Studies On the Anti-Proliferative Effects and Molecular Mechanism of a Novel Small Molecular BOC26P in Breast Cancer

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Abstract:

Background To explore the pharmacodynamic evaluation and mechanism research of BOC26P against breast cancer, and to provide a basis for the treatment of breast cancer.

Method MTT assay was used to detect the cytotoxicity of BOC26P against 4 breast cancer cell lines (MCF-7/TAX, MDA-MB-231/PT, MDA-MB-231 and MCF-7), and as well as the non-tumor cell lines MCF-10A, in various drug concentrations (from 0.004 to 1 μM). Western Blotting and Real-Time PCR assay were used to detect the relative protein and gene expression level after treatment with BOC26P in MCF-7/TAX. The effect of BOC26P on Specific fluorescent P-gp substrate accumulation in MCF-7/TAX was analyzed by flow cytometry; Molecular docking was used to analyze the binding capacity between BOC26P, Cyclosporine A, and Verapamil. FCM assay staining with Annexin V-FITC/PI and Propidium iodide was used to measure the apoptosis and the cell cycle after treatment with BOC26P in MCF-7/TAX, MDA-MB-231/PT, MDA-MB-231, and MCF-7; Detection of mitochondrial membrane potential after treatment with BOC26P inMCF-7/TAX, MDA-MB-231/PT, MDA-MB-231 and MCF-7; Western Blotting and Real-Time PCR assay was used to detect the apoptosis relative protein and gene expression level after treatment with BOC26P in MDA-MB-231, MCF-7, MDA-MB-231/PT, and MCF-7/ADR.

Results Cytotoxicity assay showed that BOC26P could effectively suppress 4 breast cancer cell lines (MCF-7/TAX, MDA-MB-231/PT, MDA-MB-231, and MCF-7) with an IC50 value of under 0.5 μM. The IC50 value of BOC26P on non-tumor cells MCF-10A was 32.29 μM. The binding ability of BOC26P to P-gp in breast cancer cells was weak. There was no significant effect on the intracellular accumulation of Rhodamin 123(Rh123), P-gp binding specific fluorescence substrate, and multi-drug resistance protein P-gp expression in MCF-7/ADR and MDA-MB-231/PT tumor cells; BOC26P induced MCF-7/TAX, MDA-MB-231/PT, MDA-MB-231 and MCF-7 cells cycle arrest at G2/M phase and lead to cell apoptosis. BOC26P induced significant activation of p53 protein in MCF-7/ADR and MAD-MB-231/TAX cells. Under the same conditions, BOC26P promoted Bax expression while inhibited Bcl-2 expression, and could significantly cause activation of Cleveland PARP and Cleaved Caspase3. The results demonstrated that BOC26P may induce apoptosis through the death receptor apoptosis pathway.

Conclusion It is known that BOC26P has a significant proliferation inhibitory effect on breast cancer cells without serious side effects. BOC26P has the Potential to be developed into a clinical substitute drug for triple-
negative breast cancer and drug-resistance breast cancer. BOC26P, a cytotoxic antitumor candidate compound but not a P-gp substrate, does not interfere with P-gp expression and efflux function in the P-gp-positive drug-resistant breast cancer cells, that is, it can avoid P-gp mediated multidrug resistance pathway in breast cancer. BOC26P activates the membrane stability protein Bcl-2 family in MDA-MB-231/PT, MCF-7/ADR and MA-MB-231 mitochondrial pathways of drug-resistant human breast cancer cells and their maternally sensitive cells, causing mitochondrial rupture, releasing P53, and activating the caspase-mediated apoptosis pathway, thus causing apoptosis of breast cancer cells. The significant inhibitory activity of BOC26P on breast cancer resistant cells may be related to P53, Activation of P53 and other tumor suppressor genes alters the expression of Bcl-2 and Bax, further activates the Caspase cascade initiates apoptosis pathway, and cause apoptosis eventually.

Key words BOC26P, breast cancer, multidrug resistance.

