

IN VITRO EFFECTS OF FLUOXETINE AND NORFLUOXETINE ON BREAST CANCER PROLIFERATION

DIANA LUPU[#], ANCA POP[#], IONEL FIZEȘAN*, BELA KISS, FELICIA LOGHIN

"Iuliu Hațieganu" University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Toxicology, Cluj-Napoca, Romania

*corresponding author: ionel.fizesan@umfcluj.ro

[#]Authors with equal contribution to this research paper.

Manuscript received: February 2017

Abstract

Pharmacological interventions in depressed cancer patients entail the use of selective serotonin reuptake inhibitors (SSRIs), of which the most prescribed is fluoxetine. This compound has been shown to interfere with oestrogen-dependent gene transcription *in vitro* and also affect the proliferation of various cancer cells. Therefore, this study aimed to assess the capacity of fluoxetine and its main metabolite, norfluoxetine, to influence breast cancer proliferation *in vitro*, using the MCF-7 cell line. Both compounds exhibited anti-proliferative activity on MCF-7 cells, either alone or in combination with 17 β -estradiol. However, norfluoxetine displayed weak co-proliferative effects at (sub)micromolar, biologically relevant concentrations.

Rezumat

Farmacoterapia în cazul depresiei la pacienții cu cancer implică utilizarea inhibitorilor selectivi ai recaptării serotoninei (ISRS), dintre care cel mai prescris este fluoxetina. Studiul *in vitro* au arătat că acest compus interferează cu transcripția genică dependentă de estrogen și, de asemenea, inhibă proliferarea diferitelor celule canceroase. Prin urmare, acest studiu a avut drept obiectiv evaluarea capacității fluoxetinei și a principalului său metabolit, norfluoxetina, de a influența proliferarea cancerului mamar *in vitro*, utilizând linia celulară MCF-7. Ambii compuși au prezentat activitate anti-proliferativă asupra celulelor MCF-7, atât individual, cât și în amestec cu 17 β -estradiol. Cu toate acestea, norfluoxetina a prezentat efecte co-proliferative slabe la concentrații (sub)micromolare, relevante din punct de vedere biologic.

Keywords: fluoxetine, norfluoxetine, proliferation, MCF-7

Introduction

Depressive disorders are highly prevalent mental disorders estimated to affect over 300 million people world-wide [21]. These disorders encompass several syndromes with common features such as sadness, irritability and feelings of emptiness, associated with clinical changes in cognition and behaviour that cause significant functional disability [2]. Depressive disorders affect individuals of all ages and are consistently more prevalent in women than in men [1]. Depression can also be a side effect of certain medications and, furthermore, can also be associated with several other chronic diseases such as cardiovascular disease, diabetes, fibromyalgia, cancer etc. [2-4, 9, 10]. Depressive disorders are common in cancer patients, with a prevalence ranging between 5% and 60%, depending on the diagnostic criteria and tools, but also the stage and type of cancer [7]. In breast cancer patients, the prevalence of depression ranges from 11% to 20% [11] and has also been associated with chemotherapy and anti-estrogenic treatment with tamoxifen [13, 22].

The treatment of depression usually entails the use of psychotherapy and pharmacotherapy, either alone or in combination, depending on the severity of the disease [2]. There are no specific guidelines for pharmacological interventions in depressed cancer patients, therefore the first-line pharmacotherapy is represented by selective serotonin reuptake inhibitors (SSRIs) [8]. Fluoxetine is a widely prescribed SSRI and has been used since the 1980s [16]. However, this molecule has been linked to reproductive and endocrine toxicity *in vitro* and *in vivo* [14, 17].

Our previous *in vitro* studies using T47D-kbluc breast cancer cells have shown that both fluoxetine (FLX) and its main metabolite, norfluoxetine (NFLX), have the capacity to interfere with oestrogen-dependent gene transcription *in vitro*, as assessed using a luciferase reporter gene [14, 17]. Similar results were previously shown for fluoxetine using a MCF-7 breast cancer cell line transfected with the luciferase reporter [15]. Furthermore, fluoxetine was able to induce an uterotrophic response in immature rats, suggesting that this molecule can disrupt oestrogen-mediated pathways *in vivo* [15].

