Hsp90 inhibitor SNX-2112 inhibits cell growth by regulating Bcl-2 family proteins and cyclin proteins in MCF-7 cells

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Abstract

Breast cancer has been a major threat for human health. Tens of Millions of people are die of breast cancer in annual year. Breast cancer has been being one of the most important malignancies, and it is also the second most responsible for cancer mortality all the word, especially among femal cancer patients. Heat shock protein 90 (Hsp90) is highly expressed in tumor cells, and is an ATP-dependent molecular chaperone that can interrelate and interact with numerous proteins. Hsp90 can play a crucial role in numerous cell signaling pathway which are involved in cell proliferation and survival, cell metabolism. SNX-2112, a novel and selective Hsp90 inhibitor, exhibits a favourable anticancer activity. SNX-2112 can effectively inhibit cell growth in diversified cancers, including breast cancer. However, little is known about its mechanism of action in breast cancer and needs to be further elaborated. In this study, we investigated the effects of SNX-2112 in MCF-7 cells. SNX-2112 inhibited cell growth via mediating expression levels of mitochondrial proteins, downregulated Bcl-2 and Bcl-xl, upregulated Bim. Further study suggested that SNX-2112 also regulated cyclin proteins in breast cancer MCF-7 cells, and may be a promosing targeted therapy drug.

Keywords

Hsp90 inbibitor, SNX-2112, Bcl-2 family proteins, cyclin proteins.

1. Introduction

Breast cancer is a arresting health problems in Public Health because of its high and increasing prevalence wordwide. Meanwhile, Breast cancer has been being one of the major causes of morbidity and mortality and giving rise to a noteworthy decrease in life quality[1]. Besides, in accordance with researches, breast cancer has been the most common tumor in female patients[2]. And it is also highly heterogenic and consist of multiple histological subtypes, such as luminal A, luminal B, human epidermal growth factor 2 and basal-like tumors, and breast tumor also has distinct molecular characteristics, including estrogen receptor (ER), progesterone receptor 9 (PR) and human epidermal epidermal growth factor receptor 2 (HER2) expression[3]. Treatment of cancer is regard as be complicated, particulary for breast cancer. Despite promising therapies against breast cancer having been presented and obvious advances having been achieved in the past decades[4]. However, traditional treatment still is the main approach for breast cancer, such as surgical operation, chemotherapy and radiation therapy. And obtaining therapeutic effect still is unsatisfactory, thereby targeted drugs has been proposeed[5].

Mitochondria plays an important role in eukaryotic cells, and their changes of functional are closely related to the occurrence of many diseases[6]. As an important organelle, mitochondria is not only the main source of energy for eukaryotic cells, but also an important carrier of extranuclear DNA (non-nuclear DNA)[7]. Mutation of mitochondrial DNA is closely related to the occurrence of many diseases, such as tumors, neurodegenerative diseases and aging[8-9]. Eukaryotic cells contain approximately 1,000 different proteins in their mitochondria. A small portion of proteins is encoded by mitochondrial DNA, most of the rest are encoded by a nuclear gene[10-11]. Studies have reported

that the functional abnormalities of some nuclear-encoded mitochondrial genes can lead to changes in protein expression, which in turn affect the physiological metabolic processes of cells, including cell proliferation[12].

Heat shock protein 90 (Hsp90) is a ATP-dependent molecular chaperone protein that shows a important role in a series of physiological activity[13]. Hsp90 plays a important roles in protein folding, assembly and degradation processes[14-15], and also serves as a crosstalk to maint the conformation, stability and function of its client proteins, such as Akt, Erk and IKKα[16]. However, these client proteins are involved in cell proliferation and survival which are contributed to development and progression of tumor[16-18]. Meanwhile, Hsp90 is highly expressed in eukaryotes and comprises over 1% of eukaryote total cellular content[17]. A large number of studies have indicated that the expression levels of Hsp90 in tumor tissues is 2-10 fold compared to normal tissues, and which is considered to a distinct charactistic for tumor[19]. Given these functions of Hsp90, inhibition of Hsp90 has become an attractive therapeutic strategy for cancer.

