新型甲磺酰胺类肿瘤抑制剂靶向AKT诱导BT549细胞凋亡的作用机制

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摘要 PI3K/AKT信号通路的异常与肿瘤的发生、转移具有非常密切的关系, 在乳腺癌细胞中更为常见。该研究从自主设计合成的小分子样品库中筛选出可有效诱导乳腺癌细胞BT549凋亡的新甲磺酰胺类肿瘤抑制剂DHW-51。选择*PTEN/p53*双基因敲除的人乳腺癌细胞株BT549, 通过细胞增殖实验发现, DHW-51可抑制BT549细胞增殖并且呈剂量和时间依赖关系。溶血实验结果表明, DHW-51浓度为40 μmol/L时, 血红细胞的溶血活性低于10%, DHW-51对正常细胞没有毒性作用。此外, 流式细胞术实验证明了DHW-51可以诱导BT549细胞凋亡, DHW-51浓度为20 μmol/L时caspase3的活性增加, pro-caspase3蛋白表达下降。Western blot结果显示, BT549细胞中AKT的磷酸化水平显著下降, 但AKT总蛋白的表达水平未受影响, 证明DHW-51诱导BT549细胞凋亡的靶点可能是AKT蛋白, 提示DHW-51可能是以AKT为靶点的肿瘤抑制剂。

关键词 甲磺酰胺; AKT; 凋亡; 人乳腺癌

Cellular Mechanisms of Targeting AKT of New Sulfonamide Tumor Inhibitor That Induces Apoptosis in BT549 Cells

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Abstract The abnormal PI3K/AKT signaling pathway accounts for tumorigenesis, metastasis and multidrug resistance, especially in breast cancer. In this article, we discovered a new sulfonamide tumor inhibitor, DHW-51, which induced the apoptosis in breast cancer cell line BT549. When *p53* and *PTEN* were both knockdown in BT549, it showed that DHW-51 effectively inhibited the survival of BT549 cell in both dose-and time-dependent manner. The hemolytic test result indicated that DHW-51 had inconsiderable effect on normal cells when hemolytic activity of red blood cell showed less than 10% with 40 μmol/L of DHW-51. In addition, FACS results also suggested that DHW-51 induced apoptosis of BT549 cells significantly. They used flow cytometry to show that DHW-51 could significantly induce apoptosis of BT549 cells, the activity of caspase3 increased with 20 μmol/L of DHW-51, however, the expression of pro-caspase3 protein decreased. As showed in the results of Western blot, the phosphorylation level of AKT in BT549 cells decreased significantly, nevertheless, the expression

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level of the AKT protein was not affected. We speculated that DHW-51 induced apoptosis of BT540 by targeting AKT, and suggested DHW-51 was an AKT-targeted tumor inhibitor.

Keywords sulfonamide; AKT; apoptosis; human breast cancer

As the mainly common malignancy in women worldwide, human breast cancer (HBC) is one of the major diseases threatening women's life, with strong evolution, rapid growth mutations and lacking of the ability to form stable and normal functional structures. Although there has been a continued decrease in death rates in decades, the amount of the women dying from HBC still accounts for a large proportion of the women dying from cancer[1-6]. Under such a severe situation, scientists consistently focused on studying the treatment of HBC, in which the research on the effective anti-tumor small-molecule targeted drugs became a hot topic around the world.