INTRODUCTION

At present, the most common malignant tumor in highly heterogeneous women worldwide is breast cancer, which occurs in the epithelium of mammary glands. According to the relevant clinical statistic, the global incidence of breast cancer has been on the rise since the late 1970s, and the global average incidence accounts for 7%-10% of various malignant tumors disease. China is not a country with a high incidence of breast cancer, but the situation should not be optimistic. In recent years, the growth rate of breast cancer incidence in China has been 1~2 percentage points higher than that in high incidence countries. According to 2009 breast cancer incidence data published by the National Cancer Center and the Ministry of Health's Bureau of Disease Control and Prevention in 2012, the incidence of breast cancer in the national tumor registration area ranked first among women. In the past few decades, there has been great progress in chemotherapy, radiotherapy, and combination therapy, the overall survival rates of breast cancer has improved, but it is still the primary cause of threats to women's health. Therefore, there is still a need to find new drugs for breast cancer with strong specificity, significant efficacy, and fewer side effects. At present, the commonly used anti-breast cancer drugs are mainly small-molecule cytotoxic chemical drugs, such as paclitaxel, adriamycin, 5-fluorouracil, platinum agents, etc. However, such drugs have low selectivity, high toxic and side effects, and easy to drug resistance, so their clinical efficacy is very limited. Therefore, enhancing selectivity and overcoming resistance is the important direction of drug therapy for breast cancer.

With the deepening understanding of natural products, is deepening, the anti-tumor properties have been paid more and more attention. Therefore, in recent years, many scholars have turned their attention to natural products to explore compounds derived from natural products that antagonize tumor resistance. Curcumin is the main active ingredient of curcumin rhizome extract, which has a wide range of antitumor and other pharmacological activities. Some studies have proved that curcumin has significant inhibitory activity on a variety of drug-resistant tumor cells. Our research group designed and synthesized a series of derivatives by chemically modifying the structure of the curcumin nucleus. Preliminary anti-tumor activity evaluation results showed that the o-aryl chalcone compound BOC26P had significant inhibitory activity on the proliferation of various tumor cells and it also showed significant inhibitory activity to some drug-resistant tumor cells. However, the previous experimental data could not elucidate the molecular mechanism of BOC26P in inhibiting tumor and antagonizing drug resistance.

Therefore, based on the previous studies of the research group, this study used in vitro and in vivo models to investigate the inhibitory activity and mechanism of BOC26P on breast cancer cells, especially the breast cancer cells that are resistant to the current clinical first-line clinical drugs such as paclitaxel and vincristine. Therefore, it is necessary to provide druggability evaluation data to confirm whether BOC26P has the development value of anti-breast cancer drugs.

MATERIALS AND METHODS

MATERIALS
BOC26P was synthesized by the research group of Professor Bu Xian Zhang, School of Pharmacy, Sun Yat-sen University. The structure of BOC26P was confirmed by HPLC, NMR, and HRMS. Taxol was purchased from Selleck, USA (Batch No: S115009); RNAiso Plus, PrimeScript™ RT reagent Kit (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were obtained from Takara (Shenyang, China), MDA-1 (ABCB1) was purchased from Santa Cruz (California, USA); GAPDH was purchased from Chengdu zhengneng biotechnology (Chengdu, China); PI and RNaseA were purchased from Sigma, USA; Annexin V-FITC/PI staining assay kit was obtained from Proteintech Group (BD, USA); DAPI was purchased from biyunt biotechnology (Shanghai, China); P53 was purchased from wanlei biotechnology (Shenyang, China); Caspase-3 rabbit polyclonal antibody, Cleaved-Caspase-3 rabbit polyclonal antibody, Caspase-9 rabbit polyclonal antibody, Cleaved-Caspase-9 rabbit polyclonal antibody, PARP rabbit polyclonal antibody, Actin rabbit polyclonal antibody, α-tubulin rabbit polyclonal antibody was obtained from Proteintech Group (Cell Signal, USA).

**CELL CULTURE**

MCF-7, MDA-MB-231, and Human non-tumor cell lines MCF-10A were purchased from the Chinese academy of sciences (Shanghai, China). The multidrug-resistant cell line MDA-MB-231/PT and MCF-7/TAX were obtained from Sun Yat-sen University School of Medicine. Various cell lines were incubated with RPMI-1640 containing 10% fetal bovine serum (Corning, USA) and DMEM medium (Gibco, USA) in a suitable humidity incubator at a concentration of 37 ℃ with 5% CO2. Besides, cell lines were co-cultured with drug-resistant compounds to maintain their multidrug resistance.

