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Unraveling the Anticancer Potential of SSRIs in Prostate Cancer by Combining Computational Systems Biology and *In Vitro* Analyses

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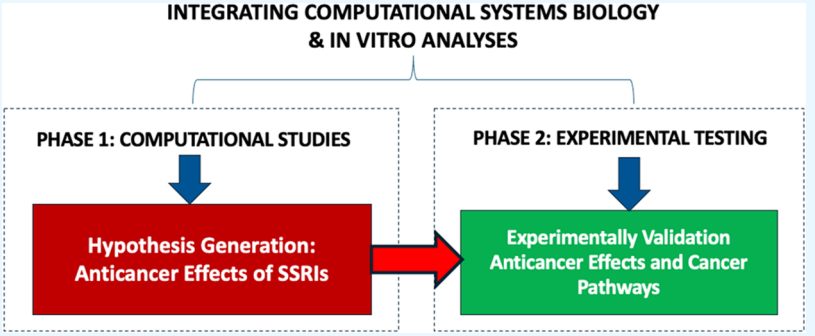

ABSTRACT: Selective serotonin reuptake inhibitors (SSRIs) are known to have anticancer activity against different types of cancer. In this study, an integrative informatics approach was applied to identify compound and genetic perturbations that produce similar effects to SSRIs to formulate systems biology hypotheses and identify biological pathways involved in the putative anticancer effects of SSRIs in prostate cancer. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay assessed the antiproliferative effects of SSRIs and drug combinations. Cell death mechanisms were studied using annexin V-FITC/PI staining, and the cell cycle analysis was carried out by counterstaining with propidium iodide. Relative gene expression was assessed using a real-time polymerase chain reaction (PCR). Computational results hypothesized that SSRIs could potentially exert anticancer effects in prostate cancer cell lines by modulating apoptotic and tumorigenesis pathways and significantly inhibiting the growth of prostate cancer cells in a time and concentration-dependent manner. The combination of SSRIs with cisplatin, 5-fluorouracil, and raloxifene resulted in either synergistic or additive effects. SSRIs resulted in a significant increase in the early and late apoptotic activity in PC3 cells. Dapoxetine, paroxetine, and sertraline resulted in cell cycle arrest at the G0/G1 phase. Treatment with either dapoxetine or paroxetine decreases the expression of *Bcl-2*, *CASP8*, *DR5*, and *VEGF*. At the same time, sertraline decreases the expression of *Bcl-2 and VEGF* and increases the expression of *CASP8* and *DR5*. Results revealed that SSRIs can potentially act as antiproliferative agents against prostate cancer cells, and their activity is mediated through different signaling pathways.

INTRODUCTION

Serotonin or 5-hydroxytryptamine (5-HT) is a biogenic amine neurotransmitter that has a major role in regulating different behavioral and neuropsychological functions such as mood, memory, cognition, perception, body temperature, sleep-wake cycle, and appetite.[1] Selective serotonin reuptake inhibitors (SSRIs) increase serotonergic activity by inhibiting serotonin reuptake.[2] They are widely used for the treatment of depression and various disorders, such as anxiety, eating disorders, and different psychiatric conditions due to their efficacy, safety, and tolerability.[3,4]

According to GLOBOCAN statistics, in 2020, prostate cancer was the second most common cancer diagnosed in men with 1.4 million cases.[5] Moreover, prostate cancer was the fifth leading cause of cancer death with an estimated 375,000 deaths worldwide.[6]

The prevalence of depressive disorders is 2−3 times higher in cancer patients than in the general population.[7] Moreover, the prevalence rate of psychological distress in cancer patients varied depending on the clinical setting, stage of the disease, and phase of treatment ranging between 5 and 49%.[7] SSRIs are considered first-line agents for the treatment of depression among cancer patients due to their general tolerability, with citalopram, escitalopram, and sertraline being the best options because they have the lowest drug−drug interactions.[8]

It has been reported that SSRIs affect multiple intracellular pathways including the Ras-Raf-MEK-ERK pathway.[9] In addition, SSRIs activate the JNK pathway and AMPA receptor

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pathway, which subsequently trigger apoptosis.[10,11] Recent studies have revealed that different SSRIs have protective effects against different types of cancer, including bladder, colorectal, hepatic, and epithelial ovarian cancer.[12] Moreover, other studies have shown that different SSRIs had potent anticancer activity against various cancer cells such as neuroblastoma, glioblastoma, breast, and prostate cancer stem cells.[13,14] The mechanism by which SSRIs exert their anticancer activities is proposed to be mediated by triggering the ATF4-AKT-mTOR signaling pathway[15] or through activating the nuclear factor kappa B (NF-kB) and the JAK/STAT signaling pathways.[16]

To the best of our knowledge, this is the first study to uncover potential underlying anticancer mechanisms of SSRIs in prostate cancer by modulating the PI3K/Akt and MAPK pathways to produce apoptotic and antitumor effects. Moreover, various combinations of SSRIs with conventional chemotherapeutic agents including 5-FU, cisplatin, and raloxifene had synergistic/additive effects when compared to monotherapy.

MATERIALS AND METHODS

Computational Systems Biology Workflow. We applied a computational systems biology workflow to study the network pharmacology of SSRIs, based on the methods developed by Hajjo et al.,[17−20] to formulate testable hypotheses regarding the putative anticancer effects and mechanisms. In this study, the computational workflow consisted of three steps: (1) identifying gene signatures representative of the chemical compound's biological action; (2) identifying genetic perturbations that result in transcriptomics profiles resembling those of SSRIs; and (3) a pathway enrichment and literature mining to elucidate the biological activities of similar gene and compound connections, respectively.

The CMap computational tool and a chemogenomics database constitute a cornerstone of the chemocentric informatics workflow developed by Hajjo et al.[17−20] It lists 1.3 million profiles of transcriptional responses of human cells to chemical and genetic perturbations. Now, there are 27,927 perturbagens (19,911 small molecules and 7494 genetic perturbagens) generating 476,251 expression signatures in 9 human cell lines: PC3, VCAP, A375, A459, HA1E, HCC515, HT29, MCF7, and HEPG2. This database uses the L1000 platform[21] which is a high-throughput gene expression assay that assesses the mRNA transcript abundance of 978 "landmark" genes from human cells. The CMap approach enables researchers to identify biological similarities based on chemogenomic effects (i.e., the gene expression profiles in response to the treatment of cancer cell lines with chemical compounds).

In this study, the Connectivity Map (CMap) was first queried to identify SSRIs using the following search terms: "compound name = citalopram, dapoxetine, escitalopram, fluvoxamine, paroxetine, and sertraline" and "compound description = serotonin reuptake inhibitor". The identified compounds and compound classes become our list of queries to identify compounds and genes with transcriptomics profiles similar to those of the queries. In the next step, the identified positive compound and gene connections from the CMAP were filtered based on their connectivity scores as described elsewhere.[21] In the last step, literature mining identified the biological activities of compound hits, and enrichment analysis using the STRING App in Cytoscope version 3.10.3 (1) identified enriched biological pathways.

Cell Viability Assay. All cell lines used in this study were purchased from the American Type Culture Collection (ATCC), ensuring the authenticity and quality of the biological materials used in our experiments. Cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) assay, as described previously.[22] All experiments were run in duplicate wells and repeated at least 3 times. The 50% inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 9 (GraphPad Software, San Diego, USA).

Drug Combination Assay. PC3 and DU-145 cells were seeded in a 96-well plate at a density of 8000 and 6000 cells/well for 48 h, respectively. To examine the combined effect of SSRIs with cisplatin, 5-fluorouracil, and raloxifene, various concentrations at different combination ratios (IC_{50} of the chemotherapy: IC_{50} of SSRIs) were applied to cells for 48 h, then an MTT assay was performed, and optical density was measured at 570 nm as previously described.[22,23] The combination index (CI) was then calculated using CompuSyn software (Combosyn Inc., Paramus, NJ, USA), which is based on Chou−Talalay's Combination Index Theorem, explained in eq 1.

$$\text{Chou} - \text{Talalay's Combination Index Theorem. } \boldsymbol{CI} = \frac{(\boldsymbol{D})\mathbf{1}}{(\boldsymbol{Dx})\mathbf{1}} + \frac{(\boldsymbol{D})\mathbf{2}}{(\boldsymbol{Dx})\mathbf{2}} \quad (1)$$

where $(Dx)1$ = dose of drug 1 to produce 50% cell kill alone, $(D)1$ = dose of drug 1 to produce 50% cell kill in combination with $(D)2$, $(Dx)2$ = dose of drug 2 to produce 50% cell kill alone, and $(D)2$ = dose of drug 2 to produce 50% cell kill in combination with $(D)1$.

Migration Assay (Wound Healing Assay). This assay was performed as previously described.[23] Images were captured at 0 and 24 h using the EVOS XL Core imaging system at 10× magnification. The wound area was measured using ImageJ software Ver. 1.53e.

Soft Agar Colony Formation Assay. This experiment was done as previously explained.[24] Images were captured within 18 days at several magnifications using the EVOS XL Core imaging system (Invitrogen, USA).

