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Maria Rosaria Ambrosio

Institute for Experimental Endocrinology and Oncology "G. Salvatore" – National Research Council (IEOS-CNR)

Elisa Magli

Department of Pharmacy - University of Naples "Federico II"

Giuseppe Caliendo

Department of Pharmacy - University of Naples "Federico II"

Rosa Sparaco

Department of Pharmacy - University of Naples "Federico II"

Paola Massarelli

Department of Medicine, surgery and neuroscience – University of Siena

Vittoria D'Esposito

Institute for Experimental Endocrinology and Oncology "G. Salvatore" – National Research Council (IEOS-CNR)

Teresa Migliaccio

Department of Translational Medicine – University of Naples "Federico II"

Giusy Mosca

Department of Translational Medicine – University of Naples "Federico II"

Ferdinando Fiorino

Department of Pharmacy - University of Naples "Federico II"

Pietro Formisano (✉ fpietro@unina.it)

Department of Pharmacy - University of Naples "Federico II"

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Serotoninerbic receptor ligands improve Tamoxifen effectiveness on breast cancer cells

Maria Rosaria Ambrosio^{1,†}, Elisa Magli^{2,†}, Giuseppe Caliendo², Rosa Sparaco², Paola Massarelli³, Vittoria D'Esposito¹, Teresa Migliaccio⁴, Giusy Mosca⁴, Ferdinando Fiorino², Pietro Formisano^{1,4}

¹ Institute for Experimental Endocrinology and Oncology "G. Salvatore" – National Research Council (IEOS-CNR), Via Pansini 5, 80131 Naples, Italy

² Department of Pharmacy - University of Naples "Federico II" (UniNa), Via Montesano 49 - 80131, Naples, Italy

³ Department of Medicine, surgery and neuroscience – University of Siena, Strada delle Scotte 6 – 53100, Siena - Italy

⁴ Department of Translational Medicine – University of Naples "Federico II" (DiSMET-UniNa), Via Pansini 5, 80131 Naples, Italy

[†] Equally contributed to the work.

Corresponding author:

Pietro Formisano

fpietro@unina.it

Tel: +39-0817464450

Abstract

Background: The tightly regulated pattern of serotonin (or 5-Hydroxytryptamine, 5-HT) in mammary gland becomes dysregulated in cancer, also contributing to proliferation, metastasis and angiogenesis. Antagonists of serotonin receptors or inhibitors of serotonin reuptake/synthesis may be co-prescribed with Tamoxifen in estrogen receptor-positive (ER⁺) tumors, and may modulate its efficacy. Thus, the discovery of novel compounds targeting serotonin signaling may contribute to tailor new therapeutic strategies usable in combination with endocrine therapies. We have previously synthesized serotonergic receptor ligands (SER) with high affinity and selectivity towards 5-HT_{2A} and 5-HT_{2C} receptors, the main mediators of mitogenic effect of serotonin in breast cancer (BC). Here, we investigated the effect of 10 SER on viability of MCF7, SKBR3 and MDA-MB231 BC cells and focused on their potential ability to affect Tamoxifen responsiveness in ER⁺ cells.

Methods: Cell viability has been assessed by sulforhodamine B assay. Cell cycle has been analyzed by flow cytometry. Gene expression of 5-HT receptors and Connective Tissue Growth Factor (CTGF) has been checked by RT-PCR; *CTGF* mRNA levels have been measured by qPCR. All data were statistically analyzed using GraphPad Prism 7.

Results: We found that SER were more effective on MCF7 ER⁺ cells (IC₅₀ range 10.2μM - 99.2μM) compared to SKBR3 (IC₅₀ range 43.3μM - 260μM) and MDA-MB231 BC cells (IC₅₀ range 91.3μM - 306μM). Next, we provided evidence that two ligands, SER79 and SER68, improved the effectiveness of Tamoxifen treatment in MCF7 BC cells and modulated the expression of *CTGF*. Moreover, in a cell model of Tamoxifen-resistance, SER79 and SER68 restored drug responsiveness and reduced CTGF levels.

Conclusions: These results identified new compounds potentially usable in combination with Tamoxifen to improve its effectiveness on ER⁺ BC patients.