It is well established that the proliferation of breast cancer cells is highly dependent on oestrogen receptor signalling [12]. Given the capacity of FLX and NFLX to interfere with oestrogen-dependent gene transcription, it is possible that the two compounds could negatively affect the proliferation of oestrogen receptor positive breast cancer cells. This study aimed to assess the capacity of these molecules to influence breast cancer proliferation *in vitro*, using the MCF-7 cell line. These breast cancer cells express nuclear oestrogen receptors (ERs) and are used as a gold standard for assessing oestrogen dependent cell proliferation *in vitro*. The two compounds were assessed both individually and in combinations with the endogenous 17 β -estradiol.

Materials and Methods

Reagents

The test compounds, namely fluoxetine (FLX) and norfluoxetine (NFLX), were purchased from LGC Standards (Germany). 17 β -estradiol (E2), resazurin, insulin (cell culture tested), 200 mM L-glutamine, 100 mM sodium pyruvate, foetal bovine serum (FBS), phenol-red free Dulbecco's Modified Eagle's Medium F-12 (DMEM/F-12) and MEM non-essential amino acid (100X) solution were all obtained from Sigma Aldrich (Steinheim, Germany). Dulbecco's Phosphate Buffered Saline (PBS) was purchased from Invitrogen, while charcoal stripped FBS and trypsin (Trypsin 0.05% + EDTA) were purchased from Gibco (Paisley, UK). All reagents and solvents used were of analytical grade.

Cell culture

MCF-7 cells (ATCC[®] HTB-22[™], human adenocarcinoma derived from mammary gland) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown at 37°C under humidified atmosphere with 5% additional CO₂ in phenol red-free DMEM/F12 medium supplemented with 1% L-glutamine, 1% sodium pyruvate, 1% MEM non-essential amino acids, 10 μ g/mL insulin and 10% FBS. Medium was changed every other day and the passage of cells was done at a confluence of 80%. During the proliferation studies FBS was substituted with charcoal-stripped FBS.

Preparation of the test compounds

500 μ L stock solutions in dimethyl sulfoxide (DMSO $\geq$ 99.5%, Riedel-de Haën, Seelze, Germany) were prepared for each compound at a concentration of 30 mM. Serial dilutions of these stock solutions were performed with DMSO in order to obtain working solutions of 0, 0.05, 0.15, 0.5, 1.5, 5, 7.5, 10 and 15 mM for both test compounds. These serial dilutions were then used to obtain the desired test concentrations (0.03 - 30 μ M) for individual chemicals.

Cell proliferation assay

Subconfluent MCF-7 cells were trypsinized and seeded in 96 well plates at a density of 8×10^4 cells/mL. Cells were let to attach for 24 h, then rinsed with PBS and exposed to increasing concentrations of individual test compounds or mixtures of compounds with a fixed concentration of estradiol (10 pM) for 72 h. 10 pM E2 was used as a positive control for both exposure scenarios. Negative controls included medium depleted of steroids with and without DMSO. After 72 h the culture medium was replaced with fresh medium without test compounds or binary mixtures and cells were incubated for another 72 h. After a total incubation time of 144 h, cell proliferation was evaluated using a resazurin-based assay, by measuring the capacity of the cells to convert the non-fluorescent resazurin to resorufin, a fluorescent product [6]. Cells were incubated for 4 h at 37°C with resazurin under humidified atmosphere, with 5% additional CO₂ and the fluorescence was measured at $\lambda_{\text{excitation}} = 530/25$; $\lambda_{\text{emission}} = 590/35$, using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek). Each experiment was performed three times on 3 - 6 replicates.

Statistical analysis

Data analyses were performed using Sigma Plot (version 12.00). Values are presented as mean $\pm$ standard deviation (SD) for the groups. Compensation for the inter-plate and inter-experiment data variability was achieved through normalization of the raw data for each plate. Normality was evaluated with the Shapiro-Wilk test ($p < 0.05$). To compare data, one-way ANOVA was applied to normally distributed data, followed by Holm-Sidak Multiple Comparison post hoc test. Differences in p values of < 0.05 were considered statistically significant.

Results and Discussion

Evaluation of proliferative activity

The proliferation of cultured cells *in vitro* can be assessed by using spectrophotometric or fluorimetric methods, involving chemical substrates that are converted by metabolically active cells [6, 19]. We used a resazurin-based assay to assess the metabolic capacity of MCF-7 cells *in vitro*, by reading the fluorescence resulted from the cell conversion of the non-fluorescent resazurin to resorufin, a fluorescent product.

