Upadacitinib and PD29 have anti-inflammatory and anti-oxidant effects on MCF-7 cells

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Abstract

Aim: Breast cancer is the most diagnosed cancer in women’s lives with a ratio of 1/8 [1]. For breast cancer huge efforts were made to develop targeted therapies to treat this cancer pathogenesis [2]. In breast cancer pathogenesis, many pathways are involved [3], such as Wnt signaling [4], PI3K/AKT/mTOR [5], Notch signaling [6], BRK pathway [7], and other cellular signal pathways. In the literature lots of evidence showed that janus kinase (jak) signal transducer and activator of transcription (stat) pathway is also known to be involved and play a pathogenesis for breast cancer. In breast cancer pathogenesis, phosphorylated Stat3 can be visualized in approximately 40% histologically [8]. In cellular metabolism, Jak-Stat signaling pathway is responsible cytokine signaling and its regulation [9].

Materials and Methods: Experiments were performed to examine the anti-proliferative, anti-oxidative and anti-inflammatory effects of Upadacitinib and PD29. Jak-1, jak-2, jak-3, and IL-6 mRNA levels were determined by the q-RTPCR method; protein of IL-6 level was observed with ELISA. In addition, total oxidant and total antioxidant status were determined by the spectrophotometric method.

Results: Compared to the control just Upadacitinib decreased cell proliferation. Upadacitinib and PD29 decreased Jak-1, Jak-2, Jak-3, and IL-6 mRNA levels significantly. Compared to the control, just Upadacitinib and Upadacitinib + PD29 decreased IL-6 protein levels significantly. There is no significance between the control and PD29 for IL-6 protein levels. In Upadacitinib group, TOS level was lower compared to control, significantly, PD29, and Upadacitinib + PD29 compared to the control. TAS level was higher in in Upadacitinib and Upadacitinib + PD29 group compared to the control. There is no significance in PD29 (0.920 ± 0.055) group for TOS levels.

Conclusion: Upadacitinib and PD29 have potential targeted therapy for anti-oxidant and anti-inflammatory effects on breast cancer. In this context, it can be said that Upadacitinib and PD29 may have effective for breast cancer.

Introduction

Breast cancer is the most diagnosed cancer in women’s lives with a ratio of 1/8 [1]. For breast cancer huge efforts were made to develop targeted therapies to treat this cancer pathogenesis [2]. In breast cancer pathogenesis, many pathways are involved [3], such as Wnt signaling [4], PI3K/AKT/mTOR [5], Notch signaling [6], BRK pathway [7], and other cellular signal pathways. In the literature lots of evidence showed that janus kinase (jak) signal transducer and activator of transcription (stat) pathway is also known to be involved and play a pathogenesis for breast cancer. In breast cancer pathogenesis, phosphorylated Stat3 can be visualized in approximately 40% histologically [8]. In cellular metabolism, Jak-Stat signaling pathway is responsible cytokine signaling and its regulation [9].

The most basic of the jak-stat family, the jak family is quite large and is commonly referred to as non-receptor tyrosine kinases [10, 11]. This family has important duties especially in autoimmune diseases and most cancer types. [10, 11]. Janus kinase-1 (Jak-1) is a member of the Janus kinase large family and is specifically involved in inflammation. In particular, Jak-1 is required for IL-6 class inflammatory cytokine signaling, plays a critical role in cancer metabolism, particularly in metastatic cancer progression. It has been shown to mediate persistent oncogenic activation of Stat-3 in breast cancer cells [3]. It was found to be more tumorigenic than wild-type cells in the absence or inhibition of Jak-1 in cell lines [12]. Also, Jak-1 works as an oncogene or tumor suppressor under certain conditions or cell contents [13]. Recent studies have shown that Jak-Stat inhibition plays a role in the tumor microenvironment to increase the production of pro-tumorigenic inflammatory factors that increase therapeutic resistance in breast cancer patients [14, 15].
Jak inhibitors (Jakinibs) play a role in competitive ATP binding and block the phosphorylation of cytokine receptors. In metabolism, it thus leads to decreased cytokine production and impaired differentiation of T-helper 1, 2, and 17 cells [16]. All jak inhibitors separated into two main groups: First one is first-generation Jak inhibitors (peficitinib, baricitinib, ruxolitinib, tofacitinib) and second-generation selective Jak inhibitors (decernotinib, filgotinib) has been developed especially for the treatment of autoimmune diseases and some malignant diseases [17]. Upadacitinib is the new third selective Jak-1 inhibitor approved for rheumatoid arthritis by FDA (August 2019) and EMA (December 2019) [18].

