Investigation of Risperidone’s anti-tumor activity on MCF-7 breast cancer cells

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Abstract
Aim: Despite increasing survival rates, breast cancer is still the cause of most cancer-related deaths after lung cancer. Risperidone is a nonconventional antipsychotic drug approved by the US Food and Drug Administration in 1994 for clinical use. It is a strong dopamine receptor D2 (DRD2) antagonist. It is also a benzisoxazole derivative with high affinity for serotonin 5-hydroxytryptophan (5-HT2a and 5-HT2c) adrenergic receptors. The stimulation of DRD2 and 5-HT2a receptors has been found to be cancerogenic in several studies. The present study aims to investigate the antitumor activity of risperidone against MCF-7 breast cancer cells, which potentially takes effect via DRD2 and 5-HT2 antagonism.

Material and Methods: A commercially available CCK-8 cell counting assay kit was used to determine the effects of risperidone on MCF-7 proliferation. To evaluate its effect on cell migration, a two-dimensional cell scratch assay was utilized. Finally, single-cell gel electrophoresis (comet assay) was performed to determine DNA damage.

Results: Risperidone significantly suppressed proliferation and migration while triggering DNA damage in MCF-7 cells.

Discussion: The potential of risperidone as an antitumor agent in breast cancer was revealed and encouraging data were obtained for future studies aiming to elucidate its predicted mechanism. This study also provides encouraging preliminary data for the development of new drugs to effectively treat various cancer types via DRD2 and 5-HT2a blockage with minimal side effects.

Keywords
5-HT2a, Breast Cancer, Dopamine, DRD2, Risperidone, Serotonin
Introduction
Currently, 1 out of every 8 women will fight breast cancer at some point in their lives. Although survival has been improved with newly developed treatment strategies and increased awareness of early diagnosis, treatment success has still not reached the desired level. It is clear that we need new treatment strategies.

Studie shows that those exposed to chronic stress are predisposed to develop cancer. A relationship between various neurotransmitters and cancer progression has also been identified [1]. Many studies report that dopamine and serotonin stimulate angiogenesis in cancer [2-9].

Exogenous serotonin has been shown to induce tumor cell proliferation in vitro [10]. It also stimulates tumor angiogenesis via the activation of serotonin receptor 1b and serotonin receptor 2b [2-4]. Serotonin 5-hydroxytryptophan (5-HT2b) was detected at higher rates in the endothelial cells in cases of breast cancer, colon cancer, and pancreatic cancer [11]. It has been determined that ritanserin, a 5-HT2a receptor antagonist, induces apoptosis in colon cancer [12]. The serotonin 5-HT2a receptor has been shown to stimulate TGF-β1 expression through ERK proliferative and fibrotic signals in mesangial cells and has also been shown to increase cell proliferation [13].

Anticancer activity through an antagonistic effect on the dopamine DRD2 receptor has also been revealed by some studies [14, 15]. Increased expression of DRD2 in cancer cells was shown by Diakatou et al. [16]. It was reported that the DRD2 antagonist exerts its antitumor activity by partial activation of the cAMP/PKA pathway [17]. Studies in which thioridazone, a well-known antipsychotic agent, was used as a DRD2 antagonist revealed its proapoptotic, antiangiogenic, and antiproliferative activities against various tumor types [18-20].

Risperidone is a selective monoaminergic antagonist with unique properties. It is a benzisoxazole derivative and a second-generation antipsychotic drug with strong affinity to 5-HT2a, 5-HT2c, D2, and H1 α1 adrenergic receptors [21].

Developing new drugs in cancer research is both costly and time-consuming. However, the design of treatment modalities on the basis of drugs that are already in clinical use can potentially shorten the process and lower the research costs. Considering its antagonistic effect on 5-HT2 and DRD2 receptors, we thought that risperidone would be a good candidate for the exploration of potential anticancer activity against a well-established breast cancer cell line, MCF-7.

Material and Methods
This study was approved by the Scientific Projects Coordinatorship of Uşak University with Decision No: 2018/ARGE002.

Maintenance of cell cultures
The MCF-7 cell line used in our experiments is a cell line originally isolated from the breast tissue of a 69-year-old Caucasian woman (ATCC HTB-22). Cells were grown and passaged in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific), 2 mM L-glutamine (Capricorn Scientific), 100 IU/mL penicillin (Capricorn Scientific), and 100 µg/mL streptomycin (Capricorn Scientific).