AKT, a serine/threonine protein kinase, which plays a key role in signaling downstream of growth factors and other stimuli and regulates critical cellular functions including proliferation and survival. When been activated, AKT needs the phosphorylation of two highly conserved residues: Thr308 in the activation loop mediated by the phosphoinositide-dependent kinase 1 (PDK-1) and Ser473 in the hydrophobic motif mediated by the mammalian target of rapamycin complex 2 (mTORC2). Termination of AKT signalling is tightly controlled and accomplished in part by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)[7]. Loss of PTEN commonly leads to hyperactivation of the PI3-K/AKT signaling pathway[8]. p53 is one of the important substrates downstream of AKT, it can coordinates the cellular response to stress, including DNA damage, hypoxia and oncogenic stress, through transcriptional mechanisms, resulting in cell cycle arrest, senescence or apoptosis[9]. AKT can also phosphorylate a wide array of additional substrates that also influence the growth of tumor, which promotes degradation of the tumor-suppressor p53[10]. Some *in vivo* studies have confirmed that AKT plays additional roles in transforming breast cancer cell invasion leading to metastatic distribution. Previous studies have developed several AKT inhibitors of human breast cancer cell lines, such as MK-2206, AZD5363 and stachydrine hydrochloride on human breast cancer cell lines, which demonstrated to successfully inhibited AKT activity *in vivo* and restrained the growth of breast tumor, particularly HER2-positive breast cancer[11-13]. Subsequent studies proved that AKT inhibited cell apoptosis, endorsed cancer cell proliferation and standardized cell metabolic pathways, which were essential for tumor growth[14-15]. Even though series of AKT inhibitors successfully induced tumor cell apoptosis, cancer cells became a resistance to AKT inhibitors by the activation of survival pathways. In addition, some studies have pointed out that some breast cancer cells have showed an increased resistance to AKT inhibitors after long-term treatment[16].

Inventing new drugs that efficiently induced apoptosis in breast cancer cells without damaging the normal cells currently has attracted high attention around the world. In this study, we assessed the efficacy of new sulfonamide tumor inhibitor DHW-51 in the human breast cancer cell line BT549 by flow cytometry and Western blot.

1 Materials and methods

1.1 Cell lines and culture

Human breast cancer cell lines for *p53*/*PTEN* dual knockdown BT549 were obtained from the Ontario Institute for Cancer Research (Toronto, Canada). The cells were cultured in Roswell Park Memorial Institute (RPMI1640) containing 12% fetal bovine serum (FBS) and then incubated in 37 °C, 5% CO_2 incubator. The new medium was replaced every 2 to 3 days and the cells were collected in the logarithmic phase for being applied to the following assays.

1.2 Test drugs and chemical reagents

DHW-51 was requested from Shenyang Pharma-

ceutical University (Shenyang, China). RPMI1640 and fetal bovine serum (FBS) were purchased from Gibco company (Grand Island, USA). PVDF membrane was obtained from Millipore company. Annexin V-FITC apoptosis detection kit was purchased from Bestbio company (Shanghai, China). 3-(4,5-dimethylthiazol-2-2 yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich company (Shanghai, China). Protein Marker was purchased from TransGen Biotech company (Beijing, China). Caspase 3 Activity Assay Kit were purchased from Beyotime company (Shanghai, China). The antibodies were purchased from Santa Cruz company (Texas, USA).

1.3 Cell proliferation assay

The MTT assay was used to detect the cytosine effect of different concentrations by DHW-51 in BT549 cells. BT549 cells were seeded at 7 000 cells/well in 96-well plates and incubated overnight. DHW-51 was added to a final concentration of 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L and 30 μmol/L in a volume of 200 μL/well. After further incubation for 12 h, 24 h or 48 h, each well was added with 20 μL MTT reagents for 4 h. Then the medium was replaced by 150 μL of DMSO. The GF-M3000 microplate reader (Shandong, China) was used to assess the absorbance of the 96-well plate reader at wavelength of 492 nm. The viability of BT549 cells was calculated with the following equation: cell viability (%)=(*D* of experimental group)/(*D* of control)[17]. Each experiment was repeated three times.

1.4 Hemolysis activity assay

The hemolytic properties of DHW-51 were examined by spectrophotometry. First, 5 mL of rabbit blood was added to 10 mL of physiological saline PS and then was centrifugated at the speed of 1 200 r/min for 8 min to isolated red blood cells (RBCs) from serum. Second, the obtained RBCs were further washed six times with 40 mL of PS, and following the last washing, the remaining RBCs were dispersed in 30 mL PS. Third, DHW-51 suspended in 0.4 mL of PS with different concentrations was separately mixed with 0.4 mL of RBCs that suspended in PS and the mixtures were then incubated at 37 °C in a thermoregulated water bath for 1.5 h. RBCs were then centrifuged at 3 000 r/min for 10 min, and 100 μL of supernatant from each sample was transferred to a 96-well plate. Free hemoglobin in the supernatant was measured with a Bio-Rad 680 microplate reader (Bio-Rad, CA, USA) at 540 nm. PBS and ddH_2O were used as negative and positive controls, respectively. All hemolysis experiments were carried out in triplicate. The hemolysis ratio[12] of RBCs was calculated with equation, HR (%)=(*D* sample–*D* negative control)/(*D* positive control–*D* negative control)×100%. In equation, *D* sample, *D* negative control, and *D* positive control denote the absorbencies of the sample, negative control, and positive control, respectively.

