Research on Mechanism of FGFR1 Inhibitor BAY1163877 against Proliferation of Breast Cancer Cells

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Abstract. To investigate the effect and mechanism of fibroblast growth factor receptor 1 (FGFR1) inhibitor BAY1163877 on proliferation and apoptosis of breast cancer cells. The expression of FGFR1 in human breast cell lines was detected by qRT-PCR and western blot. IC50 of BAY1163877 and cell viability were measured by CCK-8 method. Cell proliferation was observed by colony assay. Cell apoptosis after treatment of BAY1163877 was tested by flow cytometry. The expressions of p-FGFR1/FGFR1 and p-STAT3/STAT3 protein were detected by Western blot. According to the results of qRT-PCR and Western blot, FGFR1 high expression of breast cancer cell line MDA-MB-231 and FGFR1 low expression of breast cancer cell line MCF-7 were selected. BAY1163877 inhibits proliferation of MDA-MB-231 and MCF-7 cells and induces apoptosis of MDA-MB-231 and MCF-7 cells. The results of Western blot showed that the expression of p-FGFR1 and p-STAT3 protein in MDA-MB-231 was reduced after BAY1163877 treatment and the expression in MCF-7 was not significantly changed. BAY1163877 inhibits the proliferation and induces the apoptosis of high FGFR1 expression breast cancer cell line MDA-MB-231, and its mechanism may be related to the decrease of p-FGFR1 and p-STAT3 protein expression. BAY1163877 inhibits the proliferation and induces the apoptosis of MCF-7, but its mechanism still needs the further study.

1. Introduction

Receptor tyrosine kinase (RTK) is a series of important regulators involved in cellular physiological and biochemical processes, including apoptosis, proliferation, migration and angiogenesis.[1] Fibroblast growth factor receptor (FGFR) is a sub-family of receptor tyrosine kinase, combined with fibroblast growth factor (FGF) and then receptor homodimerization occurs, resulting in intracellular structure phosphorylation, which causes cascade and gene transcription of intracellular signals.[2] Fibroblast growth factor (FGF) is a key regulator of key cellular processes, and its deregulation may lead to the characteristics of cancer such as excessive proliferation, evasion of apoptosis, invasive behavior, and abnormal angiogenesis, so the occurrence of tumors may be related to abnormal activation of FGFR. Selective inactivation of FGFR is an important strategy for cancer treatment.[3]

FGFR1-4 are members of the FGFRs family, and they transmit signals, regulate cell growth, angiogenesis, immunity, and metabolism by binding to ligand FGFs.[4] Janus-activated kinase-signal
transducer and activator of transcriptions (JAK/STAT) pathway is a newly discovered signal transduction pathway in recent years. It is the intracellular signal transduction pathway of interferon, which is widely involved in cell proliferation, differentiation, apoptosis and immunomodulatory processes.[5] FGFR1 combined with ligands can be transmitted through multiple pathways such as RAS/RAF/MAPK and JAK/STAT to participate in biological activities such as cell proliferation and apoptosis. Transcription activator 3 (STAT3) is a key molecule in the JAK/STAT pathway. Signal transduction of FGFR1 and its downstream signaling pathways and abnormal activation of STAT3 drive cell proliferation, survival and invasion.[6-8] Experimental study on lung cancer cells suggests a decrease in the expression level of p-STAT3 in BAY1163877 sensitive lung cancer cell lines.[9] p-STAT3/STAT3 may be associated with BAY1163877 anti-cancer effects. In recent years, a variety of FGFR inhibitors have been approved by the FDA for clinical anti-cancer treatment, but so far no drugs have been approved for breast cancer treatment. FGFR targeted therapy is considered a promising breast cancer treatment strategy, especially for luminal type and three-negative breast cancer, but the current clinical trial results of FGFR targeted drugs are unsatisfactory.[10] Therefore, it is necessary to explore new targeted drugs for breast cancer. BAY1163877 is a selective and highly FGFR-1,2,3 inhibitor, and preclinical experiments in vitro have shown that BAY1163877 inhibits phosphorylation of downstream signaling molecules and proliferation of various cancer cell lines.[11] The BAY1163877 Dose Increment Experiment (NCT01976741) for lung cancer, bladder cancer and head and neck cancer showed that: FGFR positive patients with urinary tract skin cancer had the best reaction to BAY1163877.[12] There are no reports of BAY1163877 treatment of breast cancer. In this experiment, the effects and mechanism of BAY1163877 on the proliferation and apoptosis of breast cancer cells were preliminarily explored, which provided new ideas for targeted treatment of breast cancer.

