pass into breast milk, thus reaching the fetus and infant during critical developmental stages [5,6]. In humans, studies

Manuscript received: 21.05.2015
Received in revised form: 22.06.2015
Accepted: 23.06.2015
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regarding perinatal SSRI exposure and adverse outcomes in offspring have focused on risks of fetal malformations, birth weight, respiratory distress, neonatal convulsions and some neurodevelopmental effects such as psychomotor delays, behavioral changes and autism [5,7,8]. Endocrine-related reproductive effects in children exposed in utero and/or through lactation have not received attention thus far.

Fluoxetine (FLX), a major representative of SSRIs, has been linked to reproductive and/or (neuro) endocrine toxicity in aquatic organisms, rodents and humans [9-11]. Studies on fish showed that FLX can disrupt the physiological responses of the reproductive axis by interfering with the neuroendocrine regulation of steroidogenesis and gametogenesis. Furthermore, FLX can also disrupt estrogen signaling in the brain by reducing the expression of estrogen receptors in the hypothalamus [10]. In addition, a few studies on rodents suggested that FLX could interfere with sexual steroid signaling during development, as this compound was able to affect sexual brain differentiation, sexual behaviour, testicular development and sperm production in rat offspring exposed in utero and/or through lactation [12-16]. Finally, a study by Mueller et al. showed that FLX can activate estrogen receptors *in vitro* and can also induce an estrogen-dependent uterotrophic response in rodents [17].

In vivo, fluoxetine is metabolized to norfluoxetine (NFLX), an active molecule for which there is no published data regarding its endocrine effects [18]. Consequently, this study aimed at assessing the capacity of NFLX to elicit estrogenic or antiestrogenic effects *in vitro*, using a firefly luciferase-reporter assay in an estrogen receptor (ER) positive cell line.

Materials and methods

Chemicals and reagents

Norfluoxetine (NFLX) was purchased from LGC Standards (Germany). Resazurin, 17β-estradiol (E_2), EDTA, tricine, dithiothreitol (DTT), ATP and Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12) were obtained from Sigma Aldrich (Steinheim, Germany). Tris and 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) were from Fluka (Buchs, Switzerland) and luciferin was from Roth (Karlsruhe, Germany). $(MgCO_3)_4Mg(OH)_2.5H_2O$ was purchased from Acros Organics (Geel, Belgium). Dulbecco's Phosphate Buffered Saline (PBS), trypsin, RPMI1640 + GlutaMAX culture medium and charcoal stripped Fetal Bovine Serum (FBS) and were purchased from Gibco (Paisley, UK). Fetal Bovine Serum (FBS) was from Sigma (Steinheim, Germany).

Stock solutions

NFLX and E_2 were prepared as stock solutions in dimethyl sulfoxide (DMSO, Riedel-de Haën, Seelze, Germany). Subsequent serial dilutions were prepared and then used to obtain the desired test concentration range for NFLX and E_2.

Cell culture

Estrogen receptor positive T47D-KBluc human breast cancer cells were obtained from American Type Culture Collection (ATCC, USA). The cells were maintained in 75 cm^2 flasks with RPMI1640 + GlutaMAX medium, supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2, under saturating humidity. During every cell passage the cells were rinsed with PBS and were detached from plastic flasks using trypsin.

Cell viability assay

Cell viability was evaluated using a resazurin-based assay. For this test, T47D-KBluc cells were seeded at $5x10^5$ cells/mL (96 wells/plate) in RPMI1640 with GlutaMAX medium. Following a 24h incubation at 37°C, 5% CO_2, the cells were rinsed with 200 µL PBS and then exposed for 24h to NFLX in increasing concentrations or binary mixtures of NFLX in increasing concentrations and 30 pM E_2 (the lowest E_2 concentration that induced a maximum response in this cell line). The experiments were performed in triplicate. Viable cells are able to reduce the non-fluorescent resazurin reagent to a fluorescent product called resorufin. After exposure to test compounds or mixtures, the cells were rinsed with 200µL PBS and then incubated with 200 µL of a 100 µM resazurin solution, for 3h at 37°C, 5% CO_2. Using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek), the fluorescence of resorufin in viable cells was measured at $\lambda_{excitation}$=530/25; $\lambda_{emission}$=590/35.