Keywords

Serotonin

Breast cancer

Serotonergic receptor ligands

Tamoxifen resistance

Connective Tissue Growth Factor

Background

Serotonin (5-HT) is a biogenic amine acting as neurotransmitter in the nervous system both at central and peripheral level [1-4]. Besides playing a role in several physiological and pathological processes, including circadian rhythms, sexual and feeding behavior, thermoregulation and cardiovascular function [5-9], 5-HT acts as trophic, mitogenic and anti-apoptotic factor for a wide range of normal and tumor cells [10-13]. Indeed, a growth stimulatory effect of 5-HT on prostate, small-cell lung, colorectal, hepatocellular and breast carcinoma, cholangiocarcinoma, glioma, bladder cancer and ovarian tumors has been described [14]. 5-HT also promotes cancer cell migration, invasiveness and angiogenesis [15]. The multiple, sometimes opposing, actions of serotonin occur through the interaction with a wide range of receptors. Indeed, with the exception of 5-HT₃, the unique receptor involving an ion channel that regulates the flow of sodium and potassium ions, six classes of 5-HT receptors - including additional subclasses - named 5-HT₁, 5-HT₂, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇, are G-protein-coupled [16]. More often the mitogenic effect of 5-HT is mediated by 5-HT₁ and 5-HT₂ receptors while less frequently through 5-HT₄ and 5-HT₆ [15]. Serotonin plays a central role in mammary gland ensuring epithelial homeostasis during changes associated with pregnancy, lactation and involution [17]. Thus, an extensive alteration of 5-HT signaling may contribute to breast cancer (BC) phenotype [15]. Of note, BC cells produce and secrete high levels of serotonin that, interfering with mitochondria biogenesis, confers proliferative advantages [18]. BC is the most common cancer in women worldwide [19], with estrogen receptor positive (ER⁺) BC representing approximately 75% of all diagnosed cancers [20]. About the latter, the predominant treatment strategy consists in the inhibition of ER pathway at various levels, including the use of selective estrogen receptor modulators (SERMs), like Tamoxifen, to directly antagonize the receptor [21]. However, Tamoxifen effectiveness may be modulated by the interaction with several drugs, including those acting on serotonin signaling. Nevertheless, serotonin action has also been targeted by using 5-HT antagonists and/or uptake inhibitors to prevent cancer cell growth [15]. Our research group has been involved in the synthesis of serotonergic receptor ligands (SER) with high affinity and selectivity [22, 23, 24, 25]. Here, we analyzed a set of previously synthesized SER with affinity and selectivity binding profile towards 5-HT_{2A} and 5-HT_{2C} receptors, known as mediators of mitogenic effect of serotonin in BC cells [18, 26]. We found that some of these serotonergic receptor ligands improve Tamoxifen responsiveness in MCF7 BC cells and that such effect occurs through the modulation of *CTGF* (Connective Tissue Growth Factor) expression. Overall, these results suggest these compounds as new serotonergic receptor ligands potentially useful to ameliorate Tamoxifen effectiveness in ER⁺ BC cells.

Methods

Materials

Media, sera and antibiotics for cell culture were from Lonza (Basel, Switzerland). Reagents and substituted piperazines for synthesis of serotonergic receptor ligands, Estradiol and Tamoxifen for cell treatments and all other chemicals were from Sigma-Aldrich (St Louis, MO, USA). TRIzol solution for RNA isolation, SuperScript III Reverse Transcriptase with oligo dT primers for RNA reverse transcription and AmpliTaq Gold for RT-PCR were from Life Technologies (Carlsbad, CA, USA). iTaq Universal SYBR Green Supermix for Quantitative Real-Time PCR (qPCR) was from Biorad (Hercules, CA, USA).

Synthesis of serotonergic receptor ligands and in vitro receptor binding

All reactions were monitored by TLC, carried out on Merck 60G F₂₅₄ plates with fluorescent indicator and the plates were visualized with UV light (254 nm). Each final compound and intermediate was purified by silica gel column chromatography (Macherey-Nagel 60 0,063-0,2mm/70-230 mesh). Some final compounds were obtained in a pure form after conversion in the corresponding hydrochloride salts. ¹H-NMR and ¹³C-NMR spectra were recorded on Varian Mercury Plus 400 MHz instrument. Unless otherwise stated, all spectra were recorded in CDCl₃. Chemical shifts are reported in ppm using Me₄Si as internal standard. The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), m (multiplet), q (quartet), qt (quintet), dd (double doublet), ddd (double dd), bs (broad singlet). Mass spectra of the final products were performed on LTQ Orbitrap XL™ Fourier transform mass spectrometer (FTMS) equipped with ESI ION MAX™ source (Thermo Fisher, San José, USA). Melting points were determined using a Buchi B-540 hot-stage instrument and are uncorrected. Where analyses are indicated only by the