Both fluoxetine and norfluoxetine significantly reduced the proliferation of MCF-7 cells during individual testing, as compared to the solvent (DMSO) control (Figures 1A and 1B). The range of the tested concentrations was chosen as the plasma levels of FLX at therapeutic doses of 20 - 80 mg/day are between 0.1 - 2 μ M, but it can accumulate in tissues due to its lipophilic nature, achieving local concentrations in the range of 10 - 20 μ M [5, 20]. FLX caused a

significant reduction of the fluorescent signal between 3 - 30 μM , while its metabolite caused a significant decrease in fluorescence between 15 - 30 μM . The observed anti-proliferative effect could

be mediated through an oestrogen pathway (i.e. *via* nuclear oestrogen receptors (ER)) or other pathways involved in cell proliferation, such as the phosphokinase ERK (extracellular regulated kinase) pathway [18].

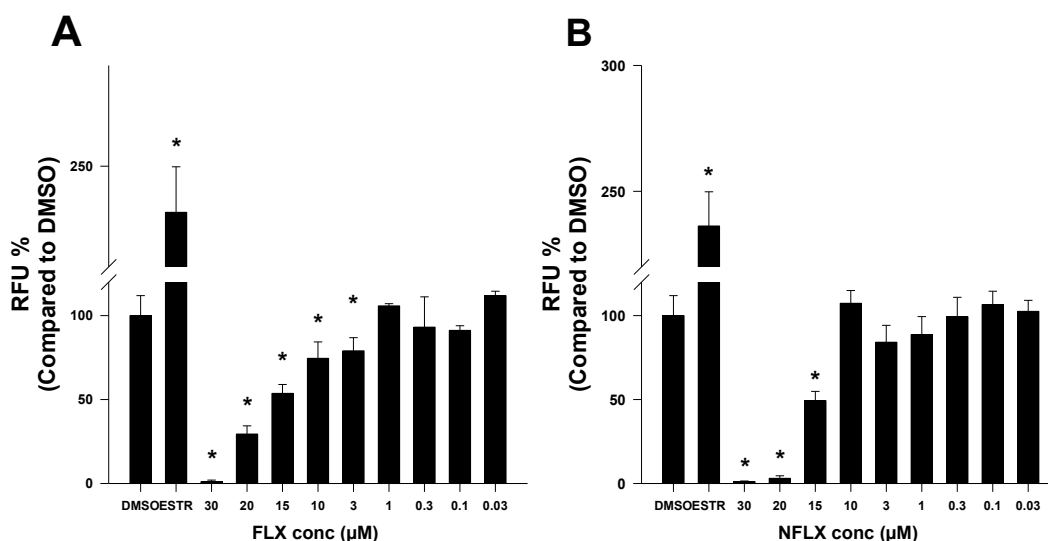


Figure 1.

Effects of fluoxetine (A) and norfluoxetine (B) on MCF-7 breast cancer cell proliferation. Results represent the mean $\pm$ SD of 3 independent experiments. Significant results (ANOVA, $p < 0.05$) as compared to the control (DMSO treated cells) are marked with asterisks (A) FLX significantly decreased cell proliferation between 3 - 30 μM (FLX 15 - 30 μM , $p < 0.001$; FLX 10 μM , $p = 0.002$; FLX 3 μM , $p = 0.009$). (B) NFLX significantly decreased cell proliferation between 15 - 30 μM ($p < 0.001$)

In our previous *in vitro* studies using resazurin-based assays on transfected T47D-Kbluc cells, we observed that fluoxetine significantly reduced the viability of breast epithelial carcinoma cells after a 24 hours exposure in concentrations above 15 μM [17]. An anti-proliferative effect of FLX was also shown in T74D breast carcinoma cells after FLX treatment up to 10 μM for 24 or 96 hours [18]. Therefore it is likely that the observed decrease in the fluorescent signal caused by 15 - 30 μM FLX is the result of a direct effect of the compound on cell viability. Our results are consistent with previous reports where FLX has been shown to induce apoptosis and inhibit the growth of various cancer cell lines [23]. Moreover, a synergic effect of FLX in combination with anticancer drugs was reported in multidrug resistant breast cancer cells, enhancing chemotherapy sensitivity mainly by increasing the intracellular accumulation of the anticancer drugs [23]. Similarly, NFLX decreased the cell viability of T47D-Kbluc cells after 24 hours incubation at concentrations above 15 μM , suggesting that the observed signal reduction for NFLX in the present study may be entirely due to a direct effect of this molecule on cell viability and/or proliferation.