Luciferase assay

The luciferase assay was used as an indicator of (anti)estrogenic activity in T47D-KBluc breast cancer cells, which express ERs and carry a stably transfected firefly luciferase-reporter. The assay is performed by measuring the luminescence intensity produced when the ER-dependent luciferase is expressed and transforms its substrate. For this purpose, T47D-KBluc cells ($8.5x10^6$ cells/mL) were seeded in 96-well plates and incubated 24h at 37°C, in phenol red-free Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12), containing 10% charcoal stripped FBS. After the 24h incubation, the medium was removed and the cells were rinsed with PBS and subsequently exposed to NFLX in increasing concentrations or binary mixtures of 30 pM E_2 and NFLX in increasing concentrations. The DMSO concentration in assay medium did not exceed 0.2%. A buffer containing 10 mM Tris, 1.99 mM DTT and 2 mM CDTA was used to lyse the cells after the 24h exposure period. The plates were incubated at -20°C for 15-minutes, then frozen at -80°C for a minimum of 30 minutes. Following a thawing period on ice, the plates were shaken for 30 mins at room temperature. Luminescence was measured using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek) with automatic injection of 100 µL luciferin FlashMix [0.47 mM luciferin, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 0.1

mM EDTA, 2 mM DTT and 5 mM ATP, pH 7.8] in each well. Light emission was stopped with 50 µL NaOH 0.2 M.

Data analysis and statistics

Experiments were performed three times in triplicate. Data was expressed either as percentages of non-treated controls (individual testing) or as percentages of the signal observed for 30 pM E_2 (testing mixtures NFLX-E_2). The normality of the distribution of data was tested with the Shapiro-Wilk test ($p<0.05$). To compare data, 1-way ANOVA was applied to normally distributed data, followed by Dunnett's Multiple Comparison post hoc test. For data that were not normally distributed we used Kruskal-Wallis test, followed by Dunnett's Multiple Comparison test. Differences in P values of <0.05 were considered statistically significant. All statistical analyses and graphs were performed using GraphPad Prism 5.

Results

Cell viability assay

To assess cell viability in the presence of NFLX, T47D-Kbluc cells were exposed to NFLX in increasing concentrations (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 15 and 20 µM) for 24 hours. NFLX significantly decreased cell viability at 15 and 20 µM (data not shown) and therefore, these concentrations were excluded from further testing. Mixtures NFLX (increasing concentrations as stated above) and E_2 (constant concentration 30 pM) were also evaluated using the same assay and incubation time. Again, mixtures that contained NFLX in concentrations above 10 µM significantly decreased cell viability (data not shown) and were excluded from further testing.

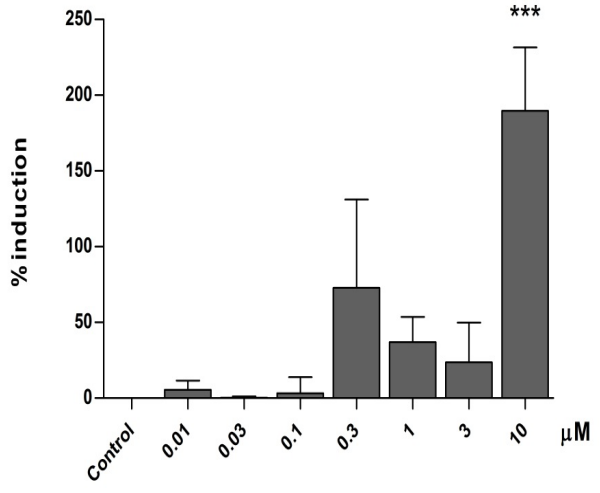

Figure 1. Luciferase induction in T47D-Kbluc cells after exposure to NFLX. Results are expressed as % induction for the luciferase assay. Results represent the mean ± SEM of 3 independent experiments. Significant results as compared to the negative control (DMSO treated cells) are marked with asterisks (*** for P-value <0.001).

Luciferase assay

To test for estrogenic effects of NFLX, T47D-Kbluc cells were incubated with increasing concentrations of NFLX (0.01, 0.03, 0.1, 0.3, 1, 3 and 10µM) for 24 hours. A significant increase in luciferase activity as compared to control was observed only at the highest concentration tested, 10 µM (Figure 1). This induction represents 10% of the E_2 response at 30 pM in T47D-Kbluc cells.

At low concentrations, mixtures NFLX (increasing concentrations as mentioned above) and E_2 (constant concentration 30 pM) yielded a significant increase in luciferase activity as compared to E_2, whereas the highest concentration tested induced a significant decrease in luciferase activity (Figure 2).