2. Materials and Methods

2.1. Materials.
MCF-10A, MCF-7, MDA-MB-231, ZR-75, SK-BR-3, MDA-MB-453(ATCC), RPMI1640, DMEM, DMEM/F-12, α-MEM (HyClone Ltd.), BAY1163877 (MCE), DMSO (Sigma Aldrich, St. Louis, MO, USA) Reverse transcription Kit (Takara Ltd.), SYBR® Premix DimerEraser ™ (Takara Ltd.), Cell Counting Kit-8 (CCK-8) (bimake.cn, Houston, USA), Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology Ltd.), Anti-FGFR1 antibodies, Anti-p-FGFR1 antibodies, Anti-STAT3 antibodies, Anti-p-STAT3 antibodies, Anti-β-Actin antibodies (Cell Signaling, Danvers, MA, USA).

2.2. Methods.

2.2.1. Cell Culture and propagation.
MCF-10A (DMEM/F-12), MCF-7 (DMEM), MDA-MB-231 (RPMI1640), ZR-75 (RPMI1640), SK-BR-3 (RPMI1640), MDA-MB-453 (α-MEM). The cells were cultured in the incubator of 37°C, 5% CO₂, and changed the medium 2-3 days. Logarithmic growth cells were used in experiments. When the cell coverage in the petri dish was 80%-90%, discarded the culture medium, washed twice with PBS buffer and then added trypsin about 300ul. When the cells detached, added 1ml medium which contained 10% FBS to terminate the digestion. The cells re-suspended with a pipette. Then transferred 1/3 of the cell suspension to a 6cm petri dish and added 3ml medium cultured in the incubator.

2.2.2. qRT-PCR.
The FGFR1 mRNA expression was detected in human breast cell lines (MCF-10A, MCF-7, MDA-MB-231, ZR-75, SK-BR-3, MDA-MB-453) by qRT-PCR. The logarithmic growth period of the above cells was used in experiments. The total RNA was extracted by trizol method, and RNA concentration was detected by the microplate reader and it was adjusted to 500-700ng/ml. The cDNA was synthesized by reverse transcription of Takara Reverse transcription kit. According to SYBR® Premix DimerEraser™ manual instructions to prepare RT-PCR System. Setting PCR conditions: pre-variable temperature 95°C, 30s; denatured temperature 95°C, 5s; annealing temperature 56°C, 30s;
extended temperature 72°C, 30s, a total of 40 cycles. Set dissolution curve, evaluated primer specificity, analyzed amplification curve and CT value, and calculated the target gene expression. FGFR1 Primer Sequence, Upstream primers: 5'-TAATGGACTCTGTGCGCC-3', Downstream primers: 5'-ATGTGTTGTTAGCTGCGTCC-3'; GAPDH used as control, Upstream primers: 5'-GAAGGCTGGGGCTCATTTGCAGGG-3', Downstream primers: 5'-GGAACGCTATTAGTTGGCAAG-3'.

2.2.3. Western blot.
Detection of the expression of FGFR1 protein in cells (MCF-10A, MCF-7, MDA-MB-231, ZR-75, SK-BR-3, MDA-MB-453) by Western blot. The above cells were cultured to close to 80% fusion degree in 6cm Petri dish. The intracellular protein was extracted by protein lysate, and the protein concentration was determined by enzyme marker with 1xProtein assay dye as a standard. 100 °C Metal bath made the protein to be tested denatured and polyacrylamide gel electrophoresis transferred protein to PVDF membrane. With 5% skim milk to block the membrane and then incubated with the primary antibody at 4 °C overnight. After washing with TBS-T, incubated with the secondary antibody at room temperature for an hour. Once again with TBS-T washing, according to the instructions using hypersensitive ECL Chemical luminescent test Kit to make the substrate color. Exposure of the film in the dark room, patches, development, and analysis of images.