1.5 Detection on the apoptotic rate of BT549 cells induced by DHW-51 with flow cytometry

The proliferative inhibition of BT549 cells by DHW-51 was observed by flow cytometry. Cells cultured in recommended medium for 24 h was marked with Annexin V-FITC and PI double labeling method for the cell apoptosis assay by flow cytometry. In brief, the BT549 cells (2×10^5 cells/mL) were seeded at 2 mL/well in 6-well plates, respectively. After incubation overnight, DHW-51 was added to a final concentration of 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L and 30 μmol/L in a volume of 200 μL/well. The treated cells were placed in the incubator with 5% CO_2 at 37 °C for 12 h and 24 h. Next, the cells were harvested and then washed with ice-cold PBS, finally being adjusted to 1×10^6 cells/mL with 1×binding buffer. The cell suspension (100 μL) was loaded into 15 μL Annexin V-FITC and 5 μL propidium iodide (PI), gently mixed, and incubated in the dark environment at 4 °C for 20 min. After staining, the cells were washed using PBS with 0.5% FBS inside. In the end, the cells were re-suspended in 400 μL binding buffer. After filtration, the suspension of each group was analyzed with FACS caliber. The experiment was repeated three times.

1.6 Determination of caspase3 activity in BT549 cells by DHW-51

DHW-51 was added to a final concentration of

10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L and 30 μmol/L in a volume of 200 μL/well after treatment for 24 h, the cells were digested with 0.125% trypsin, centrifuged (4 °C, 1 500 r/min, 3 min), suspended with 1 mL PBS, re-centrifuged (4 °C, 1500 r/min, 3 min), and re-suspended in 100 μL Cell Lysis Buffer of Caspase 3 Activity Assay Kit on ice for 15 min per 2×10^6 cells. Cytosolic extract (the supernatant) was transferred equally to 96-well plate containing 20 μL after centrifugation at 12 000 r/min for 15 min at 4 °C. Next, Bradford Protein Assay was applied to detect protein concentration (1-3 mg/mL). Finally, 70 μL reaction buffer and 10 μL Ac-DEVDpNA (2 mmol/L) were added to each sample, incubated at 37 °C for 2 h and analyzed at 405 nm with a spectrophotometer. The experiment was repeated three times.

1.7 Western blot of pro-caspase3, p-AKT and AKT

The protocol for Western blot has been described previously[18]. Cells were treated with different concentration of DHW-51, washed twice with ice-cold PBS, and gently lysed for 1 h in ice-cold cell lysis buffer (Dingguo, Beijing, China). Lysates were centrifuged at 12 000 r/min at 4 °C for 10 min. Supernatants were collected to determine the protein concentrations for Western blot analysis. An equal amount of protein was subjected to electrophoresis on an SDS-polyacrylamide gel and transferred to a PVDF membrane by electroblotting. The blots were blocked in phosphate buffered saline (PBS) containing 10% non-fat milk and 0.1% Tween-20 (blotting grade) for 3 h and then were applied to probe the desired antibodies at 4 °C overnight. Subsequently, membranes were subsequently incubated with appropriate HRP-conjugated secondary antibody for 45 min and visualized by Western blot detection reagents (Trans Gene, Beijing, China).