Measurement of cell proliferation
For the proliferation experiment, 10,000 cells were seeded per well in 90 µL of medium in a 96-well plate and incubated for 24 hours at 37 °C. By the end of this period, the cells had reached about 75% confluence. At that point, 10 µL of risperidone (Toronto Research Chemicals) solutions prepared by serial dilution in cRPMI were added to final concentrations of 800, 400, 200, 100, 50, 10, 1, 0.1, and 0.01 µM. Only methanol, the solvent of risperidone, was added as a negative control. As a positive control, cisplatin, a well-known chemotherapeutic agent, was added with a final concentration of 30 µM. Considering the possibility of not having an autocrine cycle with dopamine and serotonin synthesis in the MCF-7 cell line, 50 µM dopamine (Toronto Research Chemicals) and 10 µM serotonin (Toronto Research Chemicals) were added to some wells together with risperidone. Controls of only dopamine and only serotonin were used to investigate the risperidone-independent effects of those compounds. Cells were incubated for 12, 24, 48, and 72 hours after risperidone was added.

The Cell Counting Kit-8 (CCK-8, Sigma Aldrich) was used in accordance with the manufacturer’s instructions to metabolically measure cell viability. The CCK-8 kit determines cell viability by calorimetrically measuring the amount of soluble yellow formazin in the medium formed by the dehydrogenase enzyme as a result of metabolic activity in living cells with a principle similar to MTT assays. Briefly, 10 µL of CCK-8 solution was added to the wells and an amount of formazin proportional to the viable cells was measured at 450 nm after 2 hours of incubation at 37 °C using the Multiskan FC Microplate Photometer (Thermo Fischer Scientific). Experiments were repeated three times, each with technical duplicates, and the values were normalized to negative controls.

Measurement of cell migration
We investigated the effect of risperidone on MCF-7 cell migration at IC50/2 (80 µM), IC50 (160 µM), and IC50×2 (320 µM) concentrations by two-dimensional cell scratch assay. When MCF-7 cells reached 100% confluency on a 24-well plate, the cells were scraped with a P200 pipette tip. The scraped cells were then washed out twice with PBS to obtain a smooth, clean cut at the edges. Images were obtained at 0, 24, 48, and 72 hours from three separate points marked with a pen from each scratch using an inverted microscope (Nikon) with a camera attachment. These images were analyzed using opensource ImageJ software and the healing rate of the cuts was determined by normalizing to 0 hours. The assay was performed in triplicate and only methanol was used as a negative control.

Determination of DNA damage
Various concentrations of risperidone (IC50/2, IC50, and IC50×2) were administered to MCF-7 cells in 24-well plates for 72 hours. Cisplatin (5 µM) was used as a positive control. Cells were first washed with PBS and then pelleted by centrifugation at 700×g for 10 minutes at 0 °C. A normal-boiling agarose gel enzyme as a result of metabolic activity in living cells with a principle similar to MTT assays. When MCF-7 cells reached 100% confluency on a 24-well plate, the cells were scraped with a P200 pipette tip. The scraped cells were then washed out twice with PBS to obtain a smooth, clean cut at the edges. Images were obtained at 0, 24, 48, and 72 hours from three separate points marked with a pen from each scratch using an inverted microscope (Nikon) with a camera attachment. These images were analyzed using opensource ImageJ software and the healing rate of the cuts was determined by normalizing to 0 hours. The assay was performed in triplicate and only methanol was used as a negative control.

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agarse, the slides were placed in an ice-cold lysis buffer (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept at 4 °C for 1 hour in the dark. Before the electrophoresis process, the slides were held in an alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na2EDTA, pH 13) for 20 minutes at 4 °C to unwind the double-stranded DNA. Electrophoresis was performed in the same buffer for 20 minutes in the dark at 4 °C at 25 V (0.96 V/cm; approximately 250 mA). After electrophoresis, slides were washed 3 times for 5 minutes with 5 mL of 0.4 M Tris HCl (pH 7.5) to neutralize the alkaline buffer. Slides were then stained with 60 µL of ethidium bromide (20 µg/mL) and evaluated within 4 hours.

DNA images were captured with a fluorescence microscope (Nikon). The evaluation of images was performed semi-quantitatively in the form of visual scoring. Undamaged cells were detected by the observation of a bright center with less intense edges (no migration). In the event of DNA damage, an irregularly edged appearance is observed depending on the number of fragments or chain breaks and the level of alkali-labile regions. Depending on the severity of the damage, there is an extension from the center to the edge. This appearance is called stretch or low migration. As the damage increases, the cells will take the form of a comet (high migration). The final stage is apoptosis. DNA images were scored subjectively from 0 (no damage) to 4 (high damage) according to the degree of damage observed. The experiment was performed in triplicate and Duncan’s test was used for statistical analysis.