SNX-2112, a novel and selective Hsp90 inhibitor, can binds competitively to the N-terminal ATP binding site of Hsp90, and also exhibited anticancer activity for numerous tumors, including breast cancer[20-21]. Besides, SNX-2112 can achieve a better therapeutic effect than other Hsp90 inhibitors, like 17-AAG[22]. However, little is known about the effects of SNX-2112 on breast cancer cells, and the molecular mechanism of action also needs to be further explore. In this study, we found that SNX-2112 could inhibit cell growth in breast cancer MCF-7 cells. We reported that SNX-2112 could influence regulation of mitochondria-regulated proteins, and downregulated expression levels of anti-apoptosis proteins Bcl-2, Bcl-xl, and upregulated expression levels of pro-apoptosis protein Bim. We also confirmed that SNX-2112 could increase expression levels of cyclin proteins, like p53, p27 and p21.

2. Materials and methods

2.1 Chemicals and reagents

SNX-2112 was synthesized in our lab according to the known procedure previously[23], with the purity above 98.0% (Fig. 1), and 100 mmol/L SNX-2112 stock solutions in dimethyl sulfoxide (DMSO, Sigma) were stored at -20 °C.

All chemicals and reagents were obtained from standard commercial sources. 3-(4, 5-dimethylthiazole -2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma. Anti human or mouse p53, p27, p21, Bim, Bcl-xl, Bad, Bim, β-actin and GAPDH antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Bcl-2 was obtained from GeneTex. Anti-mouse IgG and anti-rabbit IgG were bought from Sigma (St. Louis, MO, USA).

2.2 Cell culture

The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM medium (Gibco, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin and 100 U/mL penicillin (complete medium) at 37 °C in a humidified atmosphere containing 5% CO2. The medium was routinely changed every 2 days and the cells were separated by trypsinisation before reaching confluency.

2.3 MTT assay

Breast cancer cells MCF-7 (4 × 104 cells/well) were seeded in 96-well plates in 100 μL DMEM medium. Following incubation overnight to attach at 37 °C under a 5 % CO2 atmosphere, the old medium was replaced with fresh medium containing varying concentrations of SNX-2112. After further culturing for 12, 24 and 48 h, the culture media was discarded, 10 μL MTT was added to the wells, followed by incubation at 37 °C for 4 h. The supernatant was carefully abandoned, and 100 μL dimethyl sulfoxide was added to per well to dissolve the produced formazan and the plates were

shaken for 15 min. Cell viability was assessed by measuring absorbance using a microplate reader (BIO-RAD, Hercules, CA, USA) at a wavelength of 570 nm. IC_{50} values for each cell line were determined by comparing treated and untreated cells.

2.4 Colony formation assay

Colony formation assay was carried out according to Park et al's work[24], MCF-7 cells (2×10^3 cells/well) were plated in 6-well plates in 2 mL DMEM medium, cultured overnight to attach and exposed to different concentrations of SNX-2112 (0.75, 6 μM) for 48 h. After washing with PBS, the cells were incubated in drug-free complete medium for 2 weeks. Subsequently, cell colonies were fixed with 70 % ethanol at 4 °C and stained with 5 % Gentian Violet (Sigma) at room temperature

2.5 Western blotting

MCF-7 cells were exposed to varying concentrations of SNX-2112 for 48 h previously described and processed for western blot analysis. Cells were collected and washed twice with ice-cold PBS. Then cells were lysed in RIPA buffer, cracked by ultrasonication, centrifuged at 14,000 g for 30 min, the supernatants were collected and concentrations of proteins were assessed by BCA Protein Assay Kit (Beyotime, Shanghai, China). Then equal amounts of denatured proteins were resolved on SDS-PAGE gels. Proteins were transferred to PVDF membranes (Millipore, Boston, MA), which were blocked with 5% skim milk for 1 h at room temperature, then incubated with primary antibodies overnight at 4 °C. The membranes were washed four times with Tris-buffered saline containing 0.1% Tween 20 solution (TBST) and incubated with antu-rabbit or anti-mouse IgG for 1 h at room temperature. Protein bands were visualized by enhanced chemiluminescence (Millipore, Waltham, MA, USA) and analyzed by densitometry.

2.6 Statistical analysis

In this study, all datas were Mean ± SD of triplicate and repeated for 3 times. Differences between two groups were analyzed using the two-tailed Student's t-test and groups of three or more were analyzed using one-way ANOVA multiple comparisons. *P < 0.05 and **P < 0.01 were considered statistically significant.