**CYTOTOXICITY TEST**

Cell viability was assessed using the MTT assay. The process was described below: The cells at logarithmic growth stage were digested with 0.25% trypsin (Gibco USA), the cells collected in the centrifuge tube. 3000-5000 cells per well were inoculated to 96-well plates (Corning, USA), and the control group was set at the same time. After cultured at 5% CO2 and 37 ℃ for 24 h, the cells were observed to be completely adherent to the walls and then treated with drugs. Paclitaxel and BOC26P are in the drug concentration range of 0.0041~1 μM, and the following six drug concentration gradients are set: 0.0041 μM, 0.012 μM, 0.037 μM, 0.11 μM, 0.33 μM, and 1 μM, and a negative control group (Add equal volume drug solvent), zero groups 1 (without inoculation of cells, plus the equal concentration of drugs) and zero groups 2 (without inoculation of cells, with an equal volume of vehicles). Five duplicate wells were set for each concentration above. 5 mg/mL MTT solution (Sigma, USA) 20 μL was added to each well. The culture continued in the incubator for 4 h. The culture medium on the culture plate was discarded and the action was terminated. Then 150 mL DMSO was added into each well and the solution was fully dissolved after 10 min of oscillation.

**ANNEXIN V-FITC/PI DOUBLE STAINING ASSAY**

Log-grown breast cancer cells were inoculated into a six-well plate (2 × 10^5 cells/mL), five concentrations (1 μmol, 0.5 μmol, 0.1 μmol, 0.05 μmol, 0.01 μmol) were respectively set. BOC26P was administered for 48 h and the liquid supernatant of each well was collected into a 15 mL centrifuge tube, digested with an appropriate amount of trypsin (without EDTA), the cells were collected and centrifuged (1000 rpm, 5 min). Discard the supernatant and wash the cells 3 times with PBS, and centrifuge (1000 rpm, 5 min). According to the instructions of Annexin V-FITC / PI Apoptosis Kit and immediately on the machine to analyze apoptosis.

**CELL CYCLE ANALYSIS**

Inoculate breast cancer cells into a six-well plate (2×10^5 cells/mL), set five concentrations (1 μmol, 0.5 μmol, 0.1 μmol, 0.05 μmol, 0.01 μmol). BOC26P was administered for 48 h and the liquid supernatant of each well was collected into a 15 mL centrifuge tube, digested with an appropriate amount of trypsin (without EDTA), the cells were collected and centrifuged (1000 rpm, 5 min).
rpm, 5 min). Discard the supernatant, add 300 μL of pre-chilled PBS to each tube, then slowly add 700 μL of absolute ethanol along the tube wall (-20 °C pre-cooling), gently mix the cells, and fix at -20 °C overnight. The next day, centrifuge the fixed samples at 4 °C (1000 rpm, 5 min), remove the absolute ethanol, wash with pre-chilled PBS, and centrifuge at 4 °C (1000 rpm, 10 min) to avoid diffuse attachment of cells to the tube wall. Immediately add 500μL PI staining solution (containing RNAse A), gently mix the cells, incubate at room temperature in the dark for 30 min, filter with a 300 mesh nylon mesh, and use a flow cytometer to detect the cells at an excitation wavelength of 620 nm. Period distribution.

DETECTION OF MITOCHONDRIAL MEMBRANE POTENTIAL (Δψm)

The cells Δψm were evaluated using JC-1 dye (5, 5', 6', 6'-tetrachloro-1', 3', 5'-tetrabenzimidazolocarbocyanine iodide, Beyotime, Shanghai, China). Briefly, after 48 h treatment, cells were incubated with JC-1 (5 μg/ml, dissolved in serum-free medium) for 30 min at 37 °C in an incubator. Then the cells were washed with PBS, immediately on the machine to analyze mitochondrial membrane potential.

RH123 ACCUMULATIVE EXPERIMENT

Logarithmic growth MCF-7/TAX cells were placed in a six-well cell culture plate. BOC26P cells were added respectively and a negative control group was set. After the treatment, the cells were incubated for 4 h, 12 h, and 24 h in an incubator at 37 °C and 5% CO2 saturation temperature. After the incubation time point was reached, Rh123 (10.0 M) was added to each sample treatment well for 2 h. After the incubation time of Rh123 was reached, the culture medium was discarded, rinsed with 1×PBS for 3 times, the cells were digested with 0.25% trypsin without EDTA, and the cells were resuspended with 1×PBS pre-cooled, washed and centrifuged (2000 RPM, 5 min). The flow cytometer set relevant parameters and immediately went on the machine to detect the accumulation of Rh123 in the cells.

