Effects of imatinib and 5-bromotetrandrine on the reversal of multidrug resistance of the K562/A02 cell line

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[Abstract] Background and Objective: Research has shown that 5-bromotetrandrine (BrTet) can effectively reverse multidrug resistance (MDR). Imatinib plays an important role in cell proliferation. This study explored the efficacy of the combination of Imatinib and BrTet on reversing MDR of tumor cells and its mechanism. Methods: Cytotoxicity was assessed by MTT assay. Apoptosis of K562/A02 cells was analyzed by flow cytometry. The expressions of mdr1 mRNA and P-glycoprotein (P-gp) were detected using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Results: After 48 h of treatment with 0.0625 μmol/L Imatinib, 0.5 μmol/L BrTet, or both, the 50% Inhibition Concentration (IC₅₀) of daunorubicin (DNR) for the K562/A02 cells were 5.69 mg/L, 5.41 mg/L, and 2.19 mg/L, respectively. The gray-scale values of mdr1 mRNA expression in the K562/A02 cells were 0.85 ± 0.02, 0.84 ± 0.01, and 0.25 ± 0.03, respectively. The expression levels of P-gp were 0.74 ± 0.02, 0.52 ± 0.02, and 0.29 ± 0.02, respectively. All decreased significantly in the K562/A02 cells treated with both Imatinib and BrTet compared to cells treated with Imatinib or BrTet alone (P < 0.05). The apoptosis rates of the K562/A02 cells increased without a significant difference after treatment with DNR, Imatinib, or BrTet (P > 0.05), while increased significantly after treatment with DNR combined with Imatinib, BrTet, or both (P < 0.05). Conclusions: The MDR of K562/A02 cells may be partially reversed by Imatinib or BrTet, and the mechanism may be related to the downregulation of mdr1 mRNA and P-gp expression and the upregulation of the rate of apoptosis in K562/A02 cells. Imatinib combined with BrTet showed a synergistic effect on K562/A02 cells.

Key words: Imatinib, BrTet, mdr1, bcr-abl, P-gp, P210

At present, chemotherapy is a clinically important treatment method for malignant tumors. However, the efficacy of chemotherapy is usually unsatisfying. The main reason is that tumor cells develop multidrug resistance (MDR). One principal mechanism of MDR is the overexpression of P-glycoprotein (P-gp), which can pump a variety of chemotherapy drugs out of the tumor cells, causing decreased intracellular concentrations of the drugs and inducing MDR. Studies have shown that clinically used chemotherapy drugs play roles mainly through inducing cell apoptosis [1]. Recent studies have found that the inhibition of tumor cell apoptosis has also led to MDR in tumor cells [2]. Many factors related to apoptosis affect the emergence of MDR together with P-gp.

It has been shown that imatinib induces tumor cell apoptosis and reverses MDR. 5-bromotetrandrine (BrTet) is a brominated product of Traditional Chinese Medicine reversed agent tetrandrine, which has a higher capacity for reversing drug resistance. Here, we used imatinib and BrTet alone and in combination to detect whether there was a synergistic effect on the reversal of drug resistance, and whether the expression of mdr1 mRNA and P-gp were related to tumor cell apoptosis.

Materials and Methods

Materials

Fetal bovine serum (FBS) was from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. RPMI-1640 was from GIBCO. Penicillin and streptomycin were from North China Pharmaceutical Co., Ltd. Dimethyl sulfoxide (DMSO) was from Tianjin Chemical Reagent Company. 4-methyl thiazolyl tetrazolium (MTT) was from Sigma-Aldrich. Adriamycin (ADM) was from Zhejiang Haizheng Pharmaceutical Co., Ltd. Daunorubicin (DNR) was from Farmitalia, Italy. The imatinib was supplied by Novartis. BrTet was from Chengdu Kanghong...
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Pharmaceutical Co. Anti-P-gp antibodies and anti-β-actin antibodies were purchased from Fujian Maxin Co. Ltd. All Western blot analysis reagents were purchased from Huamei Company. TRizol was from the Molecular Research Center. The TaKaRa LA PCR TM kit Ver 2.1 was purchased from TaKaRa Bio Inc.

Methods

Cell lines and cell cultures Chronic myeloid leukemia cell line K562 cells were preserved by our laboratory. Adriamycin-resistant cell line K562/A02 cells were supplied by the Chinese Academy of Medical Science Hematology Institute. The K562/A02 cells were cultured in a medium containing 1 μg/mL Adriamycin, and 2 weeks before treatment, the cells were cultured in a medium without Adriamycin. All cell lines were cultured in RPMI-1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in the humidified atmosphere of a 5% CO₂ incubator. The cells were subcultured every 2–3 days.