Evaluation of antiproliferative activity

Both FLX and NFLX significantly affected the proliferation of MCF-7 cells during testing in combination with 10 pM E2, as compared to E2

(Figures 2A-D). Fluoxetine caused a significant reduction of the fluorescent signal between 15 - 30 μM , while its metabolite presented a dual effect, reducing the E2-induced cell proliferation between 10 - 30 μM and increasing the signal at 0.3 and 1 μM .

For both compounds, the observed reduction in signal at 20 μM and 30 μM can be attributed to a reduction in cell viability (the decrease is significant compared to the solvent control). However the reductions caused by 15 μM FLX + 10 pM E2 and 15 μM or 10 μM NFLX + 10 pM E2 can be considered real anti-proliferative effects of FLX and NFLX. It is possible that the observed effects are ER-mediated considering that similar binary mixtures of 10 or 15 μM FLX/NFLX and E2 caused a significant reduction in luminescence when assessed using an ER-dependent reporter gene assay in T47D-Kbluc breast cancer cells [14, 17].

Binary mixtures of 10 pM E2 and 0.3 or 1 μM NFLX caused a significant increase in fluorescence as compared to 10 pM E2. The increase is less than 20%, but it suggests a potential dual effect on proliferation for norfluoxetine in combination with endogenous E2. Because this effect is observed at biologically relevant concentrations, our current focus is to determine if this co-proliferative effect is consistent in various ER positive and negative cancer cells and to further understand its relevance for the clinical setting.