Annexin V-FITC/Propidium Iodide Apoptosis Assay. This assay was performed as previously described.[25] PC3 cells were seeded in a 6-well plate at a density of 300,000 cells per well and allowed to attach overnight. Thereafter, the cells were treated with IC_{50} concentration of SSRIs, and 5-fluorouracil (positive control) for 48 h, with wells containing only fresh DMEM media acting as a negative control. Cells were then harvested and stained with Annexin V-FITC and propidium iodide according to the manufacturer's instructions. The samples were analyzed immediately using BD ACSCanto II flow cytometer (BD Biosciences, USA), and the results were processed with BD FACSDiva software.

Cell Cycle Analysis Assay. PC3 cells were seeded at a density of approximately 1×10^6 in a tissue culture dish until confluency and then treated with sub-IC_{50} (0.5 IC_{50}) concentration of dapoxetine, paroxetine, and sertraline and incubated for 48 h. After treatment, cells were harvested, collected, fixed with 70% ice-cold ethanol overnight at −20 °C, and stained with PI. The samples were then processed using a

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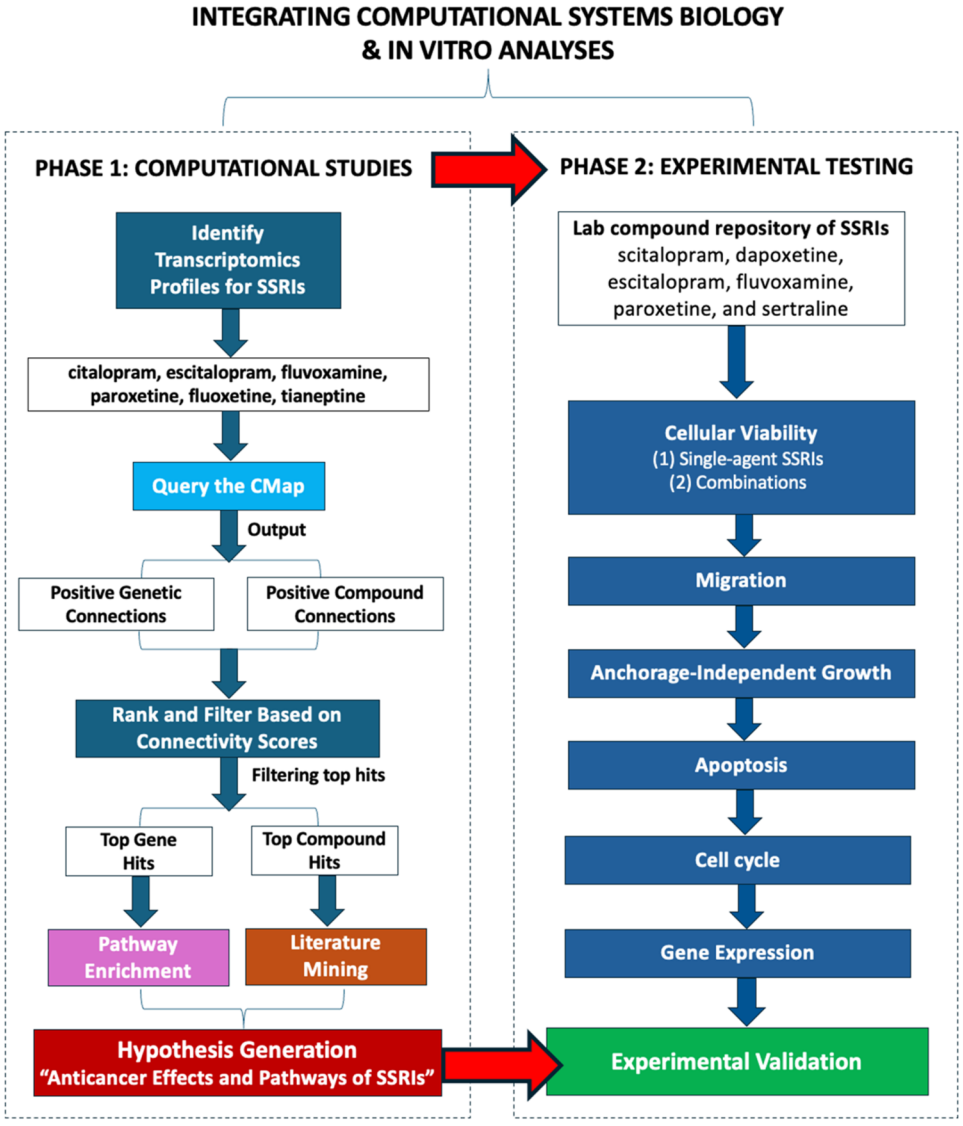

Figure 1. Integrative Computational Systems Biology and Experimental Validation Workflow for Assessing the Anticancer Activities of SSRIs.

BD FACSCanto II flow cytometer (BD Biosciences, USA), and the results were analyzed using BD FACSDiva software.[24,25]

Real-Time Polymerase Chain Reaction (RT-PCR). PC3 and DU-145 cells were seeded in a tissue culture dish at a density of 600,000 and PC3 cells were treated with 0.1 IC_{50} and 0.25 IC_{50} of SSRIs (dapoxetine, paroxetine, and sertraline) for 48 h. DU-145 was not treated and was only utilized as a control, while an untreated PC3 control was also included. After the treatment time was over, the total RNA of cells was extracted and purified using Direct-zol RNA Miniprep Plus kit according to manufacturer protocol,[22−25] moreover, nanodrop was used to measure the purity and concentration of RNA produced. Afterward, a reverse-transcription reaction solution was done using the ProtoScript^{R} First Strand cDNA Synthesis kit.[22−25] Then the reverse-transcription reaction was carried out at 25 °C for 5 min, followed by an incubation step at 42 °C for 1 h, then increased the temperature to 80 °C for 5 min to inactivate the enzyme. Quantitative real-time PCR was carried out using SYBR Green Real-Time PCR Master Mix through a 7900 Real-time PCR detection system (Applied Biosystems, USA).[22−25] The Primers' sequence and their optimized annealing temperature (Ta) are shown in Table S1.

Statistical Analysis. Data analysis was performed using GraphPad Prism software (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California, USA). The differences between treatment groups were determined by a two-way analysis of variance (ANOVA). Data are expressed as mean ± standard deviation (SD), and $p < 0.05$ was considered a statistically significant difference.

A nonlinear regression analysis was used to calculate the IC_{50} values. CompuSyn software (Combosyn Inc., Paramus, NJ, USA) was used to calculate the combination index (CI). Wound area, colony size, and colony numbers were measured using ImageJ software Ver 1.53e.

RESULTS

In this study, an integrative computational systems biology and experimental validation workflow was applied to generate and validate testable hypotheses about the systems biology effects of SSRIs. The workflow (Figure 1) comprises two phases: Phase 1 employs computational systems biology methods to predict the biological effects of SSRIs, while Phase 2 experimentally validates these predictions.

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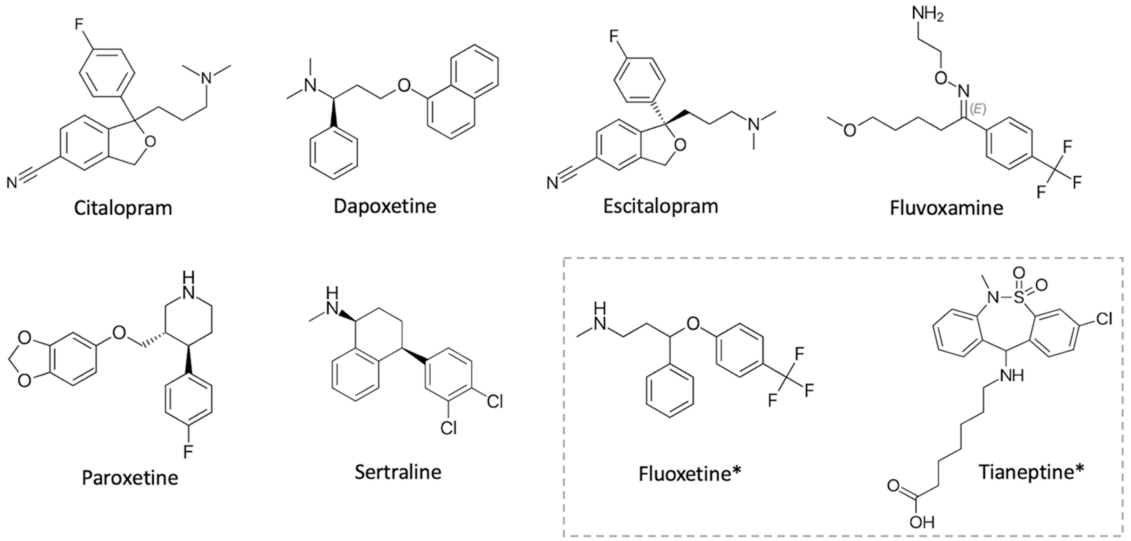

Figure 2. Chemical structures of SSRIs used in both experimental and computational studies. The two compounds denoted by asterisks and surrounded by a dotted rectangle have been included in the computational studies only.