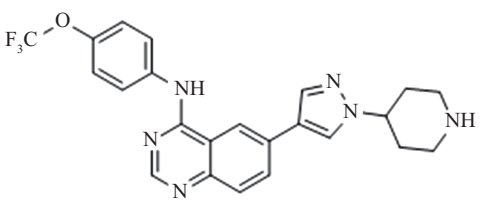


Fig.1 Chemical structure of DHW-51

1.8 Statistical analysis

All data were presented as the mean±S.D.. Significant differences among the groups were determined by the unpaired Student's *t*-test. In result, $P<0.05$ was statistically significant. In this article, the results in each figures represented at least three independent experiments.

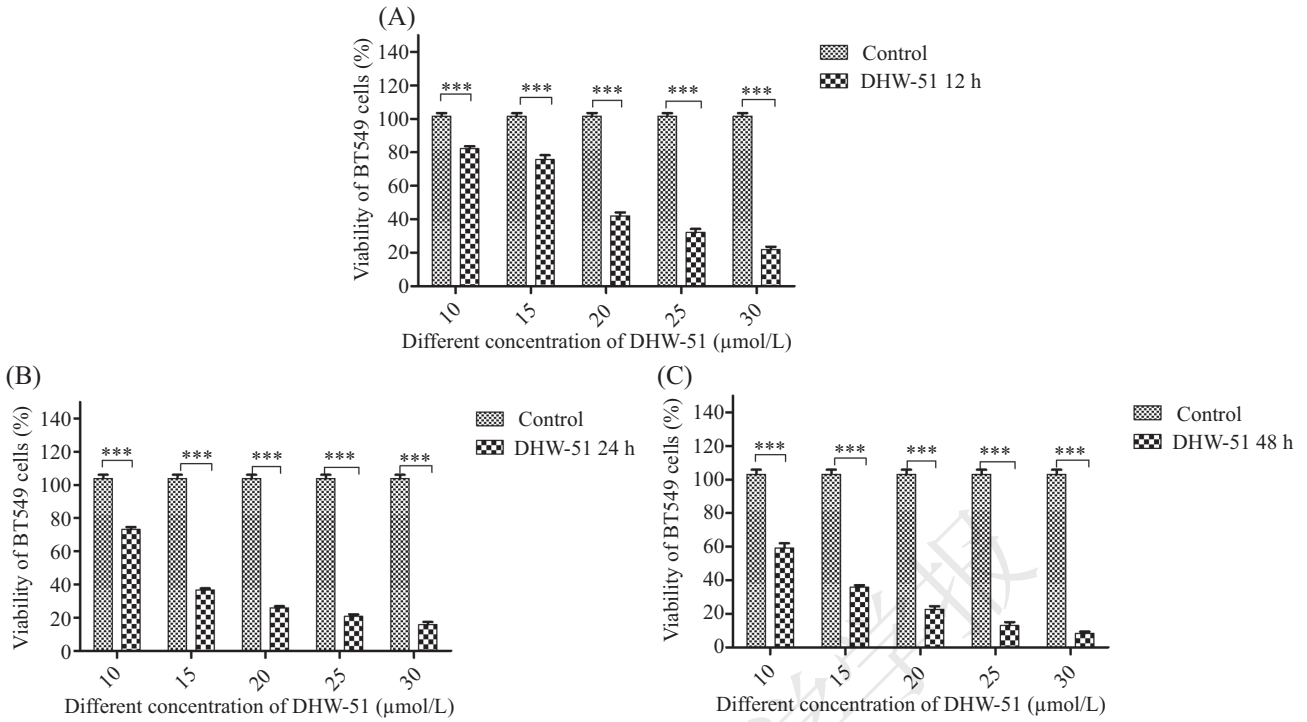
2 Results

2.1 DHW-51 inhibited BT549 cell growth

In this study, 6-(1-(piperidin-4-yl)-1H-pyrazol-4-yl)-N-(4-(trifluoromethoxy) phenyl) qui-nazolin-4-amine (DHW-51) was a new synthetic small molecular, being selected from our own synthetic library (Fig.1). Its drug concentrations included 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L and 30 μmol/L, and its durations contained 12 h, 24 h and 48 h, respectively. The drug had inhibitory effects on the growth of both *p53*/*PTEN* double knockdown human breast cancer cell lines of BT549, which depended on the dose of and duration of the drug (Fig.2). It was found that the optimal duration of the drug was between 12 h and 24 h for the breast cancer cell line BT549, indicating that DHW-51 inhibited the viability of BT549 cells in a dose-dependent manner. After treatment for 24 h, there was a significant decrease in the cell viability of BT549 with 15 μmol/L DHW-51. Its inhibition rate was 73% higher than that with 10 μmol/L DHW-51 and the IC_{50} was 13.69 μmol/L.