REAL-TIME PCR

The logarithmic liver cancer cells were spread to a 6-well plate, numbered 1, 2, and 3, respectively, and cultured in an incubator for 24 h. At the same time, BOC26P of the same concentration was added for further incubation for 0 h, 6 h, 12 h, and 24 h, respectively. The culture medium was discarded, and 1 mL of 1×PBS pre-cooled at 4 °C was added to each well. Rinse the cells by gently shaking them for 1 min, and repeat the above operation 2 times. Discard 1×PBS and place the petri dish on ice. Add 1 mL of each dish cells Trizol, heat later transferred to the EP tube, add in 400 μL chloroform extraction, oscillation after 1 min, let stand at room temperature, 4 °C after stratification centrifugal (12000 RPM, 15 min), take on 400 μL transferred to another EP in the tube, to join 500 μL isopropyl alcohol, oscillation after 1 min, let stand at room temperature, in the 4 °C refrigerators precooling 15 min, 4 °C centrifugal (12000 RPM, 15 min), in addition to the supernatant, gently add 1 mL 75% ethanol (don’t let it dissolve), the ball hits bottom, The isopropyl alcohol on the precipitated surface was thoroughly cleaned and centrifuged at 4 °C (7500 RPM, 15 min). Repeat the above operation twice. Soak up 75% ethanol in the EP tube. The concentration and purity of RNA were detected after dilution. PrimeScript™RT kits and SYBR®Premix Ex Taq™II (Tli RNaseH Plus) kits were used in BD CFX96 (Bio-RAD, CA, USA) according to the instructions.

Logarithmically grown cells were passed through three 10-cm culture dishes, numbered 1, 2, and 3, respectively. BOC26P of the same concentration was added for further incubation for 0 h, 6 h, 12 h, respectively. 1 mL 1×PBS precooled at 4 °C was added to each well cell. Rinse the cells by gently shaking them for 1 min, then discard the lotion. Repeat the above operation twice to wash away the culture medium. Discard 1×PBS and place the petri dish on ice. Add an appropriate amount of precooled lysate to each dish cell. After full lysis, the adherent cells were scraped completely with cell scraping, and the cells and lysate were transferred to the precooled EP tube gently. Centrifugation at 4 °C (12000 RPM, 20 min) was performed to adjust the centrifugal force according to different cell types. The supernatant was absorbed gently and
transferred to the newly precooled EP tube, i.e. the protein sample. The protein concentration was detected by the BCA protein quantitative kit. 5× loading buffer was adjusted to 1× loading buffer with the adjusted concentration of protein samples and heated at 80 °C constant temperature metal bath for 10 min to conduct protein denaturation. One-step gel preparation kit (Forde, China) was used to make the gel. Sample electrophoresis was performed. The PVDF membrane was activated by 100% methanol for 1-2 min and transferred into the membrane. The strips were cleaned and incubated with PBST diluted secondary antibody at room temperature for 2 h. After cleaning, the target strips and the luminescent solution were fully incubated for 1-2 min and then developed in a developer.

DATA ANALYSIS AND STATISTICS
Each experiment was repeated at least three times unless otherwise specified. Numerical data are treated as the mean ± SD. Data were analyzed by a two-tailed unpaired Student’s t-test between any two groups. All statistical analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA). p < 0.05 was considered statistically significant.

RESULTS
IN VITRO ANTI-BREAST CANCER EFFECTS OF BOC26P
MTT assay was used to investigate the inhibitory effect of BOC26P on the proliferation of breast cancer cells (MDA-MB-231, MDA-MB-231/PT, MCF-7, and MCF-7/ADR) in vitro. The result is shown in Fig. 1 and shown in Table 1, BOC26P on (MDA-MB-231, MDA-MB-231/PT, MCF-7, and MCF-7/ADR) proliferation inhibition effects of breast cancer cells, such as its IC50 all less than 2μM, including BOC26P on MDA-MB-231/PT and MCF-7/ADR proliferation inhibition significantly, according to the preclinical guideline of CFDA antitumor drug IC50 < 10 M, the drug has antitumor activity, it shows that BOC26P has significant proliferation inhibitory activity on breast cancer cells. The IC50 of BOC26P on human breast epithelial cells MCF-10A is 32.29, suggesting that BOC26P has certain selectivity.