2.2.4. Detection of cell proliferation by cell counting kit-8 (CCK-8) method.
Based on the results of the above qRT-PCR and Western blot experiments, the FGFR1 low expression breast cancer cell line MCF-7 and FGFR1 high expression breast cancer cell line MDA-MB-231 were selected. The effects of FGFR1 inhibitor BAY1163877 on the proliferation of MCF-7 and MDA-MB-231 cells were studied by using the MCF-10A of non-tumor epithelial cell line as the control.

2.2.5. IC50(half maximal inhibitory concentration) and cell viability.
The MCF-10A, MCF-7 and MDA-MB-231 cells in the logarithmic growth period were inoculated on the 96-well cell culture plate (6x103 cells/well), each cell was equipped with an experimental group (different drug concentration treatment cells), a control group (no drug treatment cells) and a blank group (medium only), each group of 3 complex wells, incubated for 24h in the incubator. Discarded the old medium, with PBS washed 2 times, and added the corresponding medium to each group 100ul/well (The BAY1163877 concentrations of the medium in the experimental group were 0.001μM, 0.01μM, 0.1μM, 1.0μM, 10μM, DMSO final concentration 1‰, control group and blank group medium DMSO final concentration 1‰), incubated for 72h in the incubator. Added CCK-8 reagent 10μl to each well, incubated for 3h at room temperature, and used enzyme marker to determine the absorbance at 450nm wavelength. Cell viability of each cell (%) = (experimental group OD value-blank group OD value) / (Control group OD value-blank group OD value) x100%. In the same way, the MCF-7 and MDA-MB-231 cells were paved with 96-well cell culture plate, the BAY1163877 concentration of each cell experimental group was 0.1μM. The control group (no drug treatment cells) and blank group (culture medium only) were set up, each group of 3 complex wells, incubated in the incubator, the cell viability was detected by 24h, 48h and 72h with CCK-8 respectively.

2.2.6. Formation of plate cloning experiments.
The MCF-7 and MDA-MB-231 cells in the logarithmic growth period were digested with 0.25% trypsin to make the cell suspension and the cells were counted. The cells were inoculated evenly and dispersed in 6-well plate with 100 cells per well, and incubated for 24h. After washing with PBS 2 times, added the corresponding medium (The concentration of BAY1163877 in the experimental group was 0.1μM, DMSO final concentration of 1‰, the DMSO final concentration of control group medium was 1‰). Then continued to incubate for 48h, replaced the growth medium and incubated in the incubator for 14 days. Discarded the old medium, with PBS washed 2 times, 4% polyformaldehyde fixed and Giemsa staining. Counted the number of clones. Cloning formation rate = (number of clones) / (number of inoculated cells) x100%.
2.2.7. Detection of apoptosis by flow cytometry.
The MCF-7 and MDA-MB-231 cells were inoculated in 6cm petri dish with \(4 \times 10^5\) cells/dish, and they were hatched in the incubator. The old medium was discarded, and the corresponding medium was added (The BAY1163877 concentration of the experimental group was 0.1μM, the final concentration of DMSO was 1‰; The final concentration of DMSO in control group medium was 1‰), and continued to incubate for 72h. Transferred medium to the labeled centrifugal tubes, with PBS washed petri dishes 2 times, then transferred PBS detergent to the above labeled centrifugal tubes, each dish added trypsin 200ul to digest to the cell flake shedding. Added PBS washing 2 times, collected cell suspension to the above labeled centrifugal tubes, and collected cells with centrifugal (1000r/min, 8min). According to the experimental operating instructions, the Annexin V-FITC Apoptosis Detection Kit was added to the collected suspended cells, using aluminum foil to avoid light, and incubated for 15min at room temperature. The apoptosis rate was calculated by flow cytometry and repeated the test 2 times.

2.2.8. Western blot detection of p-FGFR1/FGFR1 and p-STAT3/STAT3 protein expression.
The mechanism of BAY1163877 inhibiting the proliferation of MCF-7 and MDA-MB-231 cells and inducing apoptosis was studied by using Western blot to detect p-FGFR1/FGFR1 and p-STAT3/STAT3 protein expression. The BAY1163877 concentration of culture medium in the experimental group was 0.1μM, the final concentration of DMSO was 1‰; The final concentration of DMSO in control group was 1‰. Detected the action of cells that used BAY1163877 after 72h, the protocol is the same as the above western blot operation method.