The Systems Biology Effects of SSRIs. The systems biology effects of SSRIs were investigated using a computational systems biology workflow that is shown in Figure 1, based on methods developed by Hajjo et al. Our data mining efforts using the CMap database identified gene expression data for citalopram, escitalopram, fluvoxamine, paroxetine, and sertraline but not for dapoxetine. We also identified gene expression data for fluoxetine and tianeptine that were not part of our chemical repository and therefore were not tested experimentally. The chemical structures for all computationally analyzed or experimentally tested compounds are shown in Figure 2.

Searching the CMap for gene expression profiles similar to those of the SSRIs used to query the database identified the following compounds as the strongest positive connections to SSRIs: sertraline, tetrindole, wiskostatin, NTNCB, berbamine, chlorprothixene, metergoline, H-89, nortriptyline, wortmannin (Figure 3A, Table 1). The top positive compound connection was sertraline, an SSRI whose gene signature was used to derive the query signature for SSRIs. Notably, eight out of the ten top compound connections have documented anticancer effects, and 3 of them (sertraline, H-89, and wortmannin) impact PI3K/Akt signaling and apoptosis pathways, while two compounds (sertraline and wiskostatin) affect the MAPK signaling pathways (Table 1).

The top ten genetic connections with positive connectivity scores (i.e., indicating high similarity in gene expression profiles) are shown in Figure 3B and they include NUDT5 kd, EGFR kd, CHMP2A kd, AKT1 kd, PTRF kd, AKT3 kd, SMAD4 kd, TUBB6 oe, KIF11kd, and TERF1 kd (Table 2). The genes were checked against pathway databases to identify common "enriched" pathways and biological processes as described in the Methods section. The most frequently identified pathways include the PI3K/AKT, MAPK, FoxO, EGFR, apoptosis, cell cycle, TGF-β, and VEGF pathways. These pathways are involved in critical cellular processes such as survival, proliferation, apoptosis, angiogenesis, and drug resistance, highlighting their relevance in cancer biology and potential therapeutic targeting. Frequently associated cancer types were also identified including pancreatic cancer, colorectal cancer, head and neck squamous cell carcinoma, gastric cancer, hepatocellular carcinoma, prostate cancer, breast cancer, and nonsmall cell lung cancer. All details are provided in Supplementary Table 1.

Effects of SSRIs on the Viability of Prostate Cancer Cell Lines. To evaluate the effects of SSRIs treatment (citalopram, dapoxetine, escitalopram, fluvoxamine, paroxetine, and sertraline) on the viability of different prostate cancer cells, an MTT assay was performed after exposing PC3 and DU-145 cell lines to increasing concentrations of SSRIs for 24, 48, and 72 h.

SSRIs have shown antiproliferative activities and treated cells had reduced cell viability compared to untreated control cells. SSRIs inhibited the growth of the cells in a time and concentration-dependent manner. The 50% inhibitory concentration (IC_{50}) values for SSRIs treatment in PC3 and DU-145 prostate cancer cells are shown in Table 3.

Effects of Combined Treatment of SSRIs and Chemotherapeutic Agents on the Viability of Prostate Cancer Cell Line. To determine the effect of combined SSRIs with different chemotherapeutic agents, SSRIs were combined with either cisplatin, 5-fluorouracil, or raloxifene at different combination ratios.

In general, the combination of SSRIs with either cisplatin, 5-fluorouracil, or raloxifene resulted in a synergistic effect in DU-145. While in PC3, the combination resulted in either a synergistic or additive effect. Combination index (CI) values were calculated using CompuSyn software. The IC_{50} values of the chemotherapy alone and in combination, the fold of reduction of the chemotherapy, and combination index values for the combined treatment of SSRIs and chemotherapeutic agents in PC3 and DU-145 are shown in Tables 4, 5, and 6.

Effect of SSRIs Treatment on the Migration of Prostate Cancer Cells. To assess the effect of SSRIs on the 2D migration of PC3 and DU-145 cells, a wound healing assay was performed using sub-IC_{50} (0.5 IC_{50}) and IC_{50} concentrations of SSRIs as a treatment. After 24 h, a complete closure (100%) of the wound in the untreated sample (control) was observed. SSRIs treatment for 24 h significantly reduced the percentage of wound closure in a concentration-dependent manner in both examined cell lines compared to untreated cells. The wound area was measured using ImageJ

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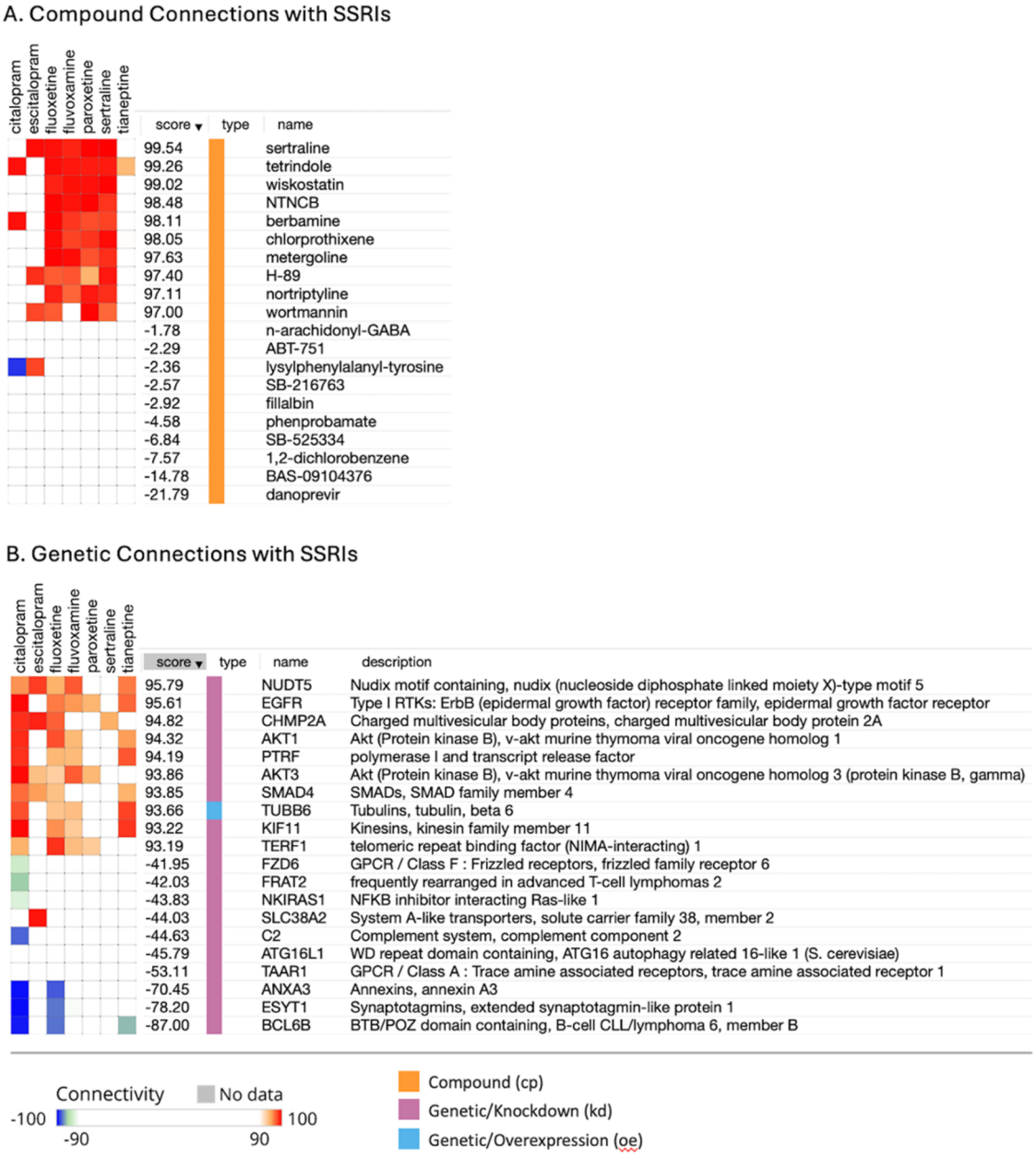

Figure 3. Compound and genetic connections with SSRIs. **A**. Compound connections. **B**. Genetic connections including knockdown and overexpression. The predicted connections are found in the "name" column.

software Ver. 1.53e. Figure 4 shows column representation, comparing the percentage of wound closure between the untreated sample and the treated one after 24 h. Images were captured at 0 and 24 h using the EVOS XL Core imaging system at 10× magnification. Wound images for PC3 and DU-145 cells are shown in Figure S1.

Effect of SSRIs on Anchorage-Independent Growth of Prostate Cancer Cells. To investigate the effect of SSRIs treatment on the colony formation capability of prostate cancer cells, PC3 cells were treated with either sub-IC_{50} (0.5 IC_{50}) or IC_{50} concentrations of SSRIs for 48 h. Then cells were allowed to grow in soft noble agar. When compared to untreated control cells, SSRIs treatment significantly reduced the number and size of colonies formed as shown in Figure 5. Representative images taken on day 18 at different magnifications; 4× and 20× are shown in Figure S2.