3. Results

3.1 Effect of SNX-2112 on cell growth of MCF-7 cells.

The chemical structure of SNX-2112 is shown in Fig. 1. And some researches have reported that SNX-2112 exhibits an anti-tumor activity, including breast cancer[25]. To explore the effect of SNX-2112 on the cell viability of human breast cancer cells, we carried out MTT assay to detect breast cancer MCF-7 cells. MCF-7 cells were exposed to various of concentrations of SNX-2112 for 12, 24 and 48 h. As shown in Table 1 and Fig. 2, SNX-2112 inhibited significantly cell proliferation in MCF-7 cells with IC_{50} values in the submicromolar range after treatment for 48 h. And the IC_{50} value of 24 and 48 h was 0.71 and 0.386 μM for MCF-7 cells,respectively.

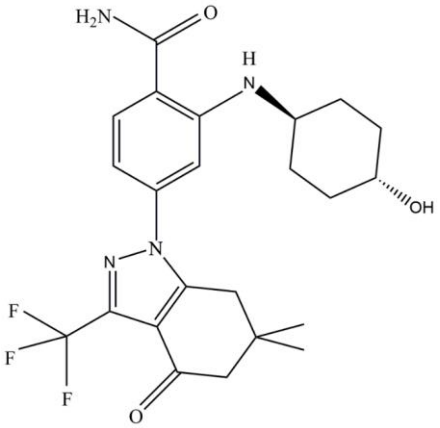

Fig. 1 Chemical structure of SNX-2112

Table 1 The IC50 values of SNX-2112 in breast cancer MCF-7 cells.

Time / Cell line	IC50 (μM)		
	12 h	24 h	48 h
MCF-7	-	0.712	0.386

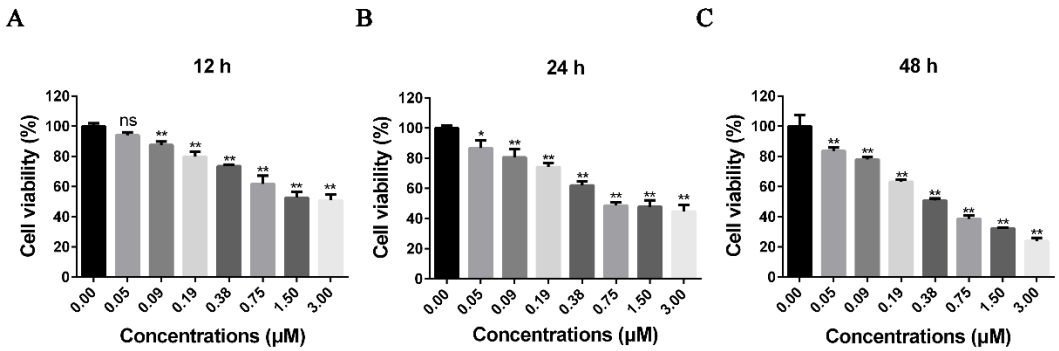

Fig. 2 Effect of SNX-2112 on cell growth of MCF-7 cells. Cells (4×10^4 cells/mL) were treated with a range of concentrations of SNX-2112 (0, 0.05, 0.09, 0.19, 0.38, 0.75, 1.50 and 3.00 μM) for 12 (A), 24 (B) and 48 h (C). The MTT assay was used to detect the cell viability of MCF-7 cells. Three independent replicates were set up and data were represented as mean ± SD (n=3).

3.2 Effect of SNX-2112 on the colony-formation ability of MCF-7 cells.

To further confirm the inhibition of SNX-2112 for the cell growth of breast cancer cells, we performed the colony formation assay to detect the colony-formation ability of human breast cancer cells. We observed that the colony-formation ability of MCF-7 cells was observably decreased after treatment with SNX-2112 (Fig. 3). Taken together, these results suggested that SNX-2112 could inhibit the cell growth in MCF-7 cells.

Fig. 3 SNX-2112 decrease the colony-formation ability of MCF-7 cells. MCF-7 cells (2000 cells/mL) were treated with different concentrations (0.1875, 0.75 μM) of SNX-2112 for 2 weeks. SNX-2112 decreased the ability of clone formation of breast cancer cells by crystal violet staining experiment. Three independent replicates were set up and data were represented as mean ± SD (n=3).