symbols of the elements, results obtained are within $\pm 0.4\%$ of the theoretical values. Solutions were dried over Na_2SO_4 and concentrated with Buchi R-114 rotavapor at low pressure. Once synthesized, serotonergic receptor ligands were tested for in vitro affinity for serotonin 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors by radioligand binding assays. The more active compounds on serotonin receptors have been selected and evaluated for their affinity for dopaminergic (D₁ and D₂) and adrenergic (α_1 and α_2) receptors. All the compounds were dissolved in 5% DMSO. The following specific radioligands and tissue sources were used: (a)serotonin 5-HT_{1A} receptor, [³H]-8-OH-DPAT, rat brain cortex; (b) serotonin 5-HT_{2A} receptor, [³H]ketanserin, rat brain cortex; (c)serotonin 5-HT_{2C} receptor, [³H]mesulergine, rat brain cortex. Non-specific binding was determined as described in the experimental section, and specific binding as the difference between total and non-specific binding. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. Competition experiments were analyzed by PRISM 5 (GraphPadPrism®, 1992-2007, GraphPad Software, Inc., La Jolla, CA, USA) to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding (IC₅₀), with six concentrations of test compounds, each performed in triplicate. The IC₅₀ values obtained were used to calculate apparent inhibition constants (K_i) by the method of Cheng and Prusoff [27], from the following equation: $K_i = \text{IC}_{50}/(1+S/K_D)$ where S represents the concentration of the hot ligand used and K_D its receptor dissociation constant (K_D values, obtained by Scatchard analysis [28], were calculated for each labeled ligand). Radioligand binding assays for 5-HT_{1A} were performed following a published procedure [29]. 5-HT_{2A} and 5-HT_{2C} binding assays were performed reported by Herndon et al. [30].

Cell cultures, treatments and survival

MCF7 (ER⁺, PR⁺, HER2⁻), SKBR3 (ER⁻, PR⁺, HER2⁺) and MDA-MB231 (ER⁻, PR⁻, HER2⁺) human BC cells were available in our laboratory. Cell culture conditions, cell treatments with Tamoxifen and cell survival assays were described by Ambrosio et al. [28].

Cytofluorimetric analysis

Cells were collected and fixed in 70% (v/v) ethanol for at least 2h at -20°C . Washed pellets were resuspended in phosphate-buffered saline (PBS) containing RNase A (1 $\mu\text{g}/1\mu\text{L}$) and Propidium Iodide (1 $\mu\text{g}/1\mu\text{L}$). The incubation was carried for 30 minutes at room temperature in a dark environment. Samples were analyzed for emission in the PE-Texas Red channel using BD LSR Fortessa (BD Biosciences, San Jose, CA, USA) and analyzed by BD FACS Diva software. 10⁴ events for each sample were acquired in all analyses.

RNA isolation, RT-PCR and Quantitative real time PCR

Total RNA was isolated from cells, quantified (NanoDrop spectrophotometer, Life Technologies, Carlsbad, CA, USA) and reverse transcribed according to the manufacturer's instructions of chemicals used. Specific primers pairs used for RT-PCR and qPCR assays were designed using Oligo 4.0. and listed in Table 2. Semiquantitative PCR and Quantitative Real-Time PCR (qPCR) assays were performed according to manufacturer's instructions for Bio-Rad T100 thermal cycler and CFX Connect Real Time system (Biorad, Hercules, CA, USA), respectively. Relative gene expression quantification was measured by $2^{-\Delta\Delta\text{Ct}}$ method normalizing for the reference sample using Rps23 (Ribosomal Protein S23) as housekeeping gene.

Statistical analysis

All the statistical analyses were carried out using GraphPad Prism 7. Kruskal Wallis test followed by Dunn's correction was applied for multiple comparisons. Mann-Whitney test was used for pairwise comparisons. Adjusted p-value<0.05 was considered statistically significant.

Results

Synthesis and in vitro receptor binding of serotonergic receptor ligands

The synthetic strategy used for SER preparation (Figure 1) was previously described [22, 23, 24, 25]. SER synthesized showed affinities in the nanomolar range towards 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors (Table 1). Besides the outstanding 5-HT_{2A} receptor affinity and selectivity of compound SER142 (0.046 nM), other interesting K_i values were those of

