SNX-2112, a Novel Hsp90 Inhibitor, Induces G2/M Cell Cycle Arrest and Apoptosis in MCF-7 Cells

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SNX-2112 is a heat shock protein 90 (Hsp90) inhibitor with anticancer properties currently in clinical trials. This study investigated the effects of SNX-2112 on inhibition of cell growth, the cell cycle, and apoptosis in MCF-7 human breast cancer cells, in addition to the various molecular mechanisms. The results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometric analysis suggest that SNX-2112 inhibits cell growth in a time- and dose-dependent manner more potently than 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a traditional Hsp90 inhibitor, probably as a result of cell-cycle arrest at the G2/M phase and the induction of apoptosis. Downregulation of Bcl-2 and Bcl-xL, upregulation of Bax, cleavage of caspase-9 and poly (ADP-ribose) polymerase (PARP), and degradation of the breast cancer-related client proteins human epidermal growth factor receptor-2 (HER2), Akt, Raf-1, and inhibitor of nuclear factor kappa-B kinase (IKK) were observed in SNX-2112 treated cells by Western blot assay. These findings suggest that the molecular mechanisms of cell-growth inhibition by SNX-2112 involve activation of the mitochondrial apoptotic pathway and the degradation of breast cancer-related proteins.

Key words: hsp90 shock protein 90 (Hsp90); SNX-2112; MCF-7; cell cycle arrest; apoptosis

Heat shock protein 90 (Hsp90) is a molecular chaperone with a key role in the maintenance of the conformation, stability, and function of its client proteins.1 Most of the client proteins associated with Hsp90 are protein kinases (e.g., human epidermal growth factor receptor-2 [HER2], Akt, Raf-1, and inhibitor of nuclear factor kappa-B kinase alpha [IKKα] or transcription factors that are important in cellular carcinogenesis.2) Given the crucial roles of Hsp90 client proteins in cell-growth arrest and apoptosis in cancer cells, inhibition of Hsp90 is an attractive therapeutic strategy for cancer.

Ansamycin antibiotic geldanamycin (GA), the first natural Hsp90 inhibitor, binds to an ATP pocket in the N-terminal domain of the protein.3 17-Allylamino-17-demethoxygeldanamycin (17-AAG), a derived inhibitor of GA, has entered phase III clinical trials, highlighting the potential use of Hsp90 inhibitors as cancer therapies.4,5 GA and its analog, 17-AAG, inhibit cell growth and induce cell-cycle arrest and apoptosis, which are associated with the degradation of Hsp90 client proteins in various tumor cells,5 but GA is too toxic for clinical use and 17-AAG has several potential limitations, including poor solubility, limited bioavailability, and hepatotoxicity.6,7 This has led to efforts to identify more effective clinical agents. Recently, a novel class of small-molecule Hsp90 inhibitors was reported, including SNX-2112. SNX-2112 binds competitively to the N-terminal ATP pocket of Hsp90 and is highly potent against various cancers when delivered orally via its prodrug, SNX-5422.7 Compared with 17-AAG, whose poor solubility has necessitated the use of DMSO in clinical applications, SNX-2112 has a water-soluble, oral prodrug, SNX-5542, which exhibited >37% oral bioavailability in rats and >75% in mice.8 Phase I clinical trials of SNX-5422 prepared by Pfizer are currently recruiting participants with refractory hematologic and solid tumor malignancies.9

Previously, we reported that the unique pharmacodynamic and pharmacokinetic properties of SNX-2112 account for potent antitumor activity against various cancers.10-12 Here we report evidence that SNX-2112 can inhibit cell growth by inducing apoptosis and G2/M cell-cycle arrest in MCF-7 human breast cancer cells, and that the molecular mechanisms might involve triggering of the mitochondrial apoptotic pathway and degradation of breast cancer-related client proteins.