MTT assay for determining drug concentration The logarithmic (log) phase of the K562/A02 and K562 cells were diluted to 2 × 10⁵ cells/mL. A volume of 200 μL of the cells were seeded into 96-well plates. Volumes of 0.03125 μmol/L, 0.0625 μmol/L, 0.125 μmol/L, and 0.25 μmol/L of imatinib and 0.25 μmol/L, 0.5 μmol/L, 1.0 μmol/L, and 1.5 μmol/L of BrTet were added to cells. Each concentration repeated 3 times. Cells without MDR-reversing agents served as the control groups. After 48 h of treatment, 20 μL of MTT (5 mg/mL) was added to each well and cultured for another 4 h. Lipids were discarded and 150 μL of DMOSO was added to each well and oscillated for 10 min. Absorbency (A) value was measured at 490 nm. The cell proliferation inhibition rate was calculated by following formula: cell proliferation inhibition rate = (1 - A value of the experimental group/A value of the control group) × 100%. All experiments were repeated 3 times, and the average values were as the final results.

Effect of MDR-reversing agents on DNR-induced cell toxicity Log-phase cells were treated with 0.0625 μmol/L of imatinib and 0.5 μmol/L of BrTet alone or combined for 1 h. Different concentrations of DNR were added according to the study design. Each concentration was repeated 3 times. Without MDR-reversing agents, DNR and cells served as the control groups. Treatment and measurement of the control groups were the same as the experimental groups. Cell proliferation inhibition was calculated according to subsection formula above mentioned.

An integrated method was used to calculate the half maximal inhibitory concentration (IC₅₀) according to the cell proliferation inhibition rate. Multiples of reversal = IC₅₀ of drug-resistant cells/IC₅₀ of cells treated with MDR-reversing agents. All experiments were repeated 3 times, and the average values were as the final results.

Detection of cell apoptosis The logarithmic phase of K562/A02 cells was adjusted to 2 × 10⁵ cells/mL. The negative control group did not have any drugs added. The experimental groups were divided into 6 groups as follows: (1) the DNR group, (2) the imatinib group, (3) the BrTet group, (4) the DNR + imatinib group, (5) the DNR + BrTet group, and (6) DNR + imatinib + BrTet group. After 48 h of treatment, the cells were collected, washed with 4°C-chilled phosphate buffered saline (PBS) and resuspended with 1 mL of a buffer with Ca²⁺. A volume of 10 μL of Annexin V-FITC was added to each group. Blank and FITC were used as controls. Cells were incubated at room temperature for 15 min and protected from the light. After washing, the cell apoptosis rate was measured by flow cytometry.

Detection of mdr1 expression by RT-PCR The K562/A02 cells were adjusted to 2 × 10⁵ cells/mL and treated with the drugs. Cells were collected after treatment for 48 h. A volume of 1 mL of TRizol was used for total RNA extraction. Reverse transcriptase for the synthesis of complementary DNA (cDNA) was supplied by TaKaRa LA PCR TM Kit Ver.2.1 and cDNA was synthesized according to the kit. The amount of total RNA was 2 μg and the total volume was 25 μL. The reverse conditions were as follows: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. The forward primer was 5'-TTGTTTGCCACCCAGATA-3' and reverse primer was 5'-GAACCATGTTGCGTTT-3'. The cycling conditions for amplification were as follows: the initial denaturation at 94°C for 2 min, then 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 min. The density of the bands were analyzed after electrophoresis. The relative level of expression of the target gene was indicated by the ratio of the density of the target gene PCR product to the β-actin PCR product.

Detection of P-gp and P210 expression by Western blot analysis Cells were treated with 0.0625 μmol/L of imatinib and 0.5 μmol/L of BrTet alone and in combination and collected. Protein was extracted and protein concentrations were determined by the Bradford method. Equal amounts of cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidine fluoride (PVDF) membrane and blocked for 4 h at room temperature. Mouse anti-human P-gp antibody was used as the first antibody and the membranes were incubated at 4°C overnight. After washing the membrane, a secondary antibody (goat anti-mouse IgG antibody) was added and incubated for 1 h at room temperature. The unbound secondary antibody was washed out, then a fresh visualization reagent was added and the reaction was terminated after 20 min. β-actin was used as the control. The experimental data was indicated by the ratio of the density of the target protein to β-actin. Each experiment was repeated 3 times, and the average values were as the final results.

Statistical analysis Statistical analysis was performed using SPSS version 13.0, and data were shown by mean ± standard deviation. The effect of the predictive variables was evaluated by a univariate model, and P < 0.05 was considered statistically significant.

Results

Effect of imatinib and BrTet on K562 and K562/A02 cell proliferation Concentrations of 0.0625 μmol/L of imatinib and lower did not
show obvious cytotoxicity on K562 and K562/A02 cells. Concentrations of 0.5 μmol/L and lower of BrTet did not either. Concentrations of 0.0625 μmol/L of imatinib and 0.5 μmol/L of BrTet were selected for treatment.