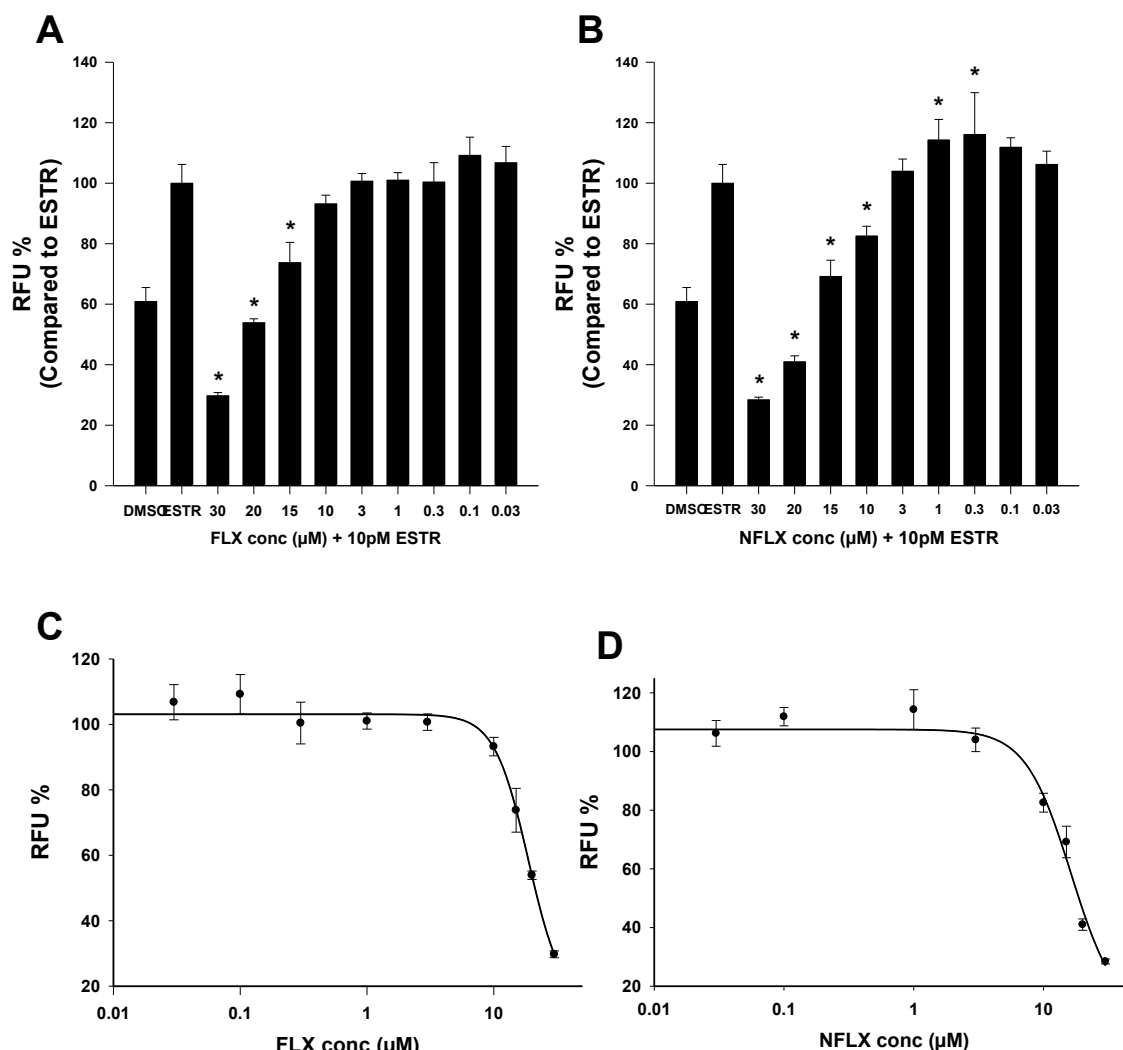


Figure 2.

Effects of estradiol and fluoxetine (A, C) or norfluoxetine (B, D) on MCF-7 breast cancer cell proliferation.

Results represent the mean $\pm$ SD of 3 independent experiments. Significant results (ANOVA, $p < 0.05$) as compared to the control (DMSO treated cells) are marked with asterisks (A) (C). FLX significantly decreased the estradiol-induced cell proliferation between 15 - 30 μM ($p < 0.001$), with an IC_{50} of 18.75 μM ; (B) (D) NFLX significantly decreased the E2-induced cell proliferation between 10 - 30 μM (NFLX 10 μM , $p < 0.002$; NFLX 15 - 30 μM $p < 0.001$) with an IC_{50} of 16.27 μM ; and significantly increased cell proliferation at 0.3 μM ($p = 0.003$) and 1 μM ($p = 0.07$)

Conclusions

Fluoxetine and its main metabolite, norfluoxetine have significant anti-proliferative effects on ER positive breast cancer cells *in vitro*, either alone or in combination with the endogenous E2. This effect is observed at micromolar concentrations, biologically relevant in the case of drug accumulation in tissues. Furthermore, norfluoxetine displayed dual effects on MCF-7 cell proliferation, in combination with E2, strongly inhibiting E2-induced proliferation at high micromolar concentrations, but weakly augmenting E2-induced proliferation at (sub)micromolar concentrations. To our knowledge this is the first instance where co-proliferative effects are reported for this compound and therefore additional studies

are required to establish if this effect is consistent in various cancer types and its implications for the clinical setting.

Acknowledgement

This article was published within the project financed by "Iuliu Hațieganu" University of Medicine and Pharmacy, Cluj Napoca, Romania, through the internal grant contract no. 4944/12/08.03.2016.