Figure 1 Effects of BOC26P on proliferative activities of various breast cancer cells.
Table 1 The inhibitory intensity of BOC26P on the proliferation activity of breast cancer cells.

<table>
<thead>
<tr>
<th></th>
<th>IC&lt;sub&gt;50±SD&lt;/sub&gt; (μM)</th>
<th>Paclitaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>1.163±0.56</td>
<td>0.812±1.42</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>0.051±1.34</td>
<td>1.612±1.24</td>
</tr>
<tr>
<td>MCF-7/ADR</td>
<td>0.0042±0.23</td>
<td>&gt;20</td>
</tr>
<tr>
<td>MDA-MB-231/PT</td>
<td>0.0141±1.3</td>
<td>&gt;20</td>
</tr>
<tr>
<td>MCF-10A</td>
<td>&gt;20</td>
<td>6.708±2.120</td>
</tr>
</tbody>
</table>

BOC26P ESCAPE P-gp MEDIATES MULTI-DRUG RESISTANCE MECHANISM

We first used Real-time PCR and Western Blot to confirm the expression of ABCB1 in paclitaxel-resistant MCF-7/ADR and MDA-MB-231/PT cells. The results showed that paclitaxel-resistant MCF-7/ADR and MDA-MB-231/PT cells have high ABCB1 expressions and are significantly higher than their corresponding wild-type cell lines MCF-7 and MDA-MB-231, while the expression levels of ABCCI and ABCG2 have no statistically significant difference compared to their corresponding wild-type cell lines (Fig. 2 (A)). P-gp protein was highly expressed in MCF-7/ADR and MDA-MB-231/PT cells, while the corresponding wild-type cell lines MCF-7 and MDA-MB-231 were almost not expressed (Fig. 2 (B)). At the same time, the fluorescence microscope results showed that MCF-7/ADR and MDA-MB-231/PT cells had significant excretion on Rh123, and Rh123 could hardly be detected in MCF-7 and MDA-MB-231 cells (Fig. 2 (C)). The above results confirm that P-gp mediates tumor resistance of MCF-7/ADR and MDA-MB-231/PT cells. Drugs and the role of P-gp is an important aspect of the two affinity ability, we use computer aided drug design software to simulate the three-dimensional conformation of P-gp, with the classic BOC26P and P-gp substrate ring spore fungus element A (Cyclosporine A, CosA) and Verapamil (Verapamil, VRP) the spatial structure of molecular simulation, from A three-dimensional, hydrophobic interaction and hydrogen bonding, factors such as comprehensive consideration and give score. The results are shown in Fig. 2 (D). Compared with P-gp classic substrates VRP (score value: 69.2569) and CosA (score value: 74.6169), BOC26P (score value: 51.9982) is comparable to P-gp classic substrates in spatial structure. P-gp protein structural affinity polar (standard: score value is proportional to affinity). Computer-aided drug design showed that Pgp and BOC26P have low affinity. In addition, the accumulation of Rh123 in paclitaxel-resistant MCF-7/ADR cells was not significantly different between 4 h, 12 h, and 24 h (Fig. 2(E)). Based on the above experimental results, we believe that BOC26P may not be recognized as efflux by P-GP due to its low affinity with P-GP, that is, BOC26P is a non-P-GP substrates candidate anti-tumor drug. Compared with paclitaxel, it can effectively avoid the most common drug resistance mechanism at present.

Figure 2 Correlation between multidrug resistance mechanism of BOC26P and P-gp.
BOC26P INDUCES CELL CYCLE ARREST AND APOPTOSIS IN BREAST CANCER CELLS