compounds SER137 (1.07 nM), SER196 (1.68 nM), SER195 (45.3 nM), SER167 (48.5 nM), and SER198 (77.8 nM) a picolinic derivative linked to bis(4-fluorophenyl) methyl piperazine moiety through to a propyl chain spacer. Instead, the analogue derivative characterized by a shorter ethyl chain spacer (SER177) showed a favorable affinity profile for 5-HT_{2C} receptors with Ki value of 0.8 nM. Other interesting Ki values towards this receptor, were those of compound SER68 characterized by 3,4-dichlorophenyl group as N-4 piperazine substituent, linked through an ethyl chain to a norbornene fragment that conferred affinity and selectivity toward 5-HT_{2C} receptor with Ki value of 1.13 nM. Instead, the norbornene derivative 4-[3-[4-(2-furoyl)piperazin-1-yl]propoxy-2-ol]-4-aza-tricyclo [5.2.1.0^{2,6}]dec-8-ene-3,5-dione (SER31) characterized by 2-hydroxy-propyl spacing unit was one of the most selective compound for the 5-HT_{2C} receptor with Ki = 5.04 nM. Moreover, the N'-cyanopicolinamidinium derivative SER79, characterized once again by the bis(4-fluorophenyl) methyl piperazine moiety, showed affinity in the nanomolar range towards 5-HT_{2C} receptor (Ki = 21.4 nM) and weak or no affinity towards 5-HT_{2A} and 5-HT_{1A} receptors respectively.

Antiproliferative effect of serotonergic receptor ligands on BC cell lines

Serotonergic receptor ligands SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68, endowed with different binding affinity for 5-HT_{2A} and 5-HT_{2C} receptors, were examined for their ability to affect BC cell viability. MCF7 (ER⁺, PR⁺, HER2⁻) cells were treated with raising concentration (1μM, 5μM, 15μM, 50μM, 100μM) of SER, in agreement with previous report [31]. We found that all compounds determined a dose-dependent growth inhibition upon both 48- and 72-hours treatment. None effect of vehicle (DMSO) on cell viability was observed (Data not shown). All serotonergic receptor ligands at 100μM dose (except for SER196) significantly reduced MCF7 cell viability upon 72 hours (60 to 80%; adjp<0.05). Of note, the effectiveness of SER79 and SER198 on reducing MCF7 cell viability was also observed at lower dose 50μM (≈70%; adjp<0.05). Consistently, IC₅₀ values were 24.11μM and 10.25μM for SER79 and SER198, respectively. For the other compounds IC₅₀ ranged between 53.28μM and 99.2μM. Finally, IC₅₀ value estimated for SER196 was 123.8μM, out of concentration range tested (Figure 2). We also evaluated the impact of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68 on BC cell lines with different molecular features: SKBR3 (ER⁻, PR⁺, HER2⁺) and MDA-MB231 (ER⁻, PR⁻, HER2⁻). We observed that all compounds at 100μM dose significantly reduced SKBR3 cell viability. On the other hand, 50μM SER79 and SER198 were able to significantly reduce SKBR3 cell viability (≈50%; adjp<0.05; Figure 3). In this case, none effect of vehicle was observed (Data not shown). Such results highlighted that SER79 and SER198 were the most effective SER in reducing not only MCF7 but also SKBR3 cell viability. Of note, IC₅₀ value estimated for SER79 and SER198 in SKBR3 cells was 53.56μM and 67.74μM, respectively. In parallel, we evaluated the effect of SER on triple negative MDA-MB231 cells. We found that only SER137, SER79, SER31, SER68 at 100μM dose were able to inhibit triple negative MDA-MB231 cell growth. In the same condition, none effect of vehicle (DMSO) was observed (Data not shown). Notably, IC₅₀ value estimated for SER79 in MDA-MB231 cells was 116.9μM, higher than those obtained for both SKBR3 and MCF7 cells (Figure 4). We also verified that the different effect of SER on BC cells was not attributable to a lack of expression of 5-HT receptors in SKBR3 and MDA-MB231 cells. Indeed, HT_{2A} and HT_{2C} receptors were detected in all cell lines suggesting that the different effect of SER on MCF7 (ER⁺, PR⁺, HER2⁻), SKBR3 (ER⁻, PR⁺, HER2⁺) and MDA-MB231 (ER⁻, PR⁻, HER2⁻) cells should be attributable to their different molecular features, which also give them a different degree of aggressiveness (Table 2 and Figure 5). Next, we analyzed the ability of selected SER - those affecting cell viability (Figures 2,3,4) - to induce cell cycle perturbation in MCF7, SKBR3 and MDA-MB231 cells, at doses corresponding to IC₅₀ values. As shown in Figure 6, all selected SER, except for SER198, caused a significant increase in the percentage of cells in G0/G1 phase, paralleled by a decrease of S phase. On the opposite, treatment with none of these compounds affected cell cycle in SKBR3 and MDA-MB231 cells (Figure 6).