**Cytotoxicity of K562 and K562/A02 cells induced by DNR before and after treatment with MDR-reversing agents**

The IC\(_{50}\) of DNR for K562 and K562/A02 cells were 12.50 mg/L and 0.36 mg/L, respectively. The multiple of drug resistance was 34.7. After the K562/A02 cells were treated with imatinib and BrTet alone or in combination, the IC\(_{50}\) was 5.69 mg/L, 5.41 mg/L, and 2.19 mg/L, respectively. The multiples of reversal were 2.19, 2.31, and 5.71, respectively. Between the single-agent treatment groups and the combined treatment groups, the differences were statistically significant (\(P < 0.05\)) (Figure 1). MDR-reversing agents alone or combination had no effect on the toxicity of K562 cells induced by DNR (\(P > 0.05\)) (Figure 2).

The rate of cell apoptosis caused by DNR, imatinib, and BrTet treatment

The rate of cell apoptosis of the K562/A02 cells was 5.47 ± 0.60. DNR, imatinib, and BrTet alone did not increase the rate of apoptosis (\(P > 0.05\)). DNR in conjunction with the MDR-reversing agents significantly increased the rate of cell apoptosis. Among them, the combined group with DNR, imatinib, and BrTet was the most significant (\(P < 0.01\)) (Figure 3).

**Effect of imatinib and BrTet on cellular mRNA expression of mdr1**

After treatment with imatinib and BrTet separately, the average mRNA expression level of mdr1 was lower than the control group in K562/A02 cells (the K562 group was 0.23 ± 0.03, the K562/A02 group was 0.96 ± 0.01, the K562/A02 + imatinib group was 0.65 ± 0.02, and the K562/A02 + BrTet group was 0.64 ± 0.01, \(P < 0.01\)). The gray-scale values of the mdr1 and BrTet combination group was 0.25 ± 0.03, which decreased significantly compared to the single-agent groups (\(P < 0.01\)) (Figure 4).

**Effect of imatinib and BrTet on cellular expression of P-gp**

The level of P-gp expression of sensitive K562 cells was 0.20
The present study showed that 0.0625 μmol/L and lower concentrations of BrTet did not show direct cytotoxicity on K562 or K562/A02 cells; 0.5 μmol/L and lower concentrations of BrTet did not either. Single and combined concentrations of 0.0625 μmol/L of imatinib and 0.5 μmol/L of BrTet significantly enhanced cell apoptosis induced by DNR in K562/A02 cells. More marked effects were found in the combination of imatinib and BrTet, which enhanced cell apoptosis induced by DNR in K562/A02 cells correspondingly.

P210 was overexpressed in K562 cells with an abnormally high level of tyrosine kinase activity, which activated a series of anti-apoptosis signal transduction pathways, such as RAS-MAPK, PBK/AKT, and JAK/STAT. The anti-apoptotic ability was further strengthened in the K562 cells, the chemotherapeutic drug sensitivity decreased, and anti-apoptosis for multiple drugs was produced. Wang et al. found that BrTet, as a tetrandrine brominated product, had a greater ability to reverse drug resistance than tetrandrine. One of the main mechanisms was that BrTet increased the sensitivity to chemotherapeutic drugs. As shown by flow cytometry, the imatinib, BrTet, and DNR single-agent groups did not increase the rate of cell apoptosis for K562/A02 cells. While treated with the combinations, the rate of cell apoptosis increased significantly, and the triple-drug combination group was the highest.

Imatinib is a selective inhibitor of bcr-abl and other tyrosine kinases, including stem cell factor receptor c-kit (CD117) and platelet derived growth factor receptor (PDGF-R). Previous studies have shown that decreased the expression of bcr-abl in K562 cells may be associated with the reversal of drug resistance [9]. Wang et al. also found that another mechanism of drug resistance reversed by BrTet decreased the expression of P-gp and increased the cellular drug concentration. Therefore, we presumed that both imatinib and BrTet could reverse drug resistance, and the mechanism was not only related to cell apoptosis, but also related to P-gp expression. As shown by RT-PCR, after treatment with imatinib and BrTet separately, the expression of P-gp decreased, and decreased more significantly in combination, showing a synergic effect. Additionally, Western blot analysis showed that the protein expression of P-gp also decreased, as did mRNA expression, which confirmed that the combination of imatinib and BrTet was significantly better than the single drug.

In summary, imatinib and BrTet reversed MDR of K562/A02 cells induced by DNR, which was not only related to decreasing mRNA and protein expressions of mdr-1, but also related to increased drug sensitivity and cell apoptosis. However, the mechanism needs to be studied further. Our study supplied a good basis for multiple mechanisms of MDR-reversing agents for the treatment of relapsed and refractory leukemia.

**References**