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Abbreviations: 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HER2, human epidermal growth factor receptor-2; Hsp90, heat shock protein 90; IKK, nuclear factor kappa-B kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase
Materials and Methods

Reagents, antibodies, and cells. SNX-2112 was synthesized as described previously, with a purity of more than 98.0% as measured by HPLC. 17-AAG was purchased from Alexis Biochemicals (San Diego, CA). SNX-2112 and 17-AAG, each 10 mM in dimethyl sulfoxide (DMSO), were stored at 4°C and −20°C respectively. The following antibodies were purchased as indicated: anti-Bcl-2, Bax, Bcl-xl, cleaved caspase-9, poly (ADP-ribose) polymerase (PARP), and HER2 from Cell Signaling Technology (Beverly, MA); anti-Akt, anti-IKK, and anti-Raf-1 from Epitomics (Burlingame, CA); and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Millipore (Billerica, MA). Human breast cancer MCF-7 cells (ATCC, Manassas, VA) were grown in minimum essential medium (MEM) with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL of penicillin and streptomycin.

MTT assay. Anti-proliferation effects on the MCF-7 cell line were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MCF-7 cells were seeded into 96-well plates (5 × 10^3 cells/well) and allowed to adhere overnight. The cells were then incubated with SNX-2112 or 17-AAG at various concentrations (0–10 μM) for 24, 48, or 72 h, and then 20 μL of MEM with 10% FBS and 5 mg/mL MTT was added. The precipitated formazan crystals were dissolved in 100 μL of DMSO. Cell viability was assessed at 570 nm for each well, with 630 nm as reference wavelength, and calculated as follows: (optical density of experimental sample/optical density of control) × 100%.

Cell-cycle analysis. Cell-cycle analyses were performed by DNA staining with propidium iodide (PI). Briefly, cells were incubated in culture medium alone or in culture medium containing 0–10 μM SNX-2112 or 17-AAG for 48 h. They were harvested in cold PBS, fixed in 70%, ethanol and stored at 4°C overnight. The fixed cells were washed once with PBS and resuspended in 1 mL of PI staining reagent (50 mg/mL containing 100 μg/mL RNase), and then incubated in the dark for 30 min. The percentage of cells in each phase of the cell cycle was measured by fluorescence activated cell sorting using a FACScan flow cytometer (Becton Dickinson) and WinList software (Verity, Topsham, ME).

Assessment of apoptosis. Apoptosis was measured by staining with annexin V-fluorescein isothiocyanate (FITC) (an apoptotic cell marker) and PI (a necrotic cell marker). Briefly, cells were incubated in culture medium alone or in culture medium containing 0–10 μM SNX-2112 or 17-AAG for 48 h, harvested, washed twice, and resuspended in 500 μL of PBS plus annexin V-FITC and PI (BioVision, Mountain View, CA). By this method, at least 1 × 10^4 cells per sample should be analyzable on flow cytometry. The degree of apoptosis was determined as the percentage of cells stained with annexin V-FITC/PI.

Western blot analysis. Proteins from MCF-7 cells incubated with 1.0 μM SNX-2112 for 0, 12, 24, or 48 h were extracted in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). The total protein concentrations of whole-cell lysates were determined using a BCA Protein Assay Kit (Beyotime). Equal amounts of protein samples from whole-cell lysates were loaded on an SDS–PAGE gel (8%–15%). After electrophoresis, the protein was transferred to a PVDF membrane (Millipore, Boston, MA), probed with primary antibodies, and then incubated with horseradish peroxidase conjugated secondary antibodies. Specific protein bands were visualized using an enhanced chemiluminescence technique (Beyotime) and imaged by autoradiography. Any differences in protein loading were normalized to the GAPDH control.

Statistical analysis. Data were expressed as mean ± standard deviation (SD). Statistical analysis of the data was performed by one-way ANOVA followed by Tukey’s multiple comparison test. Results were expressed as the mean ± SD and were considered significant at p < 0.05 or p < 0.01.