Effect of SSRIs on Apoptosis of Prostate Cancer Cells. Annexin V-FITC/PI double staining was performed to examine whether SSRIs affected prostate cancer cell viability by either inducing apoptosis or necrosis. As shown in Figure 6, treatment of PC3 cells with SSRIs at IC_{50} concentrations resulted in increased early and late apoptotic effects (except with citalopram) (Q2 and Q4 regions presented in the dot plot) compared to the untreated control group. As demonstrated in Figure 7 a quantitative comparison of each cell phase was performed. The most significant increase in the number of apoptotic cells was observed in response to treatment with dapoxetine (44.2%), followed by fluvoxamine (31.6%).

Effect of SSRIs on Prostate Cancer Cell Cycle. To examine whether SSRIs (dapoxetine, paroxetine, and sertraline) affect the prostate cancer cell cycle, PC3 cells were cultured in a tissue culture plate and left overnight to ensure

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Table 1. Top Ten Positive Compound Connections Predicted from the CMap

Compound	Description	Anticancer Effects
Sertraline	SSRI	Targets important cellular pathways involved in tumorigenesis such as the TNF-MAP4K4-JNK pathway, the antiapoptotic pathway PI3K/Akt/mTOR, and the AMPK/mTOR axis.[26]
Tetrindol	Monoamine oxidase inhibitor	Unclear/Unknown.
Wiskostatin	Neural Wiskott-Aldrich syndrome protein inhibitor	Anticancer effects (breast and ovarian) by modulating via p38 MAPK/JNK signaling pathway.[27]
NTNCB	Neuropeptide receptor antagonist	Reverses the expression of several genes overexpressed in various cancers.[28]
Berbamine	Calmodulin antagonist	Has antitumor and antimetastasis effects, and works as STAT3 inhibitor[29]
Chlorprothixene	Dopamine receptor antagonist	Anticancer and antiapoptotic effects by elevating the expression levels of apoptosis-related genes[30]
Metergoline	Dopamine receptor agonist, serotonin receptor antagonist, prolactin inhibitor	Unclear/Unknown.
H-89	AKT inhibitor, cAMP dependent protein kinase inhibitor, PKA inhibitor, voltage-gated potassium channel blocker	Anticancer effects, stimulates apoptosis by inhibiting the PI3K/Akt pathway.[31]
Nortryptyline	PI3K inhibitor, potassium channel blocker, tricyclic antidepressant	Powerful anticancer effects on colon cancer, melanoma and multiple myeloma, induces oxidative stress[32]
Wortmannin	PI3K inhibitor, ATM kinase inhibitor, ATR kinase inhibitor, DNA dependent protein kinase inhibitor, mTOR inhibitor, PLK inhibitor	Anticancer effects, induce apoptosis, inhibit the PI3K/Akt signal transduction pathway, and generate reactive oxygen species.[33,34]

Table 2. Top Ten Genetic Connections Predicted from the CMap

Gene	Anticancer Effects
NUDT5	Novel drug target and prognostic biomarker for ER-positive breast cancer (2).
EGFR	Known drug target and biomarker for many cancers (3).
CHMP2A	Ablation of CHMP2A can increase NK cell-mediated antitumor activity (4).
AKT1	Known drug target and biomarker for many cancers (5)
PTRF	Contributes significantly to tumor progression and metastasis in prostate cancer cells (6).
AKT3	Known drug target and biomarker for many cancers (5,6).
SMAD4	SMAD4 is a tumor initiator in some tumors, and it seems to be cancer-type dependent, and it is involved in TGF-β pathways (7).
TUBB6	Biomarker of muscle-invasion and poor prognosis in bladder cancer (8).
KIF11	Kinesin Family Member 11 elevated in solid tumors and hematologic malignancies (30)
TERF1	TERF1 downregulation promotes the migration and invasion of the PC3 prostate cancer cell line, and it affects the epithelial-mesenchymal transition (EMT) pathway (9).

adherence. then treated with sub-IC_{50} (0.5 IC_{50}) concentrations of dapoxetine, paroxetine, and sertraline for 48 h, cells were stained afterward with propidium iodide. The DNA quantity was then detected using BD FACSCanto II flow cytometer as illustrated in Figure 8. Treatment of the PC3 cells with sub-IC_{50} concentrations of dapoxetine, paroxetine, and sertraline resulted in cell cycle arrest at the G0/G1 phase. Figure 8 also shows part of the whole view that was used to determine cell cycle components for each sample.

Effect of SSRIs on Multiple Gene Expression of Prostate Cancer Cells. The expression levels of *Bcl-2*, *CASP8*, *DR5*, and *VEGF* were detected by qPCR in the untreated PC3 and DU-145 cells. The results suggest that *Bcl-2* is under-expressed in DU-145, while the expression levels of *CASP8* and *DR-5* were comparable in both cell lines. In addition, *VEGF* is overexpressed in DU-154 as shown in Figure 9.

The effect of SSRIs on PC3 cells expression levels of *Bcl-2*, *CASP8*, *DR5*, and *VEGF* genes was assessed using qPCR after treatment with 0.1 and 0.25 IC_{50} concentrations of dapoxetine, paroxetine, and sertraline for 48 h. Compared to the untreated PC3 cells, the treatment with dapoxetine, paroxetine, and sertraline resulted in a reduction in the expression of *Bcl-2* and *VEGF*. Moreover, the treatment with dapoxetine and paroxetine decreases the expression of *CASP8* and *DR5*. The expression levels of *CASP8* and *DR5* in response to increasing concentrations of sertraline were comparable to their levels in untreated PC3 cells (control) as shown in Figure 10.

DISCUSSION

The utilization of an informatics approach[17−20] that applies gene expression profiles to identify significant biological similarities with SSRIs has yielded significant insights into

Table 3. 50% Inhibitory Concentration (IC_{50}) Values for SSRIs and Chemotherapeutic Agents in PC3 and DU-145 Prostate Cancer Cells at 24, 48, and 72 h of Treatment Duration[a]

Time (hours)	Citalopram	Dapoxetine	Escitalopram	Fluvoxamine	Paroxetine	Sertraline	Cisplatin	5-FU	Raloxifene
				PC3 IC_{50} μM (±SD)					
24	122.50 (±2.89)	72.65 (±3.98)	290.60 (±2.69)	142.40 (±4.38)	27.24 (±0.27)	23.31 (±1.07)	-	-	-
48	102.50 (±0.07)	27.41 (±0.76)	200 (±3.75)	89.08 (±4.30)	15.29 (±1.72)	13.47 (±0.42)	16.57	19.10	20.51
72	98.89 (±0.11)	20.31 (±4.75)	126.30 (±2.83)	88.02 (±0.85)	9.31 (±4.64)	9.39 (±3.54)	-	-	-
				DU-145 IC_{50} μM (±SD)					
24	147.60 (±1.07)	57.88 (±4.4)	337.30 (±1.48)	152.40 (±0.14)	26.20 (±1.93)	24.51 (±2.07)	-	-	-
48	134.80 (±2.52)	43.28 (±1.53)	290.70 (±4.38)	126.50 (±0.85)	23.26 (±0.84)	24.34 (±0.61)	12.63	119.10	60.54
72	104.50 (±3.65)	23.11 (±2.96)	276.10 (±1.98)	119.80 (±0.92)	17.27 (±2.25)	16.31 (±1.73)	-	-	-

[a]μM: micromolar, SD: standard deviation, 5-FU: 5-fluorouracil.

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Table 4. 50% Inhibitory Concentration (IC_{50}) Values for Cisplatin Alone and in Combination with SSRIs, the Fold of Reduction in the IC_{50} of Cisplatin, Combination Ratio Used, and Combination Index (CI) Values in PC3 and DU-145

Drug	Cisplatin alone	Cisplatin: Citalopram	Cisplatin: Dapoxetine	Cisplatin: Escitalopram	Cisplatin: Fluvoxamine	Cisplatin: Paroxetine	Cisplatin: Sertraline
				PC3			
IC_{50} (μM)	16.57	7.92	7.75	8.47	8.59	7.60	7.91
Fold reduction	-	2.09	2.14	1.95	1.93	2.18	2.09
Combination ratio	-	1:6	1:2	1:12	1:5	1:1	1:1
Combination index[a]	-	0.99	1.07	1.06	1.03	0.96	0.98
				DU-145			
IC_{50} (μM)	12.63	4.67	3.53	5.85	6.80	5.18	2.98
Fold reduction	-	2.70	3.58	2.16	1.86	2.44	4.24
Combination ratio	-	1:11	1:4	1:23	1:10	1:2	1:2
Combination index[a]	-	0.75	0.62	0.94	0.98	0.79	0.46

[a]CI < 1, = 1, >1 indicates synergism, additive, and antagonistic effects, respectively. CI was calculated using CompuSyn software

Table 5. 50% Inhibitory Concentration (IC_{50}) Values for Fluorouracil Alone and in Combination with SSRIs, the Fold of Reduction in the IC_{50} of 5-FU Combination Ratio Used, and Combination Index (CI) Values in PC3 and DU-145

Drug	5-FU alone	5-FU: Citalopram	5-FU: Dapoxetine	5-FU: Escitalopram	5-FU: Fluvoxamine	5-FU: Paroxetine	5-FU: Sertraline
				PC3			
IC_{50} (μM)	19.10	9.69	10.35	9.05	9.14	7.72	7.33
Fold reduction	-	1.97	1.81	2.11	2.09	2.47	2.61
Combination ratio	-	1:5	1:1	1:11	1:5	1:1	1:1
Combination index[a]	-	0.98	0.94	0.97	0.99	0.88	0.91
				DU-145			
IC_{50} (μM)	119.10	47.99	35.78	47.59	57.99	38.30	35.53
Fold reduction	-	2.48	3.33	2.50	2.05	3.11	3.35
Combination ratio	-	1:1	3:1	1:2	1:1	5:1	5:1
Combination index[a]	-	0.76	0.57	0.73	0.95	0.64	0.59

[a]CI < 1, = 1, >1 indicates synergism, additive, and antagonistic effects, respectively. CI was calculated using CompuSyn software.