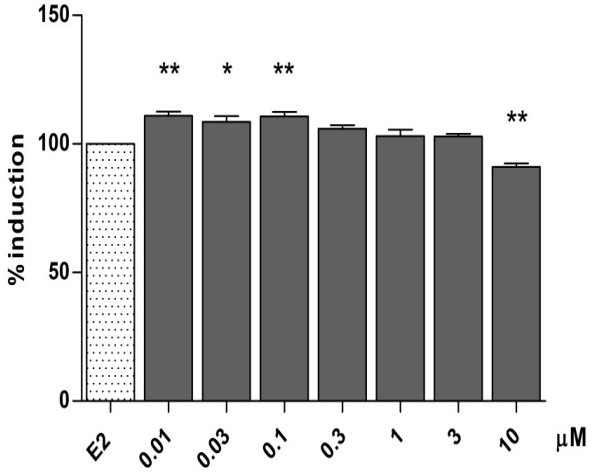

Figure 2. Luciferase induction in T47D-Kbluc cells after exposure to mixtures of NFLX (increasing concentrations) + E_2 (30 pM). Results are expressed as % induction for the luciferase assay. Results represent the mean ± SEM of 3 independent experiments. Significant results as compared to the control (E_2 30 pM treated cells) are marked with asterisks (* for P-value <0.05, ** for P-value <0.01).

Discussion

In the light of the recent findings on FLX interactions with the nuclear estrogenic receptors (ERs), we aimed at assessing if the main metabolite, NFLX, shares the same capacity to interfere with estrogenic signaling [17]. As the parent compound, NFLX is highly lipophilic and crosses the blood-brain barrier to accumulate in the brain, where it acts as an antidepressant by selectively blocking the serotonin transporter [18]. However, the structural and functional similarities of the two compounds suggest that NFLX might also be an active ligand for nuclear ERs.

Indeed, the results of the *in vitro* firefly-luciferase assay show that NFLX alone can induce estrogenic effects by activating the nuclear estrogenic receptors and thus affecting the ER-regulated gene expression. However,

the small magnitude of the observed effect (10% of E_2 induction) suggests that NFLX is a weak ER agonist at high concentrations.

We further tested mixtures of NFLX and E_2 to simulate the biological scenario where this compound would be present at the ERs alongside their natural ligand. In this case, NFLX caused an increase in the E_2-induced transcriptional activity at low (submicromolar) concentrations. Considering the fact that no effect was observed at these concentrations during individual testing, we hypothesize that NFLX can act in a synergistic manner with E_2. Conversely, at 10 μM we observed a significant decrease in signal as compared to E_2. This is in line with the previous finding from individual testing and suggests that NFLX can displace the natural ligand from the ERs, thus resulting in a decrease in signal due to the weak agonist properties of NFLX.

These results may raise concern regarding the endocrine disruptive potential of NFLX, since the synergistic effect observed at submicromolar concentrations suggests that this compound may interfere with estrogenic signaling at therapeutic plasma concentrations, which are reported to be below 1 μM, with variations depending on FLX dose and CYP2D6/CYP2C9 genotype [19,20]. In the fetus and newborns, NFLX concentrations reach aproximately 70 to 80% of the maternal plasma levels [21]. Also, during breastfeeding, the infants receive less than 1% of the maternal FLX dose through breast milk [21]. However, FLX, as well as NFLX are highly bound to plasma proteins (~95%) and therefore less compound may be available for receptor interaction *in vivo* compared to our *in vitro* studies [22,23]. Little is known about the level of FLX or NFLX protein binding and metabolism in the fetus and newborn [24]. Although higher concentrations can be achieved in brain tissue due to accumulation of FLX and NFLX, the biological significance of the observed effect of NFLX at 10 μM remains unclear due to the fact that only total FLX-NFLX concentrations have been reported for the human brain (~ 13 μM) [25]. Further *in vitro* and *in vivo* testing would be useful to understand the relevance of our present findings in the context of disrupted estrogenic signaling during development.

A limitation of the present study lies in the fact that the observed interaction with the nuclear ERs was not confirmed through tests in the presence of total ER antagonists.

Conclusion

In conclusion, this is the first study to report that NFLX has the potential to interfere with estrogenic signaling *in vitro*, either by increasing or decreasing the ER-mediated activity of 17β-estradiol.

Acknowledgement

This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/136893.

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