Results

SNX-2112 was a more potent inhibitor of MCF-7 cell growth than 17-AAG

Inhibition of cell growth by SNX-2112 and 17-AAG was measured by MTT assay using human breast cancer MCF-7 cells treated with concentrations of 0.08–10 μM for 24, 48, or 72 h. As shown in Fig. 1, both SNX-2112 and 17-AAG significantly inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. MCF-7 cells were more sensitive to SNX-2112 than to 17-AAG, with half-maximal inhibitory concentrations of 1.65 and 4.07 μM at 72 h respectively. Cell viabilities were 64.59%, 58.23%, and 51.03% following treatment with 1.25 μM SNX-2112 for 24, 48, and 72 h respectively, and 70.92%, 57.71%, and 52.47% following treatment with 17-AAG. For the remaining experiments, we adopted 1 μM as the optimal concentration. The data imply that SNX-2112 has a greater growth inhibitory effect than 17-AAG on MCF-7 cells in vitro.

SNX-2112 caused G2/M cell-cycle arrest in MCF-7 cells

To probe the mechanism of cell growth inhibition by SNX-2112, we examined its effects on the cell cycle by flow cytometry. Cells treated with SNX-2112 or 17-AAG (0.1, 1, or 10 μM) for 48 h were subjected to flow cytometric analysis after PI staining. As shown in Fig. 2, the percentage of cells in the G2/M phase increased in a dose-dependent manner with both SNX-2112 and 17-AAG. After 48 h, the percentage of G2/M phase cells in the control was 7.4%. With 1 μM SNX-2112 or 17-AAG, the percentages of G2/M phase cells significantly increased, to 30.5% (p < 0.01) and 26.9% (p < 0.01) respectively. At the highest concentration (10 μM) of SNX-2112 and of 17-AAG, the percentages of cells in the G2/M phase were 30.6% (p < 0.01) and...
27.6% (p < 0.01), and at the lowest concentration (0.1 mM) they were 28.7% (p < 0.01) and 25.5% (p < 0.01). These results indicate that cell-cycle arrest at the G2/M checkpoint is due to the growth inhibitory effect of SNX-2112 on MCF-7 cells.

SNX-2112 induced MCF-7 cell apoptosis through the mitochondrial apoptotic pathway

To determine whether SNX-2112 induces apoptosis in MCF-7 cells, annexin-V and PI double staining was done. After treatment with 0.1, 1, or 10 μM SNX-2112 or 17-AAG for 48 h, the cells were double-stained and assayed by flow cytometry. The degree of apoptosis was expressed as the sum of the percentages of cells in the lower right and upper right quadrants as shown in Fig. 3. After 48 h, the rate of apoptosis in the control was 9.7%. Of the cells treated with 0.1, 1, or 10 μM SNX-2112, 20.6% (p < 0.05), 31.2% (p < 0.01) and 50.6% (p < 0.01) respectively, were apoptotic; of those treated with 17-AAG, 23.0% (p < 0.05), 29.8% (p < 0.01), and

33.0% (p < 0.01) were apoptotic at the same doses (Fig. 3). These results suggest that SNX-2112 induced apoptosis in the MCF-7 cells and had a more potent effect than 17-AAG, except for the 0.1 mM treatment.

To explore further the molecular mechanisms involved in the induction of apoptosis by SNX-2112, we measured the levels of the Bcl-2 family proteins Bcl-2, Bcl-xL, and Bax and of the major proteins in the mitochondrial apoptotic pathway, caspase-9 and PARP. Western blot analysis indicated that Bax was upregulated, Bcl-2 and Bcl-xL were downregulated, and caspase-9 and PARP were cleaved in a time-dependent manner in the MCF-7 cells treated with 1 μM SNX-2112 (Fig. 4A). The final concentration of DMSO in this culture system was 0.01%. This had no significant effect on cell growth kinetics or apoptosis in MCF-7 cells. This suggests that SNX-2112 induces apoptosis in MCF-7 cells by regulating the expression of Bcl-2 family proteins and activation of the mitochondrial apoptotic pathway.
SNX-2112 targeted degradation of Hsp90 client proteins in MCF-7 cells