3.3 Effect of SNX-2112 on Bcl-2 family proteins in MCF-7 cells.

According to other studies, we know that changes of mitochondria-related proteins is involved in cell growth[26]. Based on the previous results, we known that SNX-2112 could surpress cell growth in

MCF-7 cells. Therefore, in order to better demonstrate that SNX-2112 inhibited cell growth by regulating mitochondria-regulated proteins in breast cancer cells, we also detected expression levels of mitochondria-related proteins by Western blot. These results of experiments suggested that SNX-2112 down-regulated expression levels of anti-apoptotic proteins Bcl-2 and Mcl-1, upregulated expression levels of pro-apoptotic proteins Bad and Bim (Fig. 4). Comprehensive analysis showed that SNX-2112 did inhibit cell growth via mediating expression levels of mitochondria-related proteins in breast cancer MCF-7 cells.

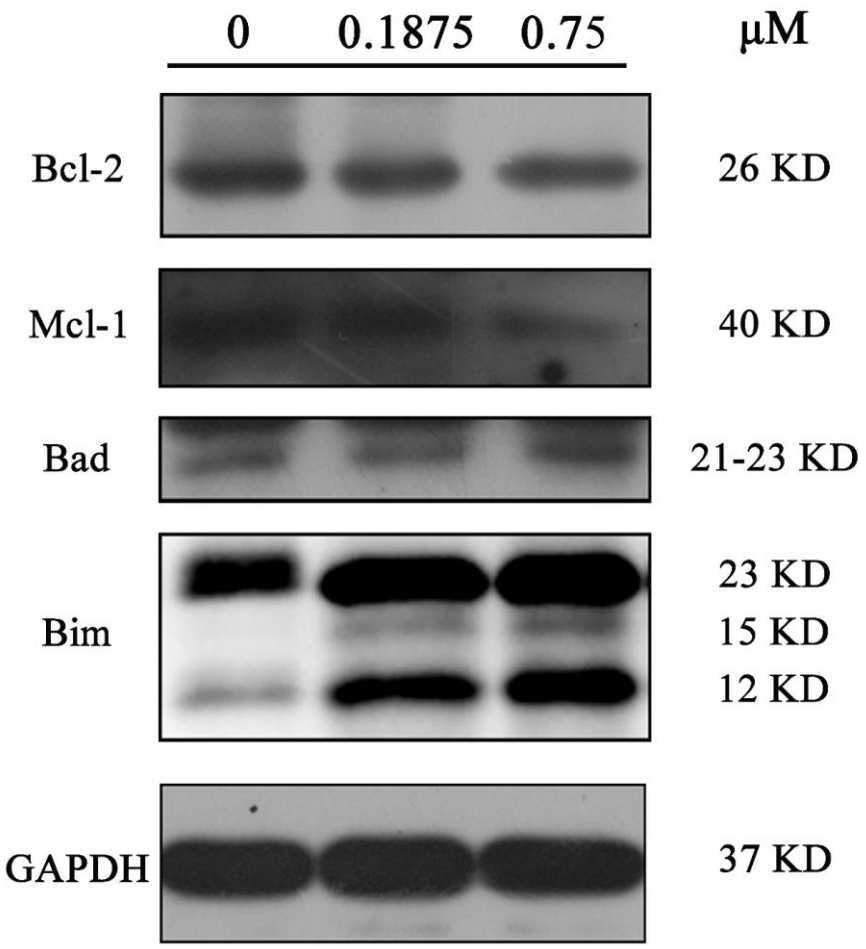

Fig.4 Effects of SNX-2112 on expression levels of mitochondria-related proteins in MCF-7 cells. SNX-2112 downregulated expression levels of anti-proteins and upregulated expression levels of pro-apoptosis proteins. Three independent replicates were set up and data were represented as mean ± SD (n=3).

3.4 Effect of SNX-2112 on the cyclin proteins in MCF-7 cells.

A large number of studies have shown that p53 is a tumor suppressor gene, which can lead to cell growth arrest, complete DNA damage, survival and apoptosis in tumor cells [27]. p21 is a cytokine-dependent kinase inhibitor in downstream of p53 and is closely related to tumor suppression. p21 and p53 together constitute the G1 / S checkpoint in the cell cycle. Once the cell is damaged, p53 stops the cell from growing and causes the cell to rest and heal itself [28]. p27 is also a negative regulator of the G1/S phase transformation, and has the same function as p21 but also binds to cyclin, leading to cycle arrest and assisting in DNA repair[29]. Therefore, we performed Western blot assay to detect expression levels of p53, p21 and p27. These results showed that SNX-2112 can up-regulate expression levels of p53, p21 and p27 (Fig. 5). Therefore, the result indicated that SNX-2112 could suppress cell growth through the p53 signaling pathway.