Table 6. 50% Inhibitory Concentration (IC_{50}) Values for Raloxifene Alone and in Combination with SSRIs, the Fold of Reduction in the IC_{50} of Raloxifene, Combination Ratio Used, and Combination Index (CI) Values in PC3 and DU-145

Drug	Raloxifene alone	Raloxifene: Citalopram	Raloxifene: Dapoxetine	Raloxifene: Escitalopram	Raloxifene: Fluvoxamine	Raloxifene: Paroxetine	Raloxifene: Sertraline
				PC3			
IC_{50} (μM)	20.51	10.29	12.5	10.33	11.66	9.24	11.51
Fold reduction	-	1.99	1.64	1.99	1.76	2.22	1.78
Combination ratio	-	1:5	1:1	1:10	1:4	1:1	2:1
Combination index[a]	-	1.01	1.07	1.02	1.09	1.02	0.98
				DU-145			
IC_{50} (μM)	60.54	30.09	11.51	27.12	31.10	27.23	14.94
Fold reduction	-	2.01	5.26	2.23	1.95	2.22	4.05
Combination ratio	-	1:2	1:1	1:5	1:2	3:1	3:1
Combination index[a]	-	0.95	0.45	0.92	0.98	0.79	0.46

[a]CI < 1, = 1, >1 indicates synergism, additive, and antagonistic effects, respectively. CI was calculated using CompuSyn software.

compounds and genetic connections with potential implications in cancer research. The CMap[21] search identified several compounds with strong positive connections to SSRIs, suggesting overlapping systems biology effects or common mechanisms of action.

Notably, sertraline emerged as the top positive connectivity compound, which is expected given that this compound was among the compound list used to derive a query signature for SSRIs. This result indicates that sertraline's gene expression profile captures the essence of the gene expression signature derived for SSRIs. As shown in Table 1, it has documented anticancer effects and affects tumorigenesis pathways (e.g., TNF-MAP4K4-JNK pathway) and antiapoptotic pathways (e.g., PI3K/Akt/mTOR pathway, and the AMPK/mTOR axis). All relevant references supporting the anticancer and cancer pathway results can be found in Table 1. Among the top ten positive compound connections, eight exhibit known anticancer properties according to the biomedical literature

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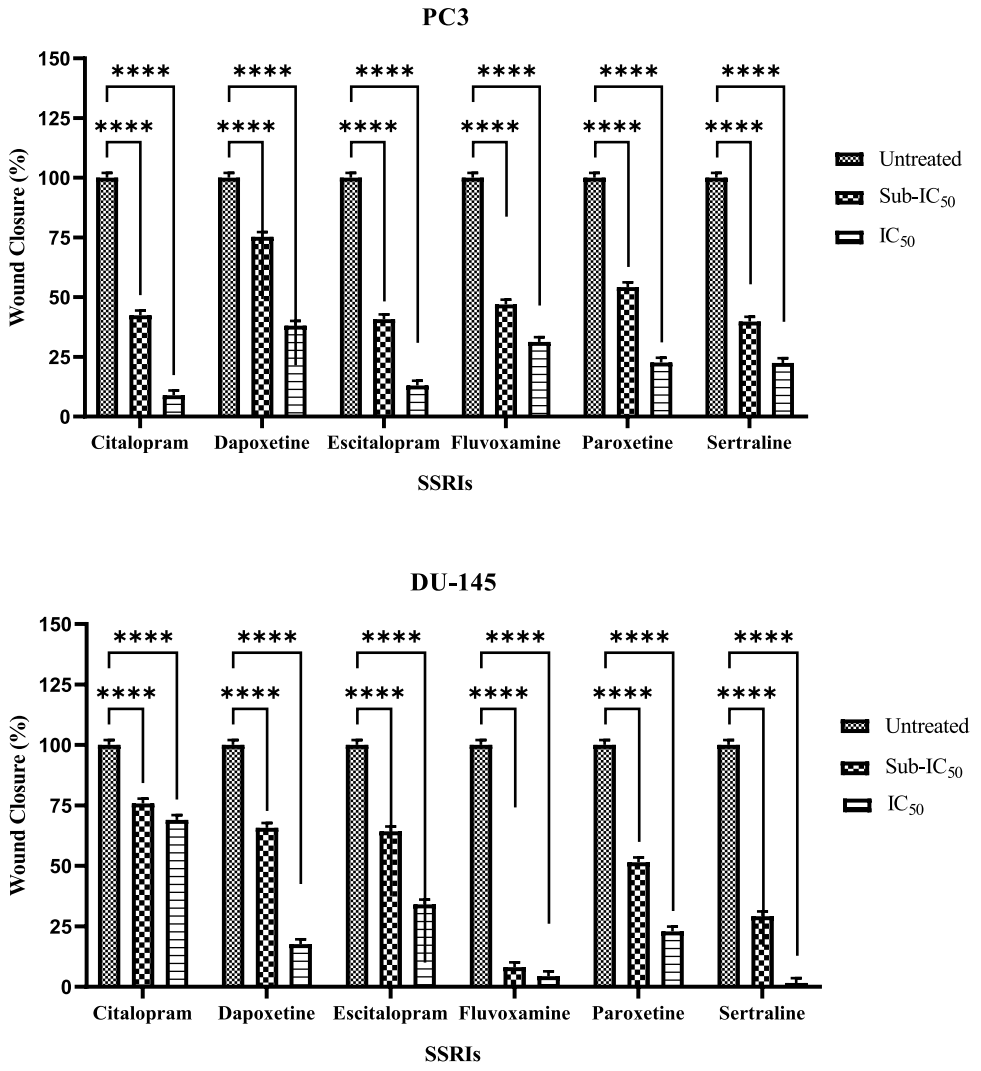

Figure 4. Effect of SSRIs on PC3 and DU-145 cell migration. The data shown represents the percentage of wound closure after 24 h of treatment duration. *P*-value <0.05 indicates statistical significance in comparison to the untreated sample. While asterisk: ns (not-significant) $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ (according to GraphPad prism 9).

and the references cited in Table 1. This finding is particularly interesting as it aligns with the broader understanding of SSRIs beyond their traditional use as antidepressants. Specifically, compounds such as sertraline, H-89, and wortmannin have been shown to affect the PI3K/Akt signaling and apoptosis pathways, which are crucial in cancer progression and treatment. Similarly, sertraline and wiskostatin impact the MAPK signaling pathways, further illustrating their potential relevance in oncology.[35−36373839404142] There is evidence that serotonin activates MAP kinase and PI3K/Akt signaling pathways in prostate cancer cell lines.[43] This makes these results highly valuable to formulate testable computational hypotheses regarding the anticancer effects of SSRIs.

Furthermore, the CMap analysis revealed several genetic connections with high similarity scores to the gene signature of SSRIs, suggesting they might share common pathways with the SSRIs and their associated compounds identified herein and reported in Table 1.

The top ten positive genetic connections in decreasing order include knockdowns of NUDT5, EGFR, CHMP2A, AKT1, PTRF, AKT3, SMAD4, and TERF1, the overexpression of TUBB6 and the knockdown of KIF11 (Table 2). All these genes are well-known for their roles in various cancer-related pathways, reinforcing the potential link between SSRIs and cancer mechanisms. These results agree with the compound connectivity results indicating a role in cancer since most of these genes are involved in cancer pathways.

Particularly, the involvement of AKT1 and AKT3 in the positive genetic connections underscores the significant role of the AKT signaling pathway in this context. The AKT pathway is a well-established player in cancer biology, regulating cell growth, survival, and metabolism. The presence of AKT1 and AKT3 in the list of top genetic connections highlights a potential mechanism through which SSRIs and related compounds might exert their anticancer effects, possibly impacting tumor growth pathways and response/resistance to treatment.