We further investigated the expression of Hsp90 client proteins HER2, Akt, Raf-1, and IKKα, which are crucial for the growth of breast cancer cells. Western blot analysis showed that the levels of these proteins fell in a time-dependent manner (Fig. 4B). After treatment with 1 μM SNX-2112 for 48 h, the expression of HER2, Akt, Raf-1, and IKKα significantly decreased, to 14.9% (p < 0.01), 2.9% (p < 0.01), 16.4% (p < 0.01), and 19.9% (p < 0.01) respectively. This indicates that inhibition of MCF-7 cell growth by SNX-2112 is associated with downregulation of HER2, Akt, Raf-1, and IKKα.

Discussion

Breast cancer is one of the most common malignancies worldwide. In our in vitro study, we found for the first time that SNX-2112, a novel Hsp90 inhibitor, caused inhibition of cell growth (Fig. 1), accumulation of cells in the G2/M phase (Fig. 2), and apoptosis (Fig. 3) in a time- and dose-dependent manner in MCF-7 human breast cancer cells. These effects were more potent than those produced by 17-AAG. Furthermore, downregulation of Bcl-2 and Bcl-xL, upregulation of Bax, and cleavage of caspase-9 and PARP were observed in the MCF-7 cells treated with SNX-2112 (Fig. 4A). This suggests that effects on the mitochondrial apoptotic pathway might be responsible for the apoptotic activity of SNX-2112. We also investigated the expression of Hsp90 client proteins HER2, Akt, Raf-1, and IKKα with regard to MCF-7 cell growth (Fig. 4B). The results indicate that the effect of SNX-2112 on MCF-7 cell growth is associated with downregulation of these proteins.

As discovered by MTT assay, SNX-2112 reduced the viability of the MCF-7 cells in a time-dependent manner and more potently than 17-AAG (Fig. 1). In addition, SNX-2112 reduced cell proliferation in a concentration-dependent manner within a range of 0.08 μM to 1 μM, but not at higher concentrations (1–10 μM). After treatment with SNX-2112 or 17-AAG, cell growth inhibition reached a plateau at doses of 1–10 μM, especially in the cells treated for 24 h. This suggests that the antitumor mechanisms of SNX-2112 and 17-AAG differ from those of cytotoxic drugs, which do not exhibit limited dose-effect relationships.

SNX-2112 causes cell-cycle arrest in the G2/M phase in MCF-7 cells. Most Hsp90 inhibitors induce G1 phase arrest, whereas others arrest the cell cycle at the G/S or the G2/M phase in various human cancer cells. To determine whether the growth inhibitory effect of SNX-2112 was caused by cell-cycle arrest, we analyzed MCF-7 cells treated with SNX-2112 or 17-AAG by flow cytometry. We found that on treatment with SNX-2112, the percentage of cells in the G2/M phase increased by more than 4-fold as compared to control. SNX-2112 had a more potent effect than 17-AAG (Fig. 2). As the G2/M percentage increased, the S phase percentage decreased and the G0/G1 percentage showed a slight change. These results are consistent with the G2/M arrest induced by SNX-2112 in MDA-468 breast cancer cells, but not with the G0/G1 arrest reported for BT474 breast cancer cells. The Hsp90 client protein p53 might play a key role in cell-cycle distribution in MCF-7 cells and might inhibit another Hsp90 client protein HER2 in BT474 cells, which might cause the cell-cycle to arrest at the G1 phase remarkably. SNX-2112 might induce G2/M arrest by similar mechanisms in MCF-7 and MDA-468 cells. This indicates that G2/M arrest...
might be a mechanism by which SNX-2112 inhibits MCF-7 cell growth. Additional studies are needed to investigate effectively the molecular mechanism arresting MCF-7 cells in the G2/M phase of the cell cycle.