Effect of serotonergic receptor ligands on MCF7 cell responsiveness to Tamoxifen

We previously demonstrated that MCF7 cell responsiveness to Tamoxifen inversely correlates with CTGF, thus identifying the Connective Tissue Growth Factor as one relevant contributor to drug effectiveness on BC [32]. Thus, we investigated the effect of SER on MCF7 cell responsiveness to Tamoxifen. Once established that the efficiency of serotonergic receptor ligands (at dose corresponding to IC₅₀ values), those able to affect cell viability and to perturb cell cycle phases (Figures 2 and 6), also occurs in presence of estradiol (E2; Figure 7a), we treated estrogen-starved MCF7 cells with Tamoxifen - and E2 - in presence of SER. As reported in Figure 7b, Tamoxifen treatment significantly reduced cell viability in presence of SER (20 to 70%; adjp<0.05; Figure 7b). Notably, SER79 and SER68 significantly decreased cell viability (≈70% with SER68 and ≈60% with SER79; adjp<0.01) also compared with cells treated with

Tamoxifen alone ($\approx 30\%$ reduction of cell viability; $\text{adjp} < 0.01$; Figure 7b), thus potentiating drug effect (Figure 7). Then, we analyzed *CTGF* mRNA levels in MCF7 cells treated with selected SER. Interestingly, we found that in presence of SER79 and SER68, while not of the other compounds, *CTGF* mRNA levels were significantly lower compared with those in untreated cells ($\approx 70\%$; $\text{adjp} < 0.01$; Figure 8a). Once checked that *CTGF* was also expressed in SKBR3 and MDA-MB231 cells (Figure 8b), we investigated whether treatment with selected SER (Figures 3,4,6), may modulate *CTGF* mRNA levels. Differently to what observed in MCF7 cells, no significant down-regulation of *CTGF* was observed in SKBR3 and MDA-MB231 cells (Figure 8c-d). To further investigate the effect of SER79 and SER68 on Tamoxifen responsiveness and *CTGF* expression in MCF7 cells, we obtained - by a continuous treatment of 10 days with $1\mu\text{M}$ Tamoxifen – a cellular model less sensitive to the drug (MCF7-R). Then, we evaluated Tamoxifen responsiveness and *CTGF* mRNA levels in absence and in presence of SER. As reported in Figure 9a, cell viability was not reduced upon Tamoxifen treatment in MCF7-R cells, and the presence of SER68 and SER79 restored Tamoxifen effect ($\text{adjp} < 0.05$; Figure 9a). In parallel, *CTGF* expression was significantly increased in MCF7-R cells ($\text{adjp} < 0.05$; Figure 9b). Both SER68 and SER79 significantly reduced *CTGF* to levels similar (SER79) or significantly lower (SER68) than those detected in Tamoxifen-responsive MCF7 cells ($\text{adjp} < 0.05$; Figure 9b).