2.2.9. Statistical analysis of data.
The measurement data are expressed by Mean±SD, the use of SPSS20.0 for statistical analysis, independent samples of the group comparison using T test, \(p<0.05\) for statistical significance, \(p<0.01\) is statistically significant.

3. Results

3.1. Expression of FGFR1 in 2.1 Breast Cell Lines.
qRT-PCR results showed that MDA-MB-231 cell line FGFR1 mRNA expression level was the highest in MDA-MB-231, ZR-75, SK-BR-3, MDA-MB-453 and MCF-7 breast cancer cell lines compared with non-tumor epithelial cell line MCF-10A, MCF-7 cell line FGFR1 mRNA expression level was the lowest (Figure. 1A). Western blot results showed high expression of FGFR1 protein in MDA-MB-231 cell line and low expression of FGFR1 protein in MCF-7 cell line (Figure. 1B). The expression level of FGFR1 in MDA-MB-231 and MCF-7 cell lines was higher than that of MCF-10A cell line, and the expression level of FGFR1 in MDA-MB-231 cell line was higher than that of MCF-7 cell line (Figure. 1). According to the results of qRT-PCR and Western blot, FGFR1 high expression breast cancer cell line MDA-MB-231 and FGFR1 low expression breast cancer cell line MCF-7 were selected, and the non-tumor epithelial cell line MCF-10A was used as the control. To study the effect and mechanism of FGFR1 inhibitor BAY1163877 on the proliferation and apoptosis of breast cancer cells.
Figure 1. FGFR1 expression in breast cancer cell lines.
A: qRT-PCR analysis of FGFR1 mRNA expression.
B: Western blot analysis of FGFR1 protein expression.

3.2. BAY1163877 Inhibits the Proliferation of MDA-MB-231 and MCF-7 in Breast Cancer Cell Lines.
The results showed that the half maximal inhibitory concentration values of BAY1163877 to MCF-10A, MDA-MB-231 and MCF-7 cell lines were 9.5μM, 0.1μM and 1.0μM (Figure. 2A). After the treatment of BAY1163877 for 24h, 48h and 72h, the viability of MCF-10A cell line was 97.7%, 93.7%, 85.3%, the viability of MDA-MB-231 cell line was 79.0%, 52.0%, 27.0%, the viability of MCF-7 cell line was 90.7%, 80.7%, 66.7% (Figure. 2B). The inhibitory effect of BAY1163877 on the proliferation of MDA-MB-231 and MCF-7 cell lines showed time and concentration dependence (Figure. 2A,2B). The formation of plate cloning experiment showed that the cloning formation of MDA-MB-231 and MCF-7 cell lines in experimental group was significantly lower than that in the control group (Figure. 2C). The cloning formation rates of MDA-MB-231 cell line in control group and experimental group were 91.3% and 44.3% respectively, and the difference was statistically significant (p<0.05) (Figure. 2D). The cloning formation rates of MCF-7 cell line in control group and experimental group were 57.0% and 35.7% respectively, and the difference was statistically significant (p<0.05) (Figure. 2E).
Figure 2. BAY1163877 inhibited the proliferation of MDA-MB-231 and MCF-7 cell lines.
A: MCF-10A(10A), MCF-7 and MDA-MB-231 cells were treated with 0.001μM, 0.01μM, 0.1μM, 1.0μM and 10μM BAY1163877 for 72h. The cell viability was measured by CCK-8 method and IC50 values were calculated.
B: The cell viability of MCF-10A, MCF-7 and MDA-MB-231 cell lines after treatment with 0.1μM BAY1163877 for 24h, 48h, 72h was measured by CCK-8 method.
C-E: MCF-7 and MDA-MB-231 cell lines were treated with 0.1μM BAY1163877 for 48h and then incubated for 14 days in the incubator. The number of clones was counted under Giemsa staining and the cloning formation rates were calculated (*p<0.05).