In vitro, experimental results showed that BOC26P inhibited the proliferation of breast cancer cells. To confirm whether the inhibitory effect of BOC26P (0.005-0.5μM) was related to apoptosis, MCF-7, MDA-MB-231, drug-resistant MCF-7/ADR and MDA-MB-231/PT breast cancer cells were treated with a certain concentration of BOC26P (0.005-0.5 M) for 48h, and the changes of apoptosis and cycle were detected by annexin V/PI staining and PI staining. Results as shown in Fig. 3, when the BOC26P concentration was 0.5μM, the G2/M cell population of MDA-MB-231, MCF-7/ADR and MDA-MB-231/PT increased significantly (up to 68%, 87.30%, and 78.89%) compared with the blank group, and the G1 cell population decreased significantly, indicating that the cell cycle arrest of MDA-MB-231 and drug-resistant MCF-7/ADR and MDA-MB-231/PT was in the G2/M phase and had an obvious dose-response relationship. Besides, the results of AnnxinV/PI staining showed that when the concentration of BOC26P was 0.1 M, the total number of apoptotic cells (the sum of early apoptotic cells and late apoptotic cells) of MDA-MB-231, MCF-7/ADR, and MDA-MB-231/PT increased by 19.78%, 38.4%, and 36.31%, and there was a significant dose-response relationship. The results indicated that BOC26P could induce apoptosis of breast cancer cells and was selective for drug-resistant breast cancer cells.

Figure 3 Effect of BOC26P on wild type breast cancer cells and their drug-resistant cell cycle and apoptosis.

EFFECTS OF BOC26P ON THE MITOCHONDRIAL MEMBRANE POTENTIAL OF MCF-7, MDA-MB-231, MCF-7/ADR, AND MDA-MB-231/PT CELLS

When a cell undergoes apoptosis, its internal mitochondrial transmembrane potential (MMP) is often destroyed. This process is widely regarded as a landmark event of early cell apoptosis. Flow cytometry was used to detect the changes of MMP in MDA-MB-231, MCF-7, MCF-7/ADR, and MDA-MB-231/PT cells after a certain concentration of BOC26P (0, 0.1, 0.5 μM). The experimental results are shown in Fig. 4. BOC26P reduced the mitochondrial membrane potential of MDA-MB-231, MCF-7, MCF-7/ADR, and MDA-MB-231/PT cells in a dose-dependent manner. It can be seen that BOC26P may induce apoptosis of breast cancer cells through the mitochondrial pathway, thereby inducing apoptosis of cells.
BOC26P ACTIVATE CYT/CASPASE-9/3 APOPTOSIS PATHWAY

The reduction of mitochondrial membrane potential often causes changes in mitochondrial membrane proteins. Therefore, the Western Blot method was used to verify the changes in the Bcl-2 protein family after BOC26P action. The results are shown in Fig. 5. After 24 hours of different doses of BOC26P (0, 0.1, 0.5 μM) in MCF-7/ADR and MDA-MB-231/PT cells, P53 expression increased, Bax expression increased, and Bcl-2 expression increased. Inhibition, while significantly causing PARP shearing, produces activated Cleaved PARP with a dose-dependent increase. Besides, BOC26P activates Caspase 3 and Caspase 9 to produce activated Cleaved Caspase 3 and Cleveland Caspase 9, causing cells to undergo mitochondrial pathway apoptosis. From the above results, it can be seen that BOC26P increases the mitochondrial membrane potential and activates the mode of the intracellular mitochondrial apoptotic pathway, resulting in the inhibition of the expression of mitochondrial stabilizing protein Bcl-2, thereby triggering mitochondrial rupture and causing apoptosis. That is, BOC26P ruptures mitochondria triggers the Caspase apoptosis pathway, and causes breast cancer drug-resistant cell apoptosis.

Figure 4 Effects of BOC26P on mitochondrial membrane potential in breast cancer cells.

Figure 5 Regulation of BOC26P on the expression of related proteins in the cascade of apoptosis in breast cancer cells.
CONCLUSION

BOC26P has obvious proliferation inhibitory activity on breast cancer cells, but no obvious inhibitory activity on non-tumor-derived breast epithelial cells MCF-10A, suggesting that BOC26P has a significant inhibitory effect on breast cancer and has good selectivity. Breast cancer may be used as one of the clinical indications of BOC26P; BOC26P is not a P-gp substrate and will not be recognized by P-gp for efflux. At the same time, it will not produce a new round of tumor resistance during the long-term intervention. Compared with drugs such as paclitaxel, BOC26P has a significant tumor-suppressing effect and can avoid the classic drug resistance mechanism, so it has a significant advantage in alternative treatment. BOC26P induces the Bcl-2 family in the mitochondrial pathway by activating the P53 protein of human breast cancer resistant cells MDA-MB-231/PT, MCF-7/ADR and their maternally sensitive cells MDA-MB-231 and MCF-7. Ruptures mitochondria, activates the apoptosis pathway mediated by the caspase family, and triggers breast cancer cell apoptosis.