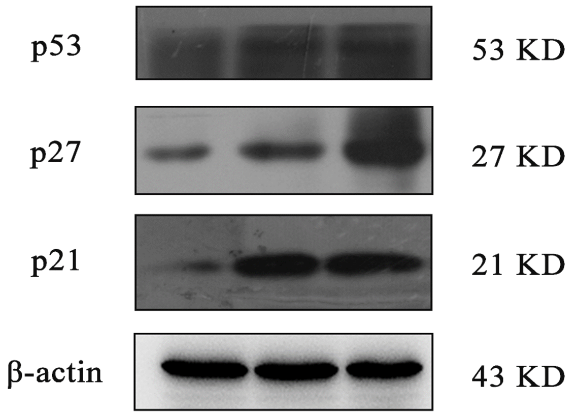

Fig. 5 Effects of SNX-2112 on cyclin proteins in MCF-7 cells. As shown, SNX-2112 increased expression levels of p53, p21 and p27. Three independent replicates were set up and data were represented as mean ± SD (n=3).

4. Discussion

Breast cancer is the most common malignant tumor in women. And traditional treatment methods have not only achieve a good effect, but also have brought great side effects for cancer patients[1]. Chemotherapy drugs, as one of the main approaches for the treatment of clinical malignant tumors, have achieved a better efficacy. And this also attracts researchers to explore[30]. At present, a large number of studies have shown that Hsp90 is highly expressed in tumor tissues, which also indicates that Hsp90 is a potential therapeutic target for tumor [31]. As a novel Hsp90 inhibitor, SNX-2112 has also become more and more attractive. It has been reported that SNX-2112 has anti-tumor activity against tumors, including breast cancer [32].

In this study, we firstly detected the anti-tumor activity of SNX-2112 by MTT assay. The result showed that SNX-2112 could significantly inhibit the cell proliferation of breast cancer cells, and further validated by the colony formation assay. Abundant studies suggest that the cell growth is correlated with the change of expression levels of anti-apoptotic and pro-apoptotic proteins in the mitochondria[33,34] Therefore, we detected these proteins by Western blot assay, we found that SNX-2112 upregulated expression levels of pro-apoptotic proteins Bim and Bad, and down-regulated anti-apoptotic proteins Bcl-2 and Mcl-1. Therefore, these results further confirmed that mitochondria-related proteins was involved in inhibition for cell growth by SNX-2112.

It is reported that p53 is a tumor suppressor gene, and the protein encoded by p53 is another important substrate of Chk2 kinase during DNA damage. When DNA damage occurs, the expression level of p53 will increases and the antitumor activity increases [35-36]. p21, a downstream protein of p53, can cause cell cycle arrest and plays an important role in DNA repairing. p27 is a protein associated with p21 that has the same role as p21 [37] Therefore, in this study, we also detected expression levels of p53, p21 and p27 by Western blot. It was found that expression levels of p53, p21 and p27 were up-regulated after cells were treated with SNX-2112. Based on these experimental results, we found that SNX-2112 displayed excellent antitumor activity in MCF-7 breast cancer cells, which could inhibit breast cancer cell proliferation through p53 signaling pathway and mediating expression of Bcl-2 familiy proteins.

5. Clnclusion

In summary, our results indicated that the Hsp90 inhibitor SNX-2112 exhibited a better antitumor activity against breast cancer cells in vitro. We confirmed that SNX-2112 could sigbificantly inhibit the cell growth in breast cancer MCF-7 cells. Morever, we detected mitochondria-related proteins to further prove these. Besides, we also found which expression levels of cyclin proteins were changed followed treatment of SNX-2112, such as p53, p21 and p27. Taken above results, we finally suggested

that SNX-2112 inhibited the cell growth of breast cancer cells via mediating expression of Bcl-2 familiy proteins and cyclin proteins, and this study shown that SNX-2112 would be a promosing agent for breast cancer therapy.

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