3.3. BAY1163877 Induces Apoptosis of MDA-MB-231 and MCF-7 in Breast Cancer Cell Lines.
The results of flow cytometry showed that there was a significant increase in apoptosis cells in the experimental group of MDA-MB-231 and MCF-7 cells compared with the control group (Figure. 3A). The apoptosis rates of MDA-MB-231 cell line in the control group and experimental group were 1.2% and 32.6% respectively, and the difference was statistically significant (p<0.01) (Figure. 3B). The apoptosis rates of MCF-7 cell line in the control group and experimental group were 1.4% and 12.8% respectively, and the difference was statistically significant (p<0.05) (Figure. 3C).

Figure 3. BAY1163877 induced the apoptosis of MDA-MB-231 and MCF-7 cell lines.
A: MDA-MB-231 and MCF-7 cells were treated with 0.1μM BAY1163877 for 72 hours and the cell apoptosis was detected by flow cytometry.
B and C: The cell apoptosis rate was calculated by cell counting (*p<0.05, **p<0.01).
3.4. Effects of BAY1163877 on the Expression of p-FGFR1/FGFR1 and p-STAT3/STAT3 Protein in Breast Cancer Cell Lines MDA-MB-231 and MCF-7.

The results of Western Blot showed that the expression level of p-FGFR1 and p-STAT3 protein in the experimental group of MDA-MB-231 cell line was lower than that in the control group, and there was no significant difference in the expression level of p-FGFR1 and p-STAT3 protein between the experimental group and the control group of MCF-7 cell line (Figure 4).

![Image of Western Blot results](image.png)

**Figure 4.** The expression of p-FGFR1/FGFR1 and p-STAT3/STAT3 protein in MDA-MB-231 and MCF-7 cell lines were detected by western blot.

4. Discussion

Breast cancer is one of the important cancers that threaten women's health, and its incidence is increasing year by year. It is reported that about 10%-15% of breast cancer has FGFR1 gene amplification.[13, 14] During embryonic occurrence, FGFR signals play a key role in the development of the nervous system, limbs, midbrain, lungs and mammary gland.[15] Abnormal expression of FGFR1 induces proliferation, anti-apoptosis, drug resistance and invasion of breast tumor cells.[16, 17] Experiments in mice showed that the expression and activation of FGFR1 induced the proliferation and invasion of breast cancer cells.[18] In vitro experiments of human breast cancer cells showed that FGFR1 amplification promoted cell proliferation and endocrine resistance, and that the FGFR1 gene knockdown breast cancer cell lines increased the sensitivity of 4-OH-Tamoxifen.[19] This may be related to poor prognosis in breast cancer patients with ER positive and FGFR1 amplification.[14] The expression of FGFR1 was associated with the adverse prognosis of triple negative breast cancer and was an independent prognostic factor for triple negative breast cancer.[20] At present, there is no effective drug treatment target for triple negative breast cancer, so it is very important to find an effective target for the treatment of triple negative breast cancer.

The main FGFR inhibitors reported for breast cancer treatment to date are ponatinib (AP24534), dovitinib (TKI258), AZD4547, BGJ398, etc. Ponatinib and dovitinib, the first generation of tyrosine kinase inhibitors, are multi-target tyrosine kinase inhibitors that act on receptors such as PDGFR and VEGFR in addition to FGFR. These drugs are mainly anti-vascular drugs and have insufficient effects on FGFR specific inhibition, and the risk of adverse events increases at the dose required for effective FGFR inhibitors.[13] Phase II clinical trials showed that dovitinib failed to improve the overall remission rate of metastatic breast cancer with FGFR1 amplification.[21] AZD4547 and BGJ398, the second generation of tyrosine kinase inhibitors, are selective tyrosine kinase inhibitors that act only on FGFR and reduce adverse reactions. But in phase II trials of patients with FGFR amplification tumors, including HER2-negative breast cancer patients, the activity of AZD4547 in FGFR1 enlarged breast cancer was found to be low.[12] Rapid resistance to single-agent therapies for inhibitors such as BGJ398 and AZD4547 has been widely observed.[22] There is an urgent need to study new FGFR targeted drugs to guide breast cancer treatment.
BAY1163877 is a selective tyrosine kinase inhibitor, which can selectively inhibit FGFR-1,2,3 and exhibit anti-tumor characteristics in clinical Phase I experimental studies. At present, there is no research report on the treatment of breast cancer with BAY1163877. In order to investigate the effect of FGFR1 inhibitor BAY1163877 on the proliferation and apoptosis of breast cancer cell lines and its mechanism, we identified MDA-MB-231 cell line with high FGFR1 expression and MCF-7 cell line with low FGFR1 expression in breast cancer lines through qRT-PCR and western blot. The cell viability, proliferation, apoptosis and expression of related signaling pathway proteins were detected by in vitro experiments. The experimental results showed that BAY1163877 inhibited the proliferation of MDA-MB-231 cell line and promoted its apoptosis, and the expression of p-FGFR1 and p-STAT3 in the experimental group was lower than that of the control group. BAY1163877 might inhibit the proliferation of MDA-MB-231 cell line and induce its apoptosis by reducing the expression of p-FGFR1 and p-STAT3. At the same time, BAY1163877 inhibited the proliferation of MCF-7 cell line and induce its apoptosis, and the expression of p-FGFR1 and p-STAT3 in the experimental group was not significantly different from that in the control group. BAY1163877 may inhibit the proliferation of MCF-7 cell line and promote its apoptosis through other pathways.