References

1. Albert P.R., Why is depression more prevalent in women?. *J. Psych. Neurosci.*, 2015; 40(4): 219-221.

2. American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Fifth Ed. American Psychiatric Association. Arlington, VA, 2013; 155-189.
3. Andreoulakis E., Hyphantis T., Kandyli D., Iacovides A., Depression in diabetes mellitus: a comprehensive review. *Hippokratia*, 2012; 16(3): 205-214.
4. Bernik M., Sampaio T.P., Gandarela L., Fibromyalgia comorbid with anxiety disorders and depression: combined medical and psychological treatment. *Curr. Pain Headache Rep.*, 2013; 17(9): 358.
5. Bolo N.R., Hodé Y., Nédélec J.F., Lainé E., Wagner G., Macher J.P., Brain pharmacokinetics and tissue distribution *in vivo* of fluvoxamine and fluoxetine by fluorine magnetic resonance spectroscopy. *Neuropsychopharmacology*, 2000; 23(4): 428-438.
6. Borra R.C., Lotufo M.A., Gaglioti S.M., Barros F.de M., Andrade P.M., A simple method to measure cell viability in proliferation and cytotoxicity assays. *Braz. Oral Res.*, 2009; 23(3): 255-262.
7. Caruso R., Nanni M.G., Riba M., Sabato S., Mitchell A.J., Croce E., Grassi L., Depressive spectrum disorders in cancer: prevalence, risk factors and screening for depression: a critical review. *Acta Oncol.*, 2017; 56(2): 146-155.
8. Gelenberg A.J., A review of the current guidelines for depression treatment. *J. Clin. Psychiatry*, 2010; 71(7): e15.
9. Kang H.J., Kim S.Y., Bae K.Y., Kim S.W., Shin I.S., Yoon J.S., Kim J.M., Comorbidity of depression with physical disorders: research and clinical implications. *Chonnam. Med. J.*, 2015; 51(1): 8-18.
10. Kolcsár M., Gáll Z., Bíró I.L., Bába L.I., Imre A., Dogaru M., Mártha O., Fluoxetine influence on body weight, serum adiponectin level and adipose tissue triglycerides levels in rats. *Farmacia*, 2016; 64(4): 599-604.
11. Krebber A.M., Buffart L.M., Kleijn G., Riepma I.C., de Bree R., Leemans C.R., Becker A., Brug J., van Straten A., Cuijpers P., Verdonck-de Leeuw I.M., Prevalence of depression in cancer patients: a meta-analysis of diagnostic interviews and self-report instruments. *Psychooncology*, 2014; 23(2): 121-130.
12. Liao X.H., Lu D.L., Wang N., Liu L.Y., Wang Y., Li Y.Q., Yan T.B., Sun X.G., Hu P., Zhang T.C., Estrogen receptor α mediates proliferation of breast cancer MCF-7 cells *via* a p21/PCNA/E2F1-dependent pathway. *FEBS J.*, 2014; 281(3): 927-942.
13. Lorizio W., Wu A.H., Beattie M.S., Rugo H., Tchu S., Kerlikowske K., Ziv E., Clinical and biomarker predictors of side effects from tamoxifen. *Breast Cancer Res. Treat.*, 2012; 132(3): 1107-1118.
14. Lupu D., Pop A., Cherfan J., Kiss B., Loghin F., *In vitro* modulation of estrogen receptor activity by norfluoxetine. *Clujul Med.*, 2015; 88(3): 386-390.
15. Müller J.C., Imazaki P.H., Boareto A.C., Lourenço E.L., Golin M., Vecchi M.F., Lombardi N.F., Minatovicz B.C., Scippo M.L., Martino-Andrade A.J., Dalsenter P.R., *In vivo* and *in vitro* estrogenic activity of the antidepressant fluoxetine. *Reprod. Toxicol.*, 2012; 34: 80-85.
16. Perez-Caballero L., Torres-Sanchez S., Bravo L., Mico J.A., Berrocoso E., Fluoxetine: a case history of its discovery and preclinical development. *Exp. Opin. Drug Discov.*, 2014; 9(5): 567-578.
17. Pop A., Lupu D.I., Cherfan J., Kiss B., Loghin F., Estrogenic/antiestrogenic activity of selected selective serotonin reuptake inhibitors. *Clujul Med.*, 2015; 88(3): 381-385.
18. Stepulak A., Rzeski W., Sifringer M., Brocke K., Gratopp A., Kupisz K., Turski L., Ikonomidou C., Fluoxetine inhibits the extracellular signal regulated kinase pathway and suppresses growth of cancer cells. *Cancer Biol. Ther.*, 2008; 7(10): 1685-1693.
19. Vlad D.C., Popescu R., Dumitrascu V., Cimporescu A., Vlad C.S., Vágvolgyi C., Krisch J., Dehelean C., Horhat F.C., Phytocomponents identification in mistletoe (*Viscum album*) young leaves and branches, by GC-MS and antiproliferative effect on HepG2 and MEC-7 cell lines. *Farmacia*, 2016; 64(1): 82-87.
20. Volpe D.A., Ellison C.D., Parchment R.E., Grieshaber C.K., Faustino P.J., Effects of amitriptyline and fluoxetine upon the *in vitro* proliferation of tumor cell lines. *J. Exp. Ther. Oncol.*, 2003; 3(4): 169-184.
21. World Health Organisation. Depression Fact Sheet. <http://www.who.int>.
22. Zainal N.Z., Nik-Jaafar N.R., Baharudin A., Sabki Z.A., Ng C.G., Prevalence of depression in breast cancer survivors: a systematic review of observational studies. *As. Pac. J. Cancer Prev.*, 2013; 14(4): 2649-2656.
23. Zhou T., Duan J., Wang Y., Chen X., Zhou G., Wang R., Fu L., Xu F., Fluoxetine synergizes with anticancer drugs to overcome multidrug resistance in breast cancer cells. *Tumour Biol.*, 2012; 33(5): 1299-12306.