In one study, the PD29 peptide, with its 29 amino acid sequence, was designed to target pulmonary fibrosis (PF) [19]. It has been shown that PD29 is responsible for, matrix metalloproteinase (MMPs) inhibition, anti-angiogenesis and inhibition of integrins in PF [19]. Again, same study, it is suggested that PD29 can treat PF by partially regulating the expression of TGF-ß1, Smad3 and Smad7 [19].

Another study demonstrated the effect of the Jak-Stat signaling pathway in MCF-7-cell lines and declared that treatment with 5-FU via the Jak-Stat pathway is more beneficial than gemcitabine [20]. Based on this information, we think that possible Jak-Stat treatments may be beneficial AZD1480 is a potential Jak-2 inhibitor that can block the persistent activity of Stat-3, thereby arresting tumorigenesis in solid tumor xenografts [21].

Peptides can offer the versatility needed for a successful oncology drug discovery approach. Peptide-drug conjugates (PDCs) are an emerging targeted therapeutic offering increased tumor penetration and selectivity. Despite these advantages, there are still limitations to the therapeutic use of peptides, exemplified by their slow progression into the clinic and limited oral bioavailability. New approaches to these problems have been studied to improve the stability of peptides and their structures. There are two molecules currently available on the market and they are bicycle-toxin conjugates and peptide-dendrimer conjugates.

The aim of this study is to investigate the possible effects of PD29, a jack inhibitor and a peptide. This study was aimed to investigate the effects of selective Jak-1 inhibitors such as upadacitinib (Upa) and PD-29 on proliferation, inflammation, and oxidative stress effects in MCF-7 breast cancer cells.

Materials and Methods

Experiments of MCF-7 cell line

MCF-7 breast cancer cell line was got from Republic of Türkiye Ministry of Agriculture and Forestry Sap Institute (Ankara, Türkiye). T25 flasks (1×10⁶ cells per flask) in RPMI-1640 (ThermoFisher, USA) with fetal bovine serum (FBS) 10% (ThermoFisher, USA), 2 mM L-glutamine, and 1% penicillin-streptomycin antibiotic (ThermoFisher, USA) were used for MCF-7 cell culture conditions. Cells incubated at 37 °C, 5% CO₂ in atmosphere in cell culture cabinet. The cell culture medium renewal has to be performed two times per week, while cells should weekly be passaged at a sub-cultivation ratio of 1:3 [22]. After the second passage, MCF-7 cells were seeded in six well plates with an average of 1 × 10⁵ cells added in each well. An average of 1 × 10⁴ cells seeded in 96 well plates for the detection of IC50 dose of PD29 and Upadacitinib with the cell proliferation assay. The seed well plates were allowed to incubate at 37 °C in a CO₂ cabinet for 24 hour in RPMI-1640 cell culture medium mix. The final concentration are 1Mol PD29 and 1Mol Upadacitinib were added MCF-7 cell culture experiments.

Cell proliferation assay

Cell proliferation tests were performed to observe the effect 1Mol PD29 and 1Mol Upadacitinib in MCF-7 cells. For IC50 and proliferation the cell-titer aqueous one solution assay (Promega, USA) was used according to the manufacturer’s instructions for MCF-7 cell proliferation assays. In 96 wells, 5 × 10⁴ cells were plated into each well and 10 µL per well of CellTiter 96 aqueous One Solution reagent was added. After this step 1h incubation in humidified 5% CO₂ atmosphere. Results were triplicate-measured by Thermo Multiscan Elisa Reader (ThermoFisher, USA ) absorbance at 490nm every 24 hour.