According to the literature, the JAK/STAT signaling pathway includes several steps that can quickly induce the transcription of downstream target genes. These include: (1) Cytokines form signal receptor complexes by combining their ligands with receptors. (2) Activate receptor-related tyrosine kinase (such as JAK) to induce STAT phosphorylation. (3) The cytoplasmic release of phosphorylated STAT, the formation of STAT two polymer, these transcription factors moved to the nucleus and the target gene binding, activating the downstream gene for transcription. STAT3 is a key signaling molecule for a variety of cytokines and growth factor receptors, which are activated by phosphorylation of tyrosine hydroxyl end (Tyr705), which in turn enters the nucleus, binds to DNA, and regulates the expression of related genes. In the course of many kinds of tumor, STAT3 showed continuous activation and increased expression. BAY1163877, as a selective inhibitor of FGFR-1,2,3, inhibits the binding of FGFRs and FGFs on cell membranes to a large extent, and then affects FGFR and the expression level of STAT in the process of subsequent signal transduction. Therefore, in JAK/STAT signaling pathway, the expression of p-STAT/STAT decreased can play a certain role in inhibiting proliferation and promoting apoptosis for the abnormal activation of tumor cells.

At the same time, we also found that MDA-MB-231 cell line expressed higher level of FGFR1 protein compared with MCF-7 cell line. BAY1163877 showed strong proliferative inhibition to MDA-MB-231 cell line, while the inhibitory effect on MCF-7 cell line was weak. This may be related to BAY1163877 selective inhibition of FGFR-1,2,3. Studies have pointed out that the FGFR2 expression level of MCF-10A cell line is significantly lower than that of MCF-7 cell line. Experiments showed that BAY1163877 inhibited the proliferation of MCF-7 cells and had no significant inhibitory effect on the proliferation of MCF-10A cell line (Figure. 2A,2B), which could not be ruled out as a correlation between the expression levels of FGFR2 protein in the two. Other studies have pointed out that MDA-MB-231 cell line expresses FGFR2 and FGFR3 protein. Abnormal proliferation of FGFR2 and FGFR3 associates with breast cancer. MCF-7 cell line expresses FGFR2 and FGFR3. It is inferred that FGFR2 and FGFR3 may be involved in the proliferation inhibition and apoptosis promotion of breast cancer cells in BAY1163877. BAY1163877 is a FGFR-1,2,3 inhibitor, FGFR1 and its ligand can be combined with multiple pathways to transmit signals, and participate in a series of biological activities of cells. The experiment failed to detect whether BAY1163877 has the role of blocking FGFR2 or FGFR3. It is not possible to determine whether BAY1163877 inhibits cell proliferation and promotes apoptosis through other pathways and other receptors in the FGFR family, which is also a limitation of this study. Further experimental verification is required.

5. Conclusion
BAY1163877 inhibits the proliferation of breast cancer cell line MDA-MB-231 and promotes cell apoptosis, and the mechanism may be related to the reduction of p-FGFR1 and p-STAT3 protein expression. BAY1163877 inhibits the proliferation of breast cancer cell line MCF-7 and promotes cell...
apoptosis, and the specific mechanism still needs to be further explored.

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