2.2 Hemolysis activity

In recent years, many cytotoxic compounds had been designed to obtain more specific anticancer drugs and minimize toxic side effects[19]. The characterization of blood compatibility *in vitro* of the DHW-51 compound was important in evaluating whether normal cells were poisoned. Therefore, hemolysis activity experiments were conducted to evaluate the damage inflicted by DHW-51 on normal cells. Hemolysis activity referred to the phenomenon of red blood cell rupture, which could be caused by many physical and chemical factors, such as bile salts, detergents, etc. To



A: cells were seeded at a density of 7 000 cells/well and then treated with 10, 15, 20, 25, 30 μmol/L of DHW-51 for 12 h; B: cells were seeded at a density of 7 000 cells/well and then treated with 10, 15, 20, 25, 30 μmol/L of DHW-51 for 24 h; C: cells were seeded at a density of 7 000 cells/well and then treated with 10, 15, 20, 25, 30 μmol/L of DHW-51 for 48 h. ****P*<0.001.

Fig.2 DHW-51 inhibited the proliferation of BT549 cells

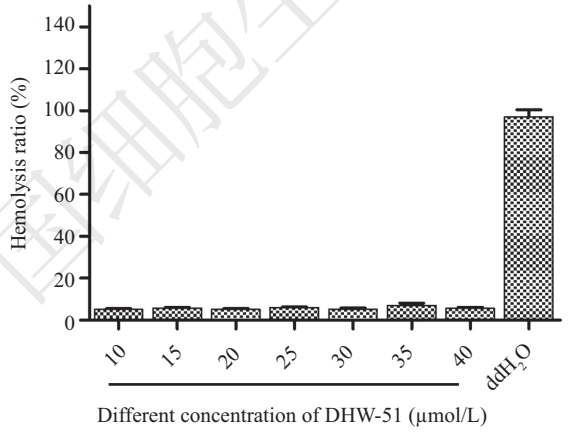
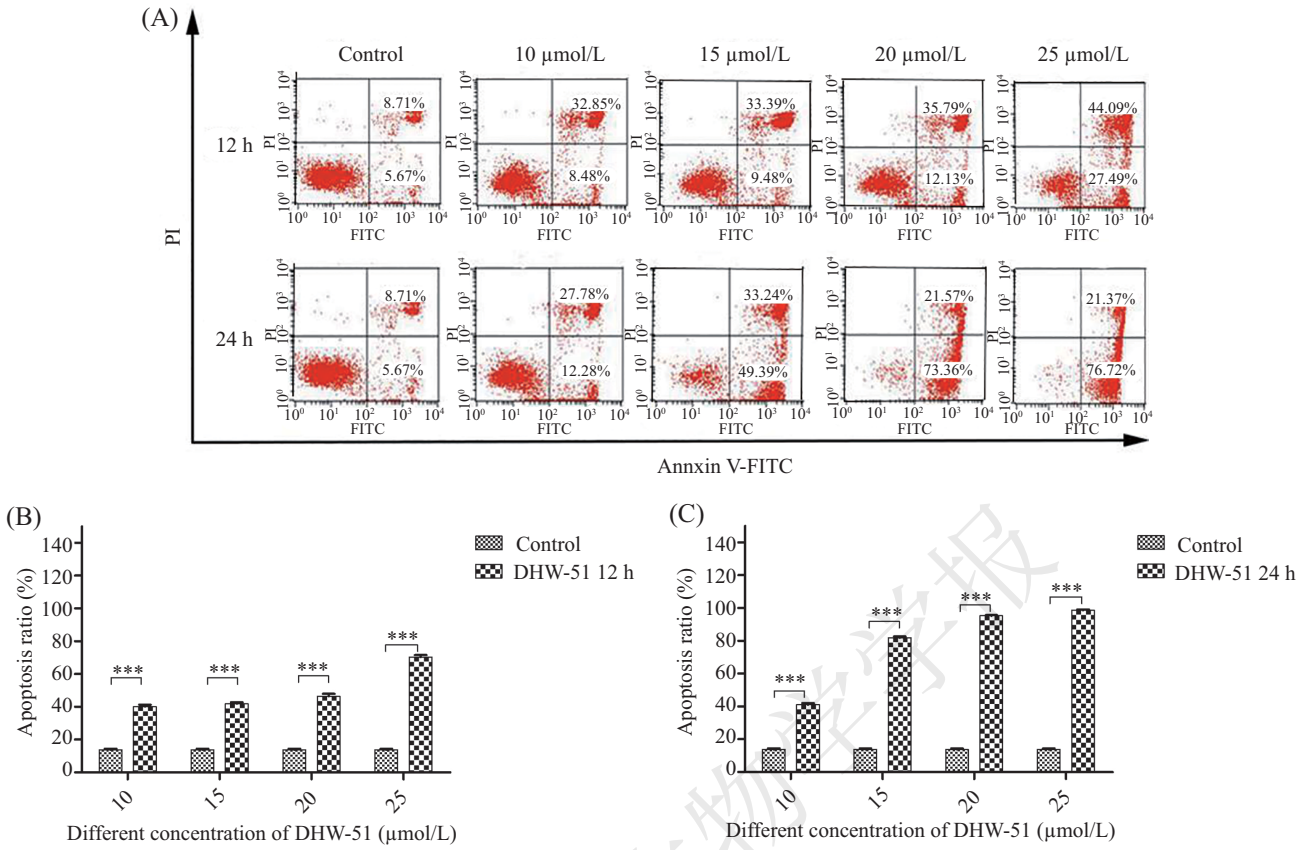