Discussion

New biological insights highlighted a role of serotonin in virtually all major organs outside the central nervous system [33]. Thus, 5-HT has numerous important peripheral functions in humans [18]. Among them, it is integral part of mammary epithelial homeostatic system in ensuring normal tissue function. Correspondingly, the tightly regulated pattern of serotonin becomes dysregulated in human breast tumor [34-35]. 5-HT signaling has been related with cancer cell growth, differentiation, angiogenesis and metastasis, suggesting an association between its levels and tumor aggressiveness and/or prognosis [15,18,35]. In particular, physiological responses to serotonin include both tumor-suppressing and tumor-promoting activities. Indeed, while controlling homeostatic regulatory mechanisms in normal mammary epithelium, 5-HT signaling appears to favor malignant progression of human BC [17]. Differences in the components of serotonin system, including the ability to synthesize 5-HT and/or the receptors pattern, may explain these opposite effects [15,17]. Of note, transcriptomic and metabolomic data from breast tumor specimens highlighted the correspondence between poor prognosis and increased tumor-specific serotonin production [36]. Seven distinct families of 5-HT receptors are expressed in a tissue-specific manner across a variety of normal and tumor cells [31]. In BC, serotonin confers proliferative advantage to tumor cells by increasing proliferation rate and decreasing programmed cell death, mainly through 5-HT_{2A} and 5-HT_{2C} receptors [18,26]. We previously synthesized serotonergic receptor ligands with high affinity and selectivity binding profile towards 5-HT_{2A} and 5-HT_{2C} receptors [22]. Here, we analyzed the effect of such compounds on BC cell survival. Interestingly, we observed that their different ability to affect MCF7 (ER⁺, PR⁺, HER2⁻), SKBR3 (ER⁻, PR⁺, HER2⁺) and MDA-MB231 (ER⁻, PR⁻, HER2⁻) BC cell growth was not due to a lack of expression of 5-HT₂ receptors but eventually attributable to their different molecular features, which also give them a different degree of aggressiveness. In addition, considering that structural analogies exist among serotonergic receptors and that SER were selective, while not exclusive, for binding 5-HT_{2A} and 5-HT_{2C}, a possible involvement of other components of the serotonergic receptors pattern, could not be excluded (Table 2 and Figure 5). Hormone receptor-positive tumors obtain substantial benefit from treatment with Tamoxifen [20-21]. We previously reported that Tamoxifen responsiveness of ER⁺ BC cells inversely correlates with Connective Tissue Growth Factor, providing additional clues to the hypothesis of its contribution to drug sensitivity in BC [32]. It has been described that inhibitors of serotonergic pathway components reduced sphere-forming activity of breast tumor cell lines in dose-dependent fashion and synergized with docetaxel to shrink breast tumor xenografts [36]. In line with this concept, we provide evidence that some serotonergic receptor ligands, namely SER79 and SER68, improve the effectiveness of Tamoxifen treatment on ER⁺ MCF7 BC cells modulating *CTGF* expression. At variance, no significant *CTGF* down-regulation was observed in SKBR3 and MDA-MB231 cells, suggesting a potential involvement of ER and/or PR in SER-mediated effects. *CTGF* may be triggered by serotonin and their association has been described [37-38]. Thus, our results identified new compounds able to target serotonin signaling, and in turn *CTGF*, and therefore potentially usable in combination with Tamoxifen improving its effectiveness on ER⁺ BC patients. Adjuvant endocrine therapies may contribute to depression and anxiety in patients with cancer [39-40]. Antidepressant medications, including antagonists of serotonin receptor and/or SSRIs, may be co-prescribed with Tamoxifen in BC [41]. However, some antidepressant agents interfere with Tamoxifen

metabolism, compromising its efficacy [42]. In this regard, the use of SSRIs has been associated with increased tumor proliferative index in patients with late-stage BC compared to patients non-users of SSRIs [35]. Nevertheless, drug interactions involving Tamoxifen and antidepressant medications remain controversial [15,23,41]. The discovery of novel compounds directly targeting serotonin signaling may contribute to tailor new therapeutic strategies usable in combination with endocrine therapies, improving their efficacy for treating cancer patients.

Conclusions

We identified serotonergic receptor ligands able to target serotonin signaling, and in turn *CTGF*, also ameliorating the sensitivity to Tamoxifen in ER⁺ BC cells. Thus, they represent new compounds potentially usable in combination with Tamoxifen improving its effectiveness on ER⁺ BC patients.

List of abbreviations

5-HT	5-Hydroxytryptamine, Serotonin
ER ⁺	Estrogen Receptor Positive
SER	Serotonergic receptor ligands
BC	Breast Cancer
CTGF	Connective Tissue Growth Factor
SERMs	Selective Estrogen Receptor Modulators
SSRIs	Selective Serotonin Reuptake Inhibitors
E2	Estradiol

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Author Contributions: M.R.A., E.M., F.F. and P.F. conceived the study and designed the work; M.R.A., G.C., R.S., T.M. and G.M. performed the experiments; M.R.A., E.M., P.M. and V.D. acquired and analyzed the data; M.R.A., E.M., F.F. and P.F. drafted the manuscript. All authors contributed to data interpretation and discussion, also edited and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Figure legends

Figure 1. Chemical structures of serotonergic receptor ligands (SER).

Figure 2. Effect of SER on MCF7 cell viability. MCF7 cells were treated with raising concentration (1μM, 5μM, 15μM, 50μM, 100μM) of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68. After 48 and 72 hours, cell viability was assessed, by sulforhodamine B assay (see Methods). The results were reported as percentage of viable cells compared to positive control (untreated cells), considered as maximum viability (100%). Data represent the mean ± SD of at least five independent triplicate experiments. * denote statistically significant values compared with positive control (*adjp<0.05, **adjp<0.01, ***adjp<0.001).

Figure 3. Effect of SER on SKBR3 cell viability. SKBR3 cells were treated with raising concentration (15 μ M, 50 μ M, 100 μ M) of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68. Cell viability was assessed, after 72 hours, by sulforhodamine B assay (see Methods). The results were reported as percentage of viable cells compared to positive control (untreated cells), considered as maximum viability (100%). Data represent the mean $\pm$ SD of at least three independent triplicate experiments. * denote statistically significant values compared with positive control (*adjp<0.05, **adjp<0.01).