Hoechst 33342 Staining

MCF-7 cells were labeled with Hoechst 33342 (Cell Signaling Technology, Inc., USA), as described previously [23]. The images were taken by Zeiss Axioscope microscope.

Quantitative real time polymerase chain reaction (q-RTPCR) experiments

To quantify level of mRNA q-RTPCR (Applied Biosystems, USA) methods was used. q-RTPCR experiments conditions were respectively; 1 min at 60 °C, 10 min at 95 °C, 15 sec at 95 °C, and 1 min at 60 °C for 40 cycles for each step). Total RNA was extracted from the MCF-7 cells using RNase Kit (Qiagen, USA). A total of 50 ng RNA samples were reverse-transcribed using a quantitect reverse transcription kit (Qiagen, USA) to obtain cDNA. Jak-1, Jak-2, Jak-3, IL-6 and β-actin primers used. Used primer sequences (Oligomer Biotechnology, Turkey) showed in Table 1. As a result of the qPCR experiment, β-actin was used as an endogenous control to normalize the targeted gene expressions. Fold values were calculated using the 2-∆∆Ct method [24].

ELISA experiments

Enzyme Linked Immuno Sorbent Assay (ELISA) kit used to measure IL-6 levels from MCF-7 cells. IL-6 ELISA Enzyme Linked Immuno Sorbent Assay (ELISA) kit used according to the user manual instructions. All samples were run in triplicate during the experiment. The 96-well plate is pre-coated with a human IL-6 antibody. 96 IL-6 coated wells were used for samples. After, the well plate incubated 370C. Three washing steps were done. The end of the experiment the absorbance was measured at 450 nm. The Standart curve was used for calculation as pg/mL.
**Figure 1.** Cell Viability (% of control) A. Hoechst 33342 Staining B. Cell proliferation experiments result, Upadacitinib decreased MCF-7 cells proliferation (*p<0.05).

**Determination of oxidative stress parameters**

Evaluation of total oxidant levels and total antioxidant levels colorimetric kits (Rel Assay, Turkey) was used. Hydrogen peroxide (H$_2$O$_2$) was used for the calibration curve. Ant he calibration curve was used TOS assay. Results were expressed as µmol H$_2$O$_2$ Equivalent/L [24]. Trolox equivalent, a vitamin E analogue, was used as a standard for measurement of serum TAS levels and results was showed as mmol Trolox Equivalent/L [24].

**Statistical analysis**

Graphpad Prism 9 (IBM, USA) program was used for the experimental findings. The Kruskal-Wallis test was used to examine the difference between the means between groups, and the Mann-Whitney U test was used to examine whether two samples came from the same distribution. A value of <0.05 for all p values was considered statistically significant. Data was given as mean ± standard deviation (SD).

**Table 1. Human qPCR primers.**

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Human Jak-1</td>
<td>GAGACAGGTCTCCCACAAACAC</td>
<td>GTGTAAGGACATCGCTTTTCG</td>
</tr>
<tr>
<td>Human Jak-2</td>
<td>CCAGATGGAACACTTGTCGCTCAG</td>
<td>GAGGTGGTACATCGAAGAACC</td>
</tr>
<tr>
<td>Human Jak-3</td>
<td>AGTGACCCCTCCTTCTGCTGT</td>
<td>GCTGAAACCAAGGATGATGC</td>
</tr>
<tr>
<td>Human IL-6</td>
<td>AGACACCCACACTCACCTCCTTCAG</td>
<td>TTCTGCGATGTCCTTGGTCT</td>
</tr>
</tbody>
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**Figure 2.** Jak-1, Jak-2, Jak-3, IL-6 q-RTPCR experiments. Upadacitinib and PD29 decreased Jak-1, Jak-2, IL-6 gene expression in MCF-7 cell. Just Upadacitinib decreased Jak-3 gene expression in MCF-7 cell (*,**,**,** p < 0.05).