Fig.3 Percentage of RBCs hemolysis incubated with DHW-51 and ddH_2O as positive controls

make sure that DHW-51 would not cause cell death, the hemolysis assay was determined as a preliminary way. The blood compatibility of DHW-51 was assessed by a hemolysis assay. The increase of hemolytic rate HR resulted in a higher damage of RBCs. The RBCs for the assay were exposed to DHW-51 at different concentrations for 1.5 h, in which ddH_2O was the positive control group. As shown in Fig.3, when the concentration of DHW-51 was 40 μmol/L, the hemolytic activity of RBCs was 10% less than that in the control group, suggesting that there was almost no damage on the normal cells. Given that we did not buy the positive drugs of testing cell toxicity, it would be better to explain the problem by coupling with the result of the positive drug control.

2.3 Induction of apoptosis by DHW-1 in BT549 cells

Apoptosis is a process of programed death, which involves a series of biochemical events. In the cytotoxic effect experiment, the flow cytometry in more detail



A: BT549 cells were treated with 10, 15, 20, 25 μmol/L DHW-51 for 12 h and 24 h; B: DHW-51 induced the BT549 cell apoptosis by 12 h; C: DHW-51 induced the BT549 cell apoptosis by 24 h. ***P<0.001.

Fig.4 DHW-51 induced the BT549 cell apoptosis by flow cytometry

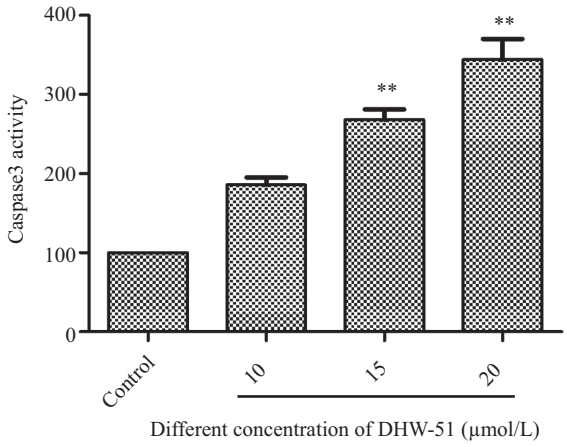
deciphered the mechanism that DHW-51 inhibited the cancer cells growth. Therefore, after being treated in DHW-51 of different concentrations for 12 h and 24 h, the cells were applied to the apoptosis analysis with flow cytometry. FACS scatter gram indicated DHW-51 could induce apoptosis of BT549 cells in a dose- and time-dependent manner (Fig.4), which was consistent with the result in previous MTT assay. Furthermore, the ratio of the late apoptotic cells increased with the increase of the concentration of DHW-51. In BT549 cell line, when the drug concentration was 20 μmol/L and the duration was 24 h, the apoptosis rate of the cell was over 90%. The drugs with the duration of 12 h significantly induced cell apoptosis, being consistent with previous research results.