Figure 4. Effect of SER on MDA-MB231 cell viability. MDA cells were treated with raising concentration (15 μ M, 50 μ M, 100 μ M) of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68. Cell viability was assessed, after 72 hours, by sulforhodamine B assay (see Methods). The results were reported as percentage of viable cells compared to positive control (untreated cells), considered as maximum viability (100%). Data represent the mean $\pm$ SD of at least three independent triplicate experiments. * denote statistically significant values compared with positive control (*adjp<0.05).

Figure 5. Representative gel images of RT-PCR assays for the 5-HT_{2A} and 5-HT_{2C} genes in MCF7, SKBR3 and MDA-MB231 cell lines. Rps23 was used as reference gene. Images have been cropped to improve the clarity of presentation.

Figure 6. Effect of SER on BC cell cycle. MCF7, SKBR3 and MDA-MB231 cells were treated with selected SER at IC₅₀ doses. After 72 hours, cell cycle was assessed by Propidium Iodide staining (see Methods). The results were reported as percentage of cells in G0/G1, S and G2/M cell cycle phase. Data represent the mean $\pm$ SD of at least three independent triplicate experiments. * denote statistically significant values compared with percentage of untreated cells in G0/G1, S and G2/M cell cycle phase (*adjp<0.05, **adjp<0.01).

Figure 7. Effect of SER on MCF7 cell responsiveness to Tamoxifen. (A) Estrogen-starved MCF7 cells (48 hours) were treated with SER137, SER142, SER167, SER195, SER196, SER79, SER177, SER31, SER68 at IC₅₀ dose in presence of Estradiol (E2; 100nM). As positive control, the cells were treated with E2 alone (dotted line). (B) Estrogen-starved MCF7 cells (48 hours) were treated with Tamoxifen (5 μ M) in presence of E2 (100nM) and SER137, SER142, SER167, SER195, SER196, SER79, SER177, SER31, SER68 at IC₅₀ dose. As positive control, the cells were treated with E2 and SER alone (without Tamoxifen, dotted line). (A-B) After 72 hours, cell viability was assessed by sulforhodamine B assay (see Methods). The results were reported as percentage of viable cells compared to positive control, considered as maximum viability (100%). Data represent the mean $\pm$ SD of at least five independent triplicate experiments. * denote statistically significant values compared with control (*adjp<0.05, **adjp<0.01); # denote statistically significant values compared with cells treated with Tamoxifen in absence of SER (#adjp<0.01).

Figure 8. Effect of SER on CTGF expression in BC cell lines. (A) Estrogen-starved MCF7 cells (48 hours) were treated with SER137, SER142, SER167, SER195, SER196, SER79, SER177, SER31, SER68 (at IC₅₀ doses) in presence of E2 (100nM). (B) Representative gel images of RT-PCR assays for CTGF gene in SKBR3 and MDA-MB231 cell lines. Rps23 was used as reference gene. Images have been cropped to improve the clarity of presentation. (C) SKBR3 cells were treated with SER 79 and SER 198 (at IC₅₀ doses). (D) MDA-MB231 cells were treated with SER137, SER79, SER31 and SER198 (at IC₅₀ doses). (A-C-D) After 72 hours, mRNA levels of CTGF were determined by qPCR (see Methods and Table 2). Data were normalized on Ribosomal Protein S23 (Rps23) gene as internal standard. Bars represent the mean $\pm$ SD of at least three independent experiments and show CTGF mRNA levels in (A) MCF7, (C) SKBR3 and (D) MDA-MB231 treated with SER relative to those in untreated cells (dotted line). * denote statistically significant values (*adjp<0.05; **adjp<0.01).

Figure 9. Effect of SER79 and SER68 on Tamoxifen responsiveness and CTGF expression in MCF7-R cells. MCF7 cells were cultured in presence of Tamoxifen (1 μ M) for 10 days (MCF7-R). (A) Estrogen-starved MCF7 and MCF7-R cells (48 hours) were treated with Tamoxifen (5 μ M) in presence of Estradiol (E2; 100nM) and SER79 or SER68 (at IC₅₀ dose). As positive control, the cells were treated with E2 alone, in absence or in presence of SER, as indicated (without Tamoxifen, dotted line). After 72 hours, cell viability was assessed by sulforhodamine B assay (see