DISCUSSION

At present, breast cancer is the most common type of highly heterogeneous female malignant tumor in the world. Among estrogen-dependent breast cancer accounts for 50-60% of total breast cancer diagnosis, and Triple negative breast cancer accounts for 15% to 20% of the total diagnosis of breast cancer, because of abnormal clinical hormone expression in breast cancer, endocrine therapy and anti-HER2 targeted therapy failed to achieve the effect. Under such a clinical background, traditional cytotoxic drugs are the main treatment for breast cancer, but traditional cytotoxic drugs can easily lead to drug resistance in long-term. Therefore, the development of cytotoxic anti-breast cancer drugs should be the development direction that can significantly inhibit the growth of breast cancer and can resist multidrug resistance 10-12.

Our study found that BOC26P had an obvious inhibitory effect on MCF-7, MDA-MB-231, MCF-7 /ADR and MDA-MB-231/PT and showed good selectivity. Research investigates the antagonism of the BOC26P resistant P-gp is mediated multi-drug resistant way has relevance, around P-gp in tumor multi-resistant played a key role in the process of development, mainly studies the BOC26P intervention in P-gp positive expression of drug resistance of breast cancer cells, P-gp fluorescent substrate specificity of Rh123 sensitivity as well as chemicals and molecular docking experiment tests the combination of P-gp and BOC26P parameters, aims to elucidate the BOC26P whether for P-gp substrate breast-cancer candidate compounds. Compared with cyclosporine A and Verapamie, BOC26P had a weak affinity for P-gp. Meanwhile, flow experiment results showed that BOC26P had no significant influence on the accumulation of rhodamine 123 (Rh123). A P-gp-specific fluorescent substrate, in MCF-7 /ADR cells, indicating that the obvious inhibitory effect of BOC26P on MCF-7 /TAX was not achieved by inhibiting the expression and function of P-gp. These results suggest that BOC26P has the potential to be developed as a clinical alternative drug for breast cancer or other tumor-like tumors that are induced by P-GP to multidrug resistance 13-14.

These results suggest that BOC26P has the potential to have been developed as a clinical alternative drug for breast cancer or other tumor-like tumors that are induced by P-gp to multidrug resistance. In the preliminary study on the mechanism of action, flow cytometry was used to detect whether BOC26P had an effect on the cell cycle and apoptosis of breast cancer, and whether it had an effect on apoptotic proteins? The results showed that BOC26P led to the above cell cycle arrest of breast cancer in the G2/M stage, which was a critical stage for cells to complete DNA and protein synthesis and enter into mitosis. If cells were blocked in this stage, they could not enter into the next stage of mitosis. The results showed that BOC26P significantly inhibited the activity of breast cancer cells, which may inhibit the expression of cancer-related protein P53 15-17 and activate the Bcl family protein. The increased mitochondrial membrane potential led to mitochondrial rupture. With the mitochondrial rupture, the Caspase apoptosis pathway resulted in the apoptosis 18-20 of the mitochondrial pathway.

In this study, objective in vivo and in vitro anti-tumor activity of BOC26P was evaluated, and on this basis, the mechanism of action of BOC26P against tumor drug resistance was
preliminarily discussed. It was found that the mitochondrial apoptosis pathway was involved in the action of BOC26P against tumor drug resistance, and it was also found that BOC26P was a non-P-GP substrate and was not affected by the over-expression of P-gp in cells. However, due to the limitation of time and relevant objective factors, there are still some deficiencies in this study, which need to be further improved: the analysis of the molecular mechanism of BOC26P against breast cancer in this study is not in-depth enough, especially the lack of some necessary experimental designs to prove it, so the mechanism of action needs to be further discussed.

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**Abbreviations:**
P-gp, P-glycoprotein; IC50, Half-inhibitory concentration; FBS, Fetal bovine serum; MTT, 3-(4,5-dimethyl-2-thiazoly1)-2,5-diphenyl-2-H-tetrazolium bromide; ABCB1, ATP-binding cassette subfamily B member 1; DMSO, Dimethy sulfoxide; PBS, Phosphate buffer salin; Rh123, Rhodamine 123; VCR Vincristine;VRP, Verapamil; TAX, Pacilatixel.