**Figure 3.** IL-6 protein expression decreased in Upadacitinib and PD29 groups (*,**,**,** p < 0.05).

**Results**

**Cell Proliferation and Hoechst 33342 Staining**

Hoechst 33342 Staining showed that Figure 1A. Compared to control (100 ± 0.00), Upadacitinib (85.8 ± 1.89) decreased the cell proliferation significantly (p < 0.05) but there is no differences between control and PD29 (91.7 ± 5.03) and Upa + PD29 (84.0 ± 1.00) (Figure 1B).
For Jak-1, compared to control (0.99 ± 0.09), Upadacitinib (0.720 ± 0.026), PD29 (0.750 ± 0.01), Upadacitinib + PD29 (0.69 ± 0.01) decreased significantly (*** p < 0.05) (Figure 2A). For Jak-2, compared to control (0.995 ± 0.01), Upadacitinib (0.840 ± 0.06), PD29 (0.890 ± 0.05), Upadacitinib + PD29 (0.803 ± 0.08) decreased significantly (*** p < 0.05) (Figure 2B). For Jak-3, compared to control (1.003 ± 0.01), just Upadacitinib (0.533 ± 0.00) decreased significantly (*, p < 0.05). There is no significance between control and PD29 (0.975 ± 0.01), Upadacitinib + PD29 (0.807 ± 0.06) (Figure 2C). For IL-6, compared to control (1.000 ± 0.00), just Upadacitinib (0.820 ± 0.04) and Upadacitinib + PD29 (0.752 ± 0.04) decreased significantly (*** p < 0.05). There is no significance between control and PD29 (0.967 ± 0.03), (Figure 2D).

**Discussion**

In women breast cancer is serious health problem their lifetime. About one in eight women are at risk for breast cancer [1]. Breast cancer five year survival rate has gradually increased to 91% due to advances in early diagnosis and new treatment methods [1]. However, breast cancer has a high recurrence rate and metastases are connected to more than 90% of breast cancer-related deaths [1]. New generation drug results are required for breast cancer treatments. Recently, FDA approved upadaticinib is Jak-1 selective inhibitor for rheumatoid arthritis treatment in August 2019. It reduces inflammation as it lowers IL-6 and TNF levels [18]. In this study, we found that Upadacitinib and PD29 reduced inflammation and oxidative stress in MCF-7 breast cancer cells. Similarly, in most clinical studies, the tested Jak were found to be safe and well tolerated. Ruxolitinib is the only accepted inhibitor to date to show a response in early-stage trials. One study showed that high CRP, ruxolitinib combined with capcitabine has been associated with improved health-related quality of life in breast cancer [25]. In addition, much data support the role of the Jak2/Stat3/IL-6 signaling pathway in breast cancer [26].

In the literature, researchers have shown that the Jak/stat signaling pathway is the responsible and widely activated pathway in breast cancer pathogenesis and that Jak signaling is involved in the reproduction, initiation, development and migration of cancer cells [27]. Here, we report a study supporting Upadacitinib as Jak-1 selective inhibitor and PD29 decreased the Jak-1, Jak-2 and Jak-3 and IL-6 mRNA levels.

In addition, researchers have shown that reactive oxygen species induction and oxidative stress play a role in the pathogenesis of breast cancer as a result of the impaired balance between anti-oxidants and pro-oxidants [28]. Breast cancer has a higher levels of ROS compared to wild-type or control cells [28]. Similar to these results, we showed that breast cells have higher oxidative stress, furthermore, upadacitinib and PD29 have antioxidant effects on MCF-7 breast cancer cells.

**Conclusion**

In conclusion, the Jak-Stat3 pathway remains a key target site in cancer pathogenesis, especially against breast cancer. Upadacitinib and PD29 have potential targeted...
therapy for antioxidant and anti-inflammatory effects on breast cancer.

Conflict of interest and financial support statement
There is no conflict of interest between the authors and no financial support had been received for this research.

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Ethical approval
Ethical approval was not required as it was a cell culture study.

References