The effects of SSRIs on the viability of prostate cancer cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The present study has shown that all six tested SSRIs (citalopram, dapoxetine, escitalopram, fluvoxamine, paroxetine, and sertraline) have significantly inhibited the growth of PC3 and DU-145 prostate cancer cells, in a concentration and time-

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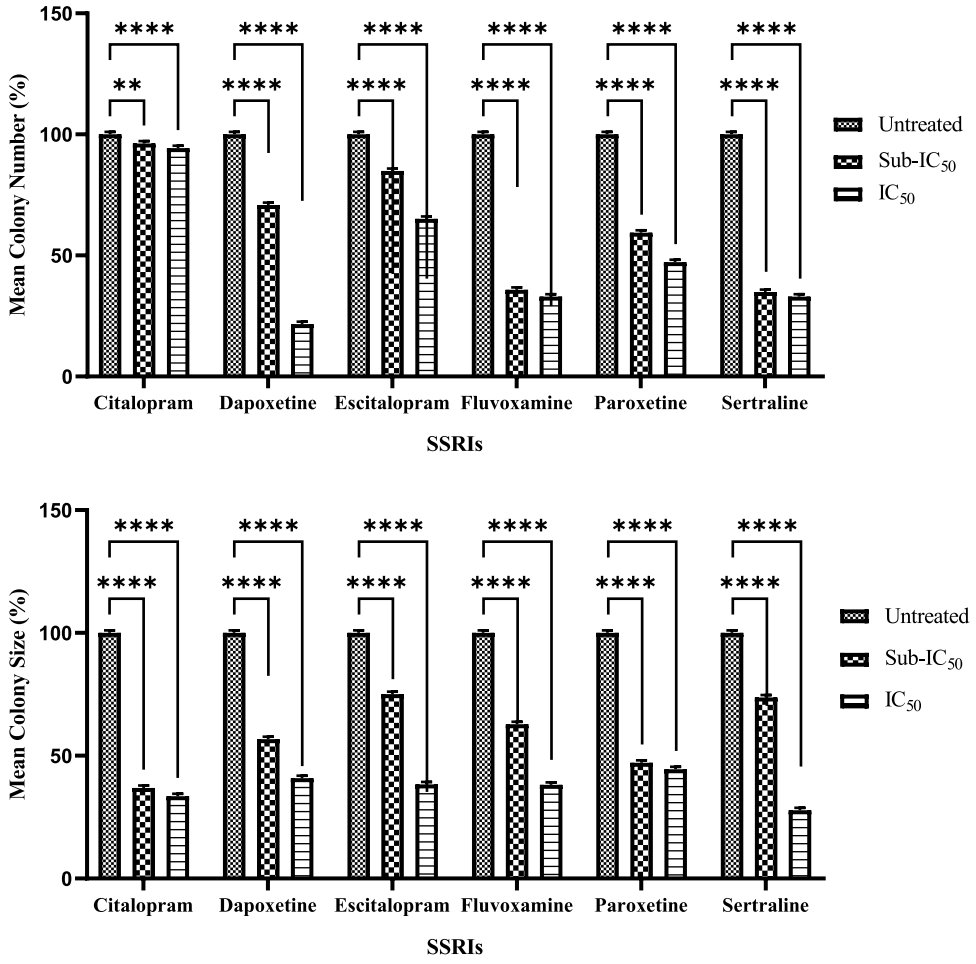

Figure 5. Effect of SSRIs on PC3 mean colony number and size using colony formation assay. The experiment was performed in duplicate and repeated in two independent trials. *P*-value <0.05 indicates statistical significance in comparison to the untreated cells. While asterisk: ns (not-significant) $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ (according to GraphPad prism 9).

dependent manner, compared to the untreated control cells. Dapoxetine, paroxetine, and sertraline showed the most potent effects. These results are compatible with previous studies for different SSRIs on various types of cancer including prostate cancer stem cells, hepatocellular, colorectal, lymphoma, and acute myeloid leukemia cells.[44] The combination effects of SSRI anticancer effects were also assessed with three popular anticancer agents: cisplatin, 5-FU, and raloxifene. Cisplatin is one of the most effective anticancer drugs widely used in the treatment of different solid tumors.[45] Interestingly, our study has shown that dapoxetine, paroxetine, and sertraline have comparable IC_{50} values with cisplatin against the PC3 cell line, while in DU-145 the IC_{50} values of paroxetine and sertraline were comparable to that of cisplatin. 5-Fluorouracil is an antimetabolite drug, that is used alone or in combination with other chemotherapeutic agents for the treatment of different types of cancer.[46,47] The combination of SSRIs with 5-fluorouracil resulted in a synergistic effect with a combination index (CI) < 1 in both PC3 and DU-145 cells. Raloxifene belongs to a group of medications called selective estrogen receptor modulators (SERMs).[48] Raloxifene was reported to have antiproliferative activity against different lung cancer lines.[24] Moreover, raloxifene has been shown to have a cytotoxic effect on prostate cancer cells.[49] In our study, the IC_{50} of raloxifene was comparable to that of SSRIs (dapoxetine, paroxetine, and sertraline) in PC3. In fact, the IC_{50} values of SSRIs (dapoxetine, paroxetine, and sertraline) were superior to those of raloxifene in DU-145. This study revealed synergistic effects for combining SSRIs with either cisplatin, 5-fluorouracil, or raloxifene; reducing the required drug concentrations for producing the desired anticancer effects in prostate cancer cells. Further studies could explore the role of export transporters in the observed synergistic effects. The anticipated effects of SSRIs on PI3K/Akt and MAPK pathways could reduce drug resistance and lead to better prognosis in some cancers (10).

Interestingly, our results demonstrated the ability of SSRIs to significantly inhibit human prostate cancer cells (PC3 and DU-145) migration in a concentration-dependent manner using a two-dimension model (wound healing).[50] Citalopram has been reported to significantly inhibit cell migration in human colorectal cancer cells.[51] In addition, escitalopram has been shown to reduce the cell motility and invasive ability of lung cancer cells, therefore inhibiting lung cell migration.[52] Human tumor cells collaborate to grow autonomously and form colonies in an anchorage-independent manner. So, the colony formation assay is used commonly to investigate the colonization potential of cancer cells.[25] According to our results, a significant reduction in the number and size of colonies of PC3 cells was observed after treatment with sub-IC_{50} and IC_{50} concentrations of SSRIs. To our knowledge, this is the first study to demonstrate the effect of SSRIs on the

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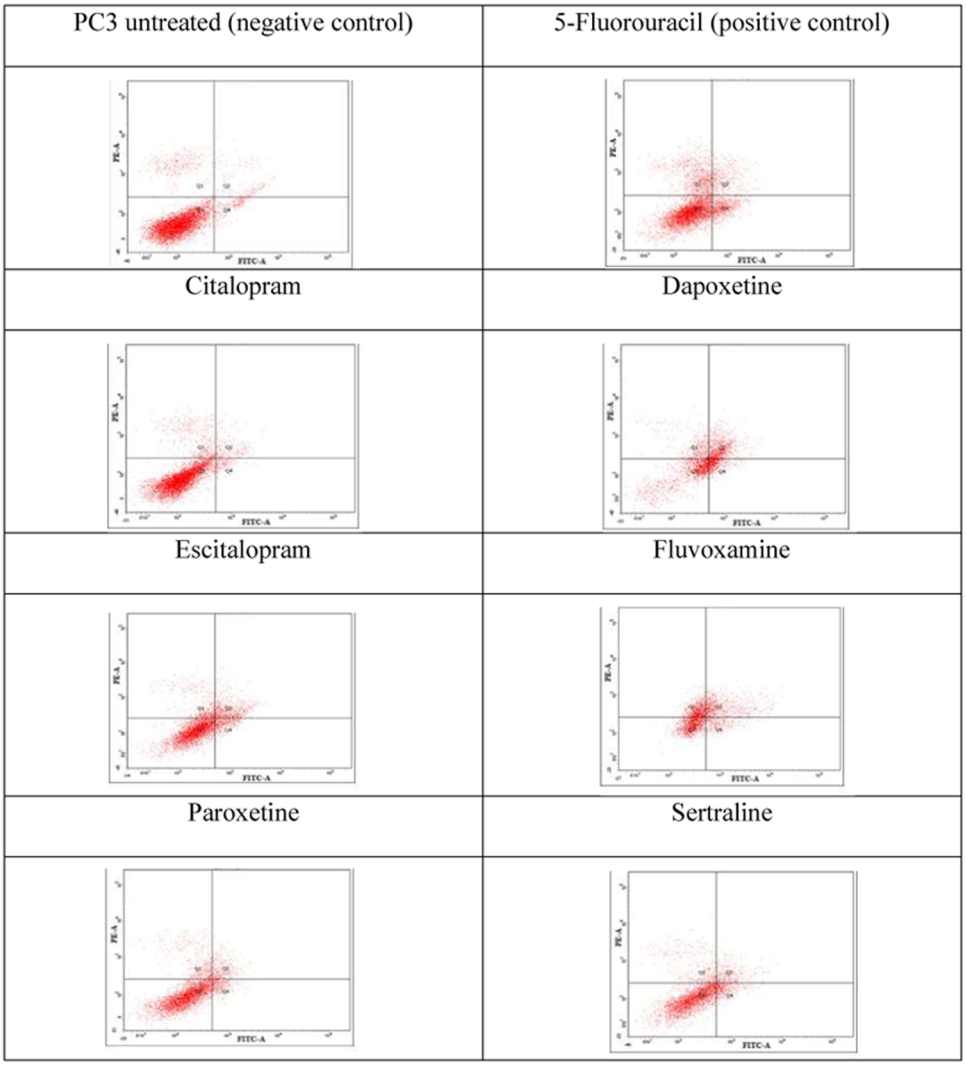

Figure 6. Dot plot for annexin V-FITC/PI staining expresses the effect of SSRIs for 48 h with IC_{50} treatment on apoptosis of PC3. Q3 shows healthy viable cells, Q1 necrotic cells, Q2 late apoptotic, and Q4 early apoptotic cells.