Various antitumor agents induce apoptosis or programmed cell death. In the present study, we found that SNX-2112 not only inhibits MCF-7 cell growth and blocks cell cycle progression, but also induces apoptosis (Fig. 3A). Members of the Bcl-2 gene family such as Bcl-2, Bcl-xL, and Bax are widely considered to be regulators of cell death and can influence the mitochondria directly.25) For example, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis.24) Hence we measured the MCF-7 cell levels of Bcl-2, Bcl-xL, and Bax and the major proteins of the mitochondrial apoptotic pathway, caspase-9 and PARP. As shown in Fig. 2, suppression of Bcl-2 and Bcl-xL activity, upregulation of Bax, and cleavage of caspase-9 and PARP were observed by Western blot analysis (Fig. 3B). Our results suggest that SNX-2112 drives apoptosis by regulating the expression of Bcl-2 family proteins and activation of the mitochondrial apoptotic pathway. Thus both G2/M arrest and the mitochondrial apoptotic pathway might be responsible for the anticancer effects of SNX-2112 in breast cancer cells.

A consequence of the inhibition of Hsp90 in cancer cells is degradation of Hsp90 client proteins. It is widely accepted that this might be the upstream mechanism of the inhibition of cell proliferation. There are many Hsp90 client proteins, but we studied the effects of SNX-2112 on the levels of HER2, Akt, Raf-1, and IKKα. These proteins are of interest because inhibition of them has been associated with inhibition of the proliferation of human breast cancer cells.25,26) For example, HER2 has oncogenic properties and is overexpressed in many human tumors, including approximately 30% of human breast cancers.27) Overexpression of HER2 might modulate bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells.28) Dysregulated signaling by the HER2 network has been causally associated with early transformation of mammary epithelial cells as well as with increased breast cancer cell proliferation and shorter survival in patients with mammary carcinomas.29,30) Akt has a wide range of downstream targets that regulate tumor-associated cell processes, including cell growth, cell-cycle progression, survival, migration, epithelial-mesenchymal transition, and angiogenesis. Overexpression of Akt might phosphorylate Bad, prevent it from binding to Bcl-XL, and inhibit the release of cytochrome c induced by paclitaxel.31) Blockage of Akt signaling results in apoptosis and inhibition of the growth of tumor cells.32) The IKK complex plays a central role in nuclear factor gamma B activation and has various biological effects on cancer cells.33) Raf isoforms are intermediaries in gamma B activation and has various biological effects.25,26) For example, the ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis.24) Hence we measured the MCF-7 cell levels of Bcl-2, Bcl-xL, and Bax and the major proteins of the mitochondrial apoptotic pathway, caspase-9 and PARP. As shown in Fig. 2, suppression of Bcl-2 and Bcl-xL activity, upregulation of Bax, and cleavage of caspase-9 and PARP were observed by Western blot analysis (Fig. 3B). Our results suggest that SNX-2112 drives apoptosis by regulating the expression of Bcl-2 family proteins and activation of the mitochondrial apoptotic pathway. Thus both G2/M arrest and the mitochondrial apoptotic pathway might be responsible for the anticancer effects of SNX-2112 in breast cancer cells.

In summary, our findings indicate that SNX-2112 has a substantially greater growth-inhibitory effect than 17-AAG on MCF-7 human breast cancer cells in vitro and thus might be more potent than 17-AAG in the treatment of human breast cancer. The superior efficacy of SNX-2112 is due to induced G2/M arrest and apoptosis, which inhibit MCF-7 cell growth through numerous mechanisms at different levels. Possible molecular mechanisms include triggering of the mitochondrial apoptotic pathway and the degradation of breast cancer-related client proteins. These mechanisms might provide a foundation for further clinical applications of SNX-2112.

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