2.4 Effects of DHW-51 on caspase3 activity assay

The mechanism of cell apoptosis induced by this drug was elucidated in terms of the protein molecule. First, BT549 cells were treated with DHW-51 of 10, 15, 20 μmol/L for 24 h, respectively. As seen in 1.6, when the drug concentration was 10 μmol/L, the activity level of caspase3 increased. In addition, at the drug concentration of 20 μmol, the activity level of caspase3 significantly increased(Fig.5). Western blot results showed that at the concentration of 20 μmol/L, the expression level of pro-caspase3 was significantly down-regulated (Fig.6), indicating that DHW-51 activated apoptosis-associated protein caspase3.

2.5 Effects of DHW-51 on AKT protein kinase and phosphorylation of BT549 cells

p53/*PTEN* double knockdown human breast cancer cell line BT549 was applied to further exploring the mechanism of BT549 cell apoptosis, finding an increased AKT expression in the cell. Next, we detected the kinetics of AKT and its phosphorylation level in BT549 cells after 20 μmol/L of DHW-51 treatment for 24 h. As shown in Fig.6, the DHW-51 obviously inhibited AKT phosphorylation in



**P<0.01 *vs* control group.

Fig.5 The activity of caspase3 in the cells treated

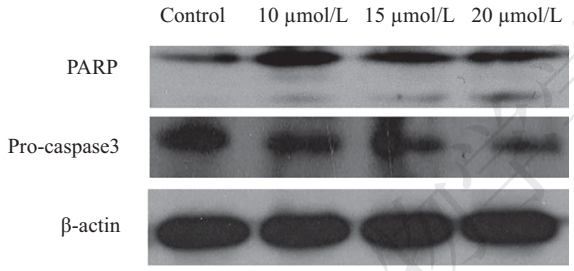


Fig.6 The expression level of pro-caspase3 protein related to apoptosis in cells by Western blot

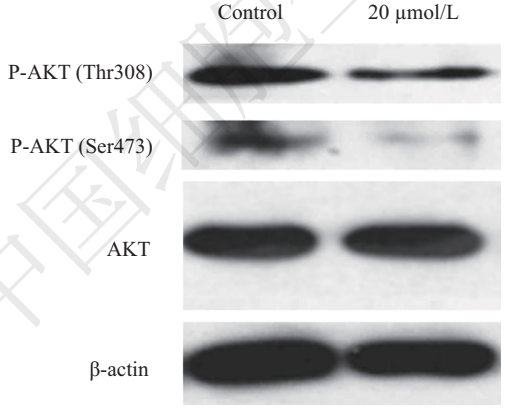


Fig.7 The expression levels of AKT phosphorylation and the AKT protein by Western blot

BT549 cells, containing Ser473 and Thr308 two phosphorylation site, but had no effect on AKT protein expression level. Given that the BT549 cells are *p53*/*PTEN* double knockdown cell lines, we concluded that DHW-51 might become a AKT protein inhibitors which could inhibit the activation of AKT (Fig.7).

3 Discussion

Cancer is one of most important causes of death, human breast cancer remains a serious public health issue in the world[20]. At present, chemotherapy, radiotherapy and surgery are the mainly common treatments in cancer therapy, but these therapies normally lead to severe side effects[21-22]. Therefore, it is significant to develop a cancer therapy that can effectively and selectively kill malignant cells without damaging the normal cells. In this article, we focused on the research on the safety of the new and small molecular compound DHW-51 in cancer treatment.