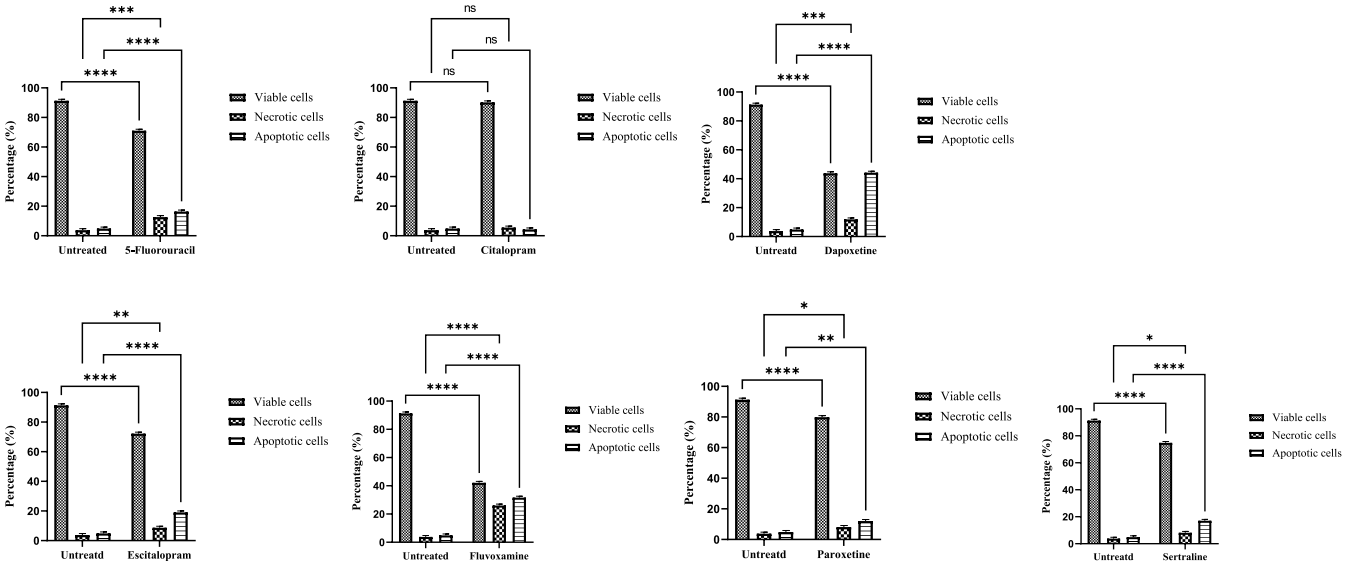

Figure 7. Percentages of healthy, apoptotic, and necrotic cells expressed as mean ± SD expressing the effect of SSRIs for 48 h of treatment on apoptosis of PC3. *P*-value <0.05 express significantly different from respective untreated cells' status; while asterisk: ns (not-significant) $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$ (according to GraphPad prism 9).

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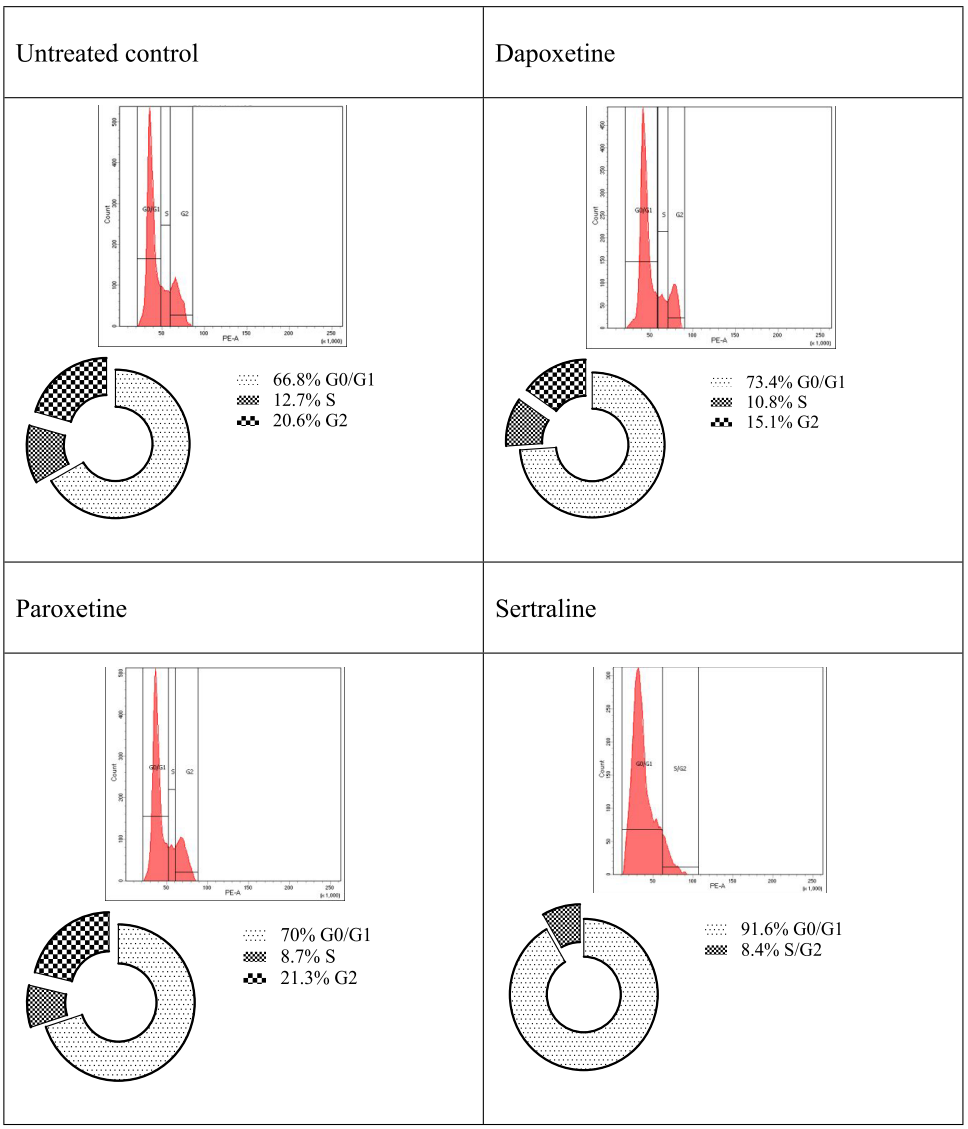

Figure 8. Effect of dapoxetine, paroxetine, and sertraline (sub-IC_{50}) for 48 h of treatment on the PC3 cell cycle. Histogram of DNA content upon PI staining of respective samples showing G0/G1, S, and G2 phases of the cell cycle. Percentages represent DNA content upon PI staining of respective samples showing G0/G1, S, and G2 phases of the cell cycle.