Methods). The results were reported as percentage of viable cells compared to positive control, considered as maximum viability (100%). Data represent the mean $\pm$ SD of three independent triplicate experiments. * denote statistically significant values compared with positive control (*adjp<0.05; ** adjp<0.01). (B) mRNA levels of *CTGF* were determined by qPCR (see Methods and Table 2) in MCF7-R cells in presence and absence of SER79 and SER68 (at IC₅₀ dose). Data were normalized on Ribosomal Protein S23 (Rps23) gene as internal standard. Bars represent the mean $\pm$ SD of three independent experiments and show *CTGF* mRNA levels in MCF7-R relative to those in MCF7 cells (dotted line; *adjp<0.05; **adjp<0.01). # denote statistically significant values compared with MCF7-R cells (##adjp<0.01. ###adjp<0.001).

FIGURE1. Chemical structures of serotonergic receptor ligands (SER).

FIGURE2. Effect of SER on MCF7 cell viability.

FIGURE3. Effect of SER on SKBR3 cell viability.

FIGURE4. Effect of SER on MDA-MB231 cell viability.

FIGURE5. Representative gel images of RT-PCR assays for the 5-HT_{2A} and 5-HT_{2C} genes in MCF7, SKBR3 and MDA-MB231 cell lines.

FIGURE6. Effect of SER on BC cell cycle.

FIGURE7. Effect of SER on MCF7 cell responsiveness to Tamoxifen.

FIGURE8. Effect of SER on *CTGF* expression in BC cell lines.

FIGURE9. Effect of SER79 and SER68 on Tamoxifen responsiveness and *CTGF* expression in MCF7-R cells.

a

b

TABLE1. Affinities of SER for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors (Ki ± SD; nM).

	5-HT _{1A} [³ H]8OH-DPAT	5-HT _{2A} [³ H]Ketanserin	5-HT _{2c} [³ H]Mesulergine
SER137	219 ± 6.3	1.07 ± 0.08	>10 ⁴
SER142	549 ± 13	0.046 ± 0.002	>10 ⁴
SER167	1320 ± 12	48.5 ± 2.7	140 ± 2.5
SER195	>10 ⁴	45.3 ± 5.7	>10 ⁴
SER196	>10 ⁴	1.68 ± 0.4	>10 ⁴
SER198	52.7 ± 2.7	77.8 ± 2.1	117 ± 6.1
SER79	>10 ⁴	504 ± 39.5	21.4 ± 1.22
SER177	>10 ⁴	218 ± 9.3	0.8 ± 0.05
SER31	1050 ± 0.231	>10 ⁴	5.04 ± 0.227
SER68	No affinity	153 ± 70	1.13 ± 0.16

TABLE2. Primer pairs for qPCR.

	Forward	Reverse	Size
5-HT _{2A}	5' CAGAATCCCATCCACCACAG 3'	5' AAGAGCCGATCAGGACAAAG 3'	187bp
5-HT _{2C}	5' GGCAGTAAGCATGGAAAAGAA 3'	5' AAAGAAATCCAGACGGGGCA 3'	171bp
Rps23	5' TCGTGGACTTCGTACTGCT 3'	5' GCTGTGATTTTCTTGCCATTC 3'	237bp
CTGF	5' GGGAAATGCTGCGAGGAGT 3'	5' GATAGGCTTGGAGATTTTGG 3'	327bp

Figures

Figure 1

Chemical structures of serotoninergic receptor ligands (SER).

Figure 2

Effect of SER on MCF7 cell viability. MCF7 cells were treated with raising concentration (1μM, 5μM, 15μM, 50μM, 100μM) of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68. After 48 and 72 hours, cell viability was assessed, by sulforhodamine B assay (see Methods). The results were reported as percentage of viable cells compared to positive control (untreated cells), considered as maximum viability (100%). Data represent the mean ± SD of at least five independent triplicate

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Effect of SER on SKBR3 cell viability. SKBR3 cells were treated with raising concentration (15μM, 50μM, 100μM) of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68. Cell viability was assessed, after 72 hours, by sulforhodamine B assay (see Methods). The results were

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Figure 4

Effect of SER on MDA-MB231 cell viability. MDA cells were treated with raising concentration (15μM, 50μM, 100μM) of SER137, SER142, SER167, SER195, SER196, SER198, SER79, SER177, SER31, SER68. Cell viability was assessed, after 72 hours, by sulforhodamine B assay (see Methods). The results were reported as percentage of viable cells compared to positive control (untreated cells), considered as maximum viability (100%). Data represent the mean ± SD of at least three independent triplicate experiments. * denote statistically significant values compared with positive control (*adjp<0.05).

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