Results

Antiproliferative effect of risperidone on MCF-7 cells

The CCK-8 proliferation assay revealed that risperidone suppresses proliferation of MCF-7 cells in a dose-dependent manner without being affected by external serotonin and dopamine supplementation (Figure 1), suggesting that an autocrine cycle was most likely established by the synthesis of dopamine and serotonin by the MCF-7 cells. Hence, dopamine and serotonin were not added exogenously in the following migration and genotoxicity experiments.

It was observed that the proliferation of MCF-7 cells was suppressed at rates of up to 100% after 72 hours in the presence of risperidone above 400 µM, which was equivalent to the effect of cisplatin at 30 µM. A similar antiproliferative effect was observed after 48 hours, but not after 12 or 24 hours (data not shown), implying that risperidone requires about 48 hours to show its cytotoxic effects.

The IC50 value of risperidone, or the concentration at which 50% of cells lose their viability, was estimated to be about 160 µM.

Table 1. Dose-dependent toxicity of risperidone on MCF-7 cells

<table>
<thead>
<tr>
<th>Application</th>
<th>Concentration (µM)</th>
<th>DNA damage (arbitrary units) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (negative control)</td>
<td>-</td>
<td>3.67±0.58</td>
</tr>
<tr>
<td>Cisplatin (positive control)</td>
<td>5</td>
<td>5±1.361*</td>
</tr>
<tr>
<td>Risperidone</td>
<td>80</td>
<td>5±1*</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>9.67±1.53*</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>13.67±0.58*</td>
</tr>
</tbody>
</table>

*: p<0.05; SD: standard deviation.
Risperidone’s anti-tumor activity

μM based on the trendline equation of the viability curve. The proliferation of MCF-7 cells was not affected by the addition of either dopamine or serotonin in the absence of risperidone, suggesting that the observed antiproliferative effect arose solely from risperidone.

**Inhibitory effect of risperidone on MCF-7 cell migration**

The effects of risperidone on the migration of MCF-7 cells after 24, 48, and 72 hours at IC50/2, IC50, and IC50×2 doses were examined by cell scratch assay. Risperidone was shown to inhibit migration at increased concentrations (Figure 1). Comparisons made with the control group containing only a solvent (methanol) showed that this difference was significant for IC50×2 (320 μM) at 48 and 72 hours (p<0.023 and p<0.008, respectively) and for IC50 at 72 hours only (p<0.039). The gap closure or migration percentage decreased from about 70% to 30% compared to the control group at the end of 72 hours in the presence of 320 μM risperidone (Figure 2).

**Genotoxic effect of risperidone on MCF-7 cells**

It was observed that risperidone at IC50/2 (80 μM), IC50 (160 μM), and IC50×2 (320 μM) concentrations caused DNA damage in MCF-7 cells in proportion to the applied concentration (Table 1). The effect was not as high as that of cisplatin at 5 μM (51±3.61 AU), but it was significant at all concentrations (5±1, 9.67±1.53, and 13.67±0.58 AU for increasing concentrations).

**Discussion**

Studies have shown that chronic stress may increase the predisposition to developing cancer, which is supported by the known associations of various neurotransmitters with cancer progression [1]. Drugs that cause DRD2 antagonism have been shown to have therapeutic effects for various cancer types. Antipsychotic agents that work as DRD2 antagonists, such as sulphuride, dexamethasone, and thioridazine, have been shown to have antitumor activities against breast cancer [23, 24]. In another study, tropisetron and ketanserin were found to suppress proliferation of the MCF-7 breast cancer cell line through 5-HT2A and 5-HT3 antagonism [25]. In the present study, risperidone, which is accepted as an antipsychotic drug in the general literature, was considered for use as an antitumor agent against breast cancer due to its well-known DRD2 and 5-HT2A antagonistic effects. Its antitumor effects on MCF-7 breast cancer cells were demonstrated in vitro by revealing its antiproliferative and genotoxic as well as migration-suppressing properties against MCF-7 cells.

Any contribution to revealing the mechanisms behind the antitumor activity of risperidone is crucial to enable more effective drug design for tumor growth intervention studies. Therefore, in future studies, the mechanistic background of risperidone’s antitumor activity needs to be elucidated by studying its effects on DRD2- and 5-HT2A-associated ERK and Akt signaling pathways. Once we have a better understanding of those underlying mechanisms, any drug currently in use or in the process of being developed that takes effect via DRD2 and/or 5-HT2A antagonism may be considered for use in potential antitumor applications. This study has provided encouraging preliminary data for the development of new drugs to effectively treat various cancer types via DRD2 and 5-HT2A blockage with minimal side effects.

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**Scientific Responsibility Statement**

The authors declare that they are responsible for the article’s scientific content including study design, data collection, analysis, and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

**Animal and human rights statement**

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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**Conflict of interest**

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**References**

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