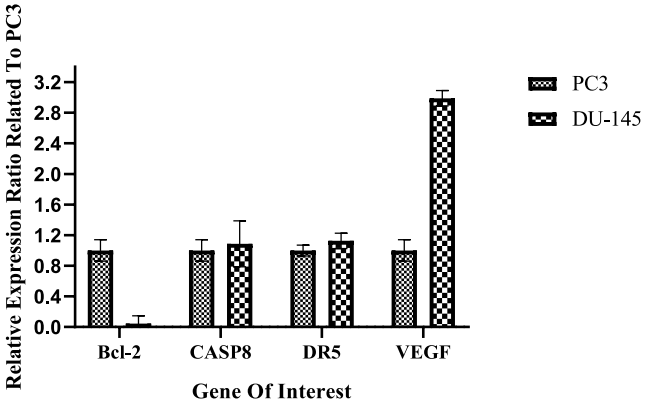

Figure 9. Relative gene expression variations between PC3 and DU-145 prostate cancer cells. Fold difference was measured using the ΔΔCt method. Bcl-2: the gene for B-cell lymphoma, CASP8: the gene for caspase 8, DR5: the gene for death receptor 5, and VEGF: the gene for vascular endothelial growth factor.

colony formation capability of prostate cancer cells. However, paroxetine has been reported to inhibit colony formation in colorectal cancer cells.[53]

Pro-apoptotic chemicals found in mitochondria are crucial for programmed cell death (apoptosis). There are two main mechanisms involved in apoptosis, the death receptor-mediated extrinsic pathway and the mitochondria-mediated intrinsic pathway.[54] The treatment of PC3 cells with SSRIs (except with citalopram) at IC_{50} concentration significantly induced apoptosis compared to the respective untreated cells. Recently Dong et al.[55] reported that citalopram exerts anticancer effects by targeting GLUT1, a major glucose transporter. By binding to GLUT1, citalopram reduces glycolytic flux, effectively reversing the Warburg effect, a metabolic adaptation that fuels cancer cell growth. This metabolic inhibition leads to tumor suppression without directly inducing apoptosis.[55]

The cell cycle is a series of events that takes place inside the cell, where the cell divides into two new daughter cells. It is controlled by a complex set of molecular and biochemical

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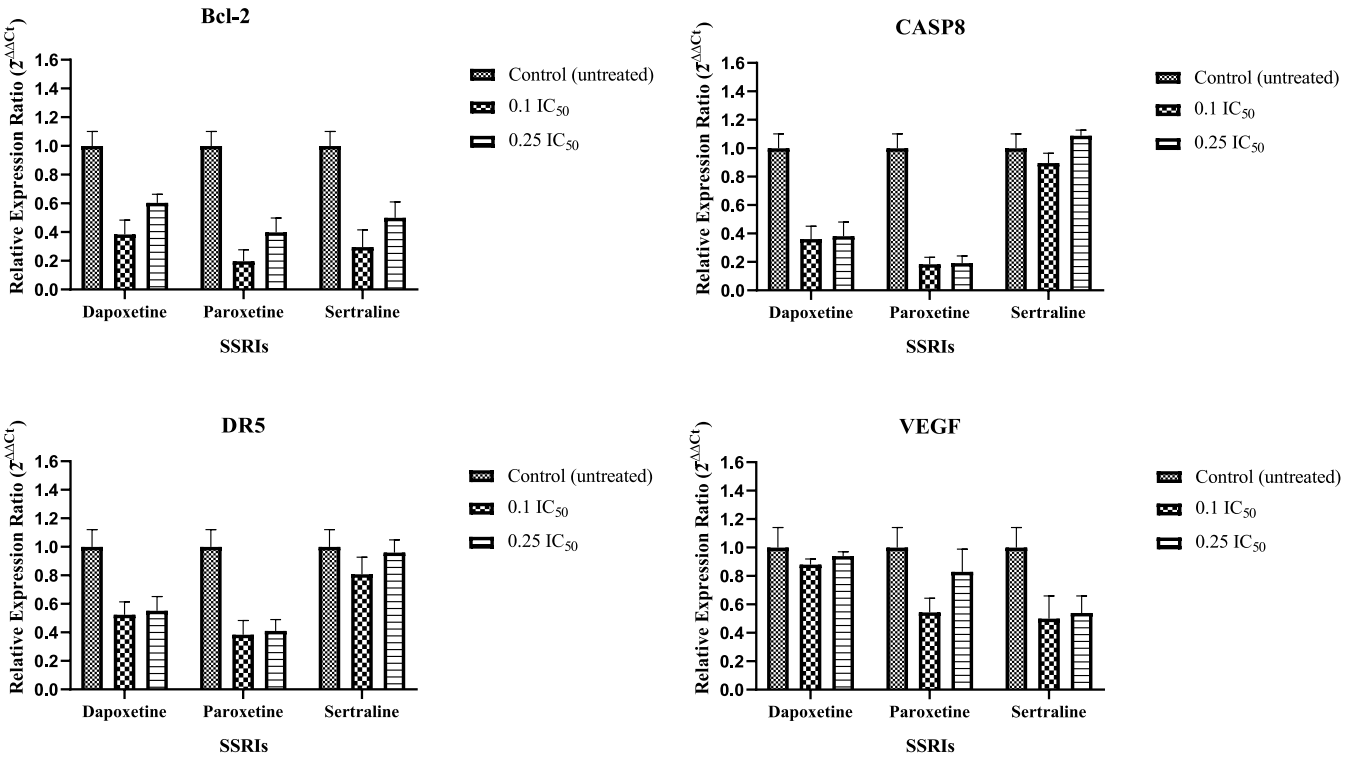

Figure 10. Effect of SSRIs (dapoxetine, paroxetine, and sertraline) on RNA expression of specific genes; Bcl-2, CASP8, DR5, and VEGF in PC3 cells. Fold difference was measured using the ΔΔCt method. Bcl-2: the gene for B-cell lymphoma, CASP8: the gene for caspase 8, DR5: the gene for death receptor 5, and VEGF: the gene for vascular endothelial growth factor.

signaling.[56] Based on the conducted analysis, upon exposure to sub-IC_{50} concentrations of dapoxetine, paroxetine, and sertraline. cell cycle arrest was evident at the G0/G1 phase. Further investigation into the expression of the cell cycle regulatory proteins would be required to ascertain the impact on cell cycle progression. In agreement with our results, Stepulak et al. showed that fluoxetine induced cell cycle arrest at the G1 phase in HT29 colorectal cells.[57]

The intrinsic apoptotic pathway is governed by the Bcl-2 protein family, which consists of pro- and antiapoptotic members.[54] Bcl-2 is an antiapoptotic oncoprotein whose oncogenic effects are mediated by preventing cell death. In many malignancies, elevated Bcl-2 expression contributes to tumorigenicity, poor clinical prognosis, and resistance to chemotherapy and radiation therapy.[58] Interestingly, SSRIs (dapoxetine, paroxetine, and sertraline) decreased *Bcl-2* gene expression. These results suggest that SSRIs induced apoptosis in PC3 cells by modulating the *Bcl-2* expression levels.

On the other hand, the extrinsic apoptotic pathway is activated when a pro-apoptotic ligand, such as a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to specific membrane receptors for apoptosis, like death receptors. The apoptotic process is initiated through the interaction of these death receptors with the Fas-associated death domain (FADD) as well as procaspases 8 and 10, this will lead to the activation of caspases 8 and 10 and eventually cause apoptosis.[59] Our analysis showed that treatment with either dapoxetine or paroxetine decreased the expression of *CASP8* and *DR5*. While treatment with sertraline increased the expression of the *CASP8* and *DR5* genes. This could indicate that sertraline induces apoptosis through the upregulation of DR5 via a TRAIL-independent manner, and activation of the CASP pathway, therefore, triggering extrinsic apoptosis. However, further experimentation should provide insights into other possible crosstalk pathways that could be involved in the underlying mechanism.

Gene expression results further revealed that SSRIs (dapoxetine, paroxetine, and sertraline) inhibited the expression of the vascular endothelial growth factor (VEGF) gene. This is consistent with the observed gene suppression effects of VEGF on the growth and metastatic potential of prostate cancer cells. Tumors often release VEGF to promote new blood vessel formation, particularly in response to hypoxia. These new capillaries not only supply the tumor with oxygen and nutrients but also allow tumor cells to enter the circulatory system, thereby promoting metastasis.[60]

Further studies, including in vivo experiments and xenograft mouse models, should validate the anticancer potential of SSRIs. Additionally, their potential synergy with chemotherapeutics (e.g., 5-fluorouracil, cisplatin) warrants further investigation. These findings may clarify the clinical applicability of SSRIs as adjunctive prostate cancer therapies and aid in optimizing treatment protocols.

CONCLUSIONS

In summary, this study suggests a compelling connection between SSRIs, certain anticancer compounds, genetic perturbations, and biological pathways involved in cancer. The identified involvement of key signaling pathways, such as PI3K/Akt and MAPK, provides a promising avenue for further anticancer drug discovery research. This could lead to new insights into how SSRIs might influence cancer biology and potentially offer novel therapeutic strategies for cancer treatment. Our in vitro study demonstrates proof of concept for the computational predictions regarding the anticancer potential of selective serotonin reuptake inhibitors (SSRIs)

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against prostate cancer. The findings provide compelling evidence that SSRIs can inhibit cancer cell proliferation and migration, while also inducing apoptosis and cell cycle arrest. Notably, dapoxetine and paroxetine were shown to trigger apoptosis through the intrinsic pathway by inhibiting Bcl-2 and downregulating VEGF expression. In contrast, sertraline induced apoptosis via both the extrinsic pathway, by activating DR5/CASP8 signaling, and the intrinsic pathway by inhibiting Bcl-2. Additionally, our study revealed that combining SSRIs with 5-fluorouracil, cisplatin, or raloxifene enhanced anticancer effects, demonstrating either synergistic or additive interactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10939.

Primers' forward and reverse sequences with their optimized annealing temperature (S1 Table); Effect of SSRIs treatment on (A) PC3 and (B) DU-145 cell migration (S1 Figure); Effect of SSRIs on anchorage-independent growth of PC3 using colony formation assay (S2 Figure) (PDF)

List of enriched biological pathways using the 10 top positive gene connections (S2 Table) (XLSX)

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Notes

The authors declare no competing financial interest.

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