In this study, 6-(1-(piperidin-4-yl)-1H-pyrazol-

4-yl)-N-(4-(trifluoromethoxy) phenyl) qui-nazolin-4-amine (DHW-51) was a new synthetic small molecular, being selected from our own synthetic library. We reported for the first time that the small molecular compound of sulfonamide classes, DHW-51, could induced the apoptosis of *p53*/*PTEN* knockdown human breast cancer cell line BT549. As shown in the data in this study, DHW-51 with different concentration and duration have the different inhibition to the proliferation in the BT549 cells, indicating that the BT549 cells was sensitive to DHW-51. DHW-51 is a small and safe molecule compound, which has no toxicity to BT549 cells. As mentioned in many literatures, the hemolytic test was a very good method for the selection of drug toxicity. For exploring the potential mechanism, we studied the effect of DHW-51 on apoptosis in BT549 cell lines and found that DHW-51 could induce the apoptosis of tumor cell in a dose- and time-dependent manner. At the same time, the intracellular activity of caspase3 had a significant increase and the protein expression level of pro-caspase3 was significantly down-regulated.

Therefore, PI3K/AKT seemed to be one of the major pathways for regulating the proliferation and apoptotic activities of DHW-51. Actually, DHW-51 obviously inhibited the kinase activity of BT549 cell lines *in vitro* toward AKT phosphorylation and the stability of apoptosis and cell survival were regulated by AKT phosphorylating proteins that were essential in apoptotic and anti-apoptotic mechanisms. It was found in this experiment that DHW-51 could effectively inhibited the protein of AKT phosphorylation in BT549 cell lines, in which there were two phosphorylation sites including Ser473 and Thr308. The above effect was likely caused by an activity of AKT protein kinase for inhibition of BT549 expression, which explained that DHW-51 inhibited BT549 expression due to a higher expression level of AKT. Previous studies also revealed that the canonical role of different drugs regulated AKT signaling pathway, and negatively regulated cell survival by inhibiting AKT, verifying the biological importance of regulatory mechanism[23-25] of microRNA-127 and miR-184 in glioma and breast cancer cells. It was necessary to note that other small molecules reported previously[26-27] could competently reduce the viability of breast cancer cells harboring p53/PTEN, which were likely achieved by targeting AKT. In addition, some other studies investigated the necessity of the AKT signal in breast cancer induced by different oncogenic signals[28-31]. Due to AKT regulates cell proliferation and apoptosis by phosphorylating several targeted proteins, abnormal high level of AKT activity is a feature of human cancer. When being treated with the AKT inhibitor, the essential pro-apoptotic protein had a significant decrease in the AKT phosphorylation[3,32-35]. However, the specific mechanism still needs further exploration.

As demonstrated in previous studies, the constitutive activation of PI3K/AKT signaling pathways cancerated in normal cells and maintained its survival time, growth and metastasis[36]. Thus, we could speculate that DHW-51 might be AKT protein inhibitor, which could achieve the effects of inhibiting tumor cell growth by inhibition of AKT catalytic activity. However, there were numerous limitations in this study. For example, although DHW-51 was supposed to affect some pathways to some extent, the exact details of these mechanisms were still unknown and also it was unclear whether the proposed role of DHW-51 was only limited to the BT549 cells. Therefore, future studies were required to conclude the perfect function of DHW-51 in tumor suppression, particularly in the perspective of activation AKT, which might provide information for the therapeutic/pharmaceutical approaches to target AKT inhibition and might be useful in designing more efficient therapeutic regimens for breast cancer. In summary, this study revealed that DHW-51 had the potential in inhibiting the activity of tumor in human breast cancer BT549 by targeting AKT, but the mechanism and the molecular targets of its chemoactivity were still unclear. We are the first one to investigate the role of DHW-51 in BT549 cell, especially with p53/PTEN. Therefore, to the best of our knowledge, BT549 is a

simple human breast cancer, which could be applied to the proportional studies with other known related cancer cell lines.

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