Combination of SNX-2112 with 5-FU exhibits antagonistic effect in esophageal cancer cells

YUTING LIU1*, XIAO WANG1*, YING WANG2, YI ZHANG1, KAI ZHENG3, HAIZHAO YAN1, LI ZHANG1, WENBO CHEN1, XIAOYAN WANG1, QIUYING LIU1, SHAOXIANG WANG3 and YIFEI WANG1

1Guangzhou Jinan Biomedicine Research and Development Center, National Engineering Research Center of Genetic Medicine,  
2Department of Biotechnology, Jinan University, Guangzhou 510632;  
3School of Medicine, Shenzhen University, Shenzhen 518060, P.R. China

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Abstract. The low efficacy of single-drug chemotherapy forms the basis for combination therapy in esophageal squamous cell carcinoma. SNX-2112, a selective heat shock protein 90 (Hsp90) inhibitor, was recently reported as being effective in combination with cisplatin and paclitaxel. In this study, we investigated the effect of SNX-2112 in combination with 5-fluorouracil (5-FU), another first-line anticancer drug, in esophageal cancer. Unexpectedly, tetrazolium assay revealed that the combination of SNX-2112 with 5-FU exhibited antagonistic effect. Flow cytometry revealed that the SNX-2112 and 5-FU combination greatly decreased the number of G2/M cells compared to SNX-2112-only treatment in Eca-109 cells. This effect might be related to the altered mRNA level of cyclin-related genes including cyclin D1, Chk2 and Cdk4. Further, 5-FU attenuated SNX-2112-induced apoptosis by decreasing poly(ADP-ribose) polymerase (PARP) cleavage and inactivating caspase-3, -8 and -9. Additionally, 5-FU suppressed the SNX-2112-induced decrease of mitochondrial membrane potential. Moreover, 5-FU partly recovered Hsp90 client proteins, including Akt, p-Akt, inhibitor of xB kinase (IKK)x, extracellular signal-regulated kinase (ERK)1/2, and glycogen synthase kinase (GSK)-3β, which SNX-2112 had downregulated. Taken together, this is the first report that the combination of SNX-2112 with 5-FU exhibited antagonistic effect in esophageal cancer cells by affecting growth inhibition, cell cycle, apoptosis, and Hsp90 client proteins, suggesting that care is required in the clinical application of combined SNX-2112 and 5-FU.

Introduction

Despite significant reductions in esophageal cancer rates in association with lifestyle changes, esophageal cancer mortality remains high worldwide, and incidence, especially that of esophageal squamous cell carcinoma (ESCC) is increasing in China (1,2). Chemotherapy is the most common treatment option for improving the poor survival rate in advanced esophageal cancer (3). The first-line drugs in the clinical treatment of esophageal cancer are, sequentially, cisplatin, 5-fluorouracil (5-FU), and paclitaxel (3). However, single-agent chemotherapy is not effective for esophageal cancer because of natural resistance and the development of resistance (4). In esophageal cancer, many studies have demonstrated that combination therapy is more effective than single-drug therapy (3,5,6).

Several heat shock protein 90 (Hsp90) inhibitors that bind to the N-terminal ATP pocket of Hsp90 have entered clinical trials. Hsp90 is a crucial molecular chaperone for protein folding and stabilization that is considered a promising target for anticancer therapy (7). Hsp90 may be a target for esophageal cancer treatment because it and its client proteins are always overexpressed in several ESCC cell lines and patient tissues (8). Previously, we reported that the novel Hsp90 inhibitor BJ-B11 had potent antitumor activity by inducing cell cycle arrest, apoptosis, and autophagy in human ESCC Eca-109 cells (9). Liu et al reported that Hsp90 antisense RNA led to cell cycle changes and increased sensitivity to various chemotherapeutic agents in the same cell line (10). Wu et al suggested that 17-AAG, a traditional Hsp90 inhibitor, effectively inhibited proliferation and viability in other ESCC lines (11). Similarly, NVP-AUY922, another novel Hsp90 inhibitor, was a potent inhibitor of proliferation in esophageal cancer TE-4 cells (12). These reports provide a rationale for current preclinical efforts targeting Hsp90 to treat ESCC.

SNX-2112, a selective Hsp90 inhibitor, has broad antitumor activity and has entered phase I clinical trials in solid tumors and lymphoma (13). In previous studies, we identified the anticancer effect of SNX-2112 in breast cancer, hepatocel-
ular carcinoma, and leukemia (14-16). Recently, SNX-2112 was reported as being effective in combination with cisplatin or paclitaxel in head and neck SCC (17). However, there have been no reports on SNX-2112 in combination with 5-FU, another widely used anticancer drug, in esophageal cancer. We examined the effects of SNX-2112 combined with 5-FU in esophageal cancer Eca-109 cells by detecting cell growth, cell cycle, apoptosis, and Hsp90 client proteins. This study may provide guidance for the clinical application of Hsp90 inhibitors.

Materials and methods

Reagents. SNX-2112 was synthesized as previously described in our lab with >98.0% purity; we stored 10 mM SNX-2112 stock solution in dimethyl sulfoxide (DMSO) at -20˚C (18). We purchased 5-FU, 3-(4, 5-diethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) from Sigma-Aldrich (St. Louis, MO, USA). An Annexin V-fluorescein isothiocyanate-propidium iodide (FITC/PI) staining kit was purchased from Beyotime (Haimen, China). RPMI-1640 medium and DMEM were purchased from Gibco (Carlsbad, CA, USA). Heat-inactivated fetal bovine serum (FBS) was provided by the Sijiqing Co. (Hangzhou, China). Antibodies against caspase-3, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), Akt, phosphorylated (p)-Akt, inhibitor of β kinase (IKK), extracellular signal-regulated kinase (ERK), β2, glycogen synthase kinase (GSK)-3β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. We cultured Eca-109 and EC-9706 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) in RPMI-1640 or DMEM medium supplemented with 10% FBS and 100 U/ml penicillin/streptomycin in 5% CO2 at 37˚C.

Cell viability assay. Cell viability was assessed with the MTT assay. Exponentially growing cells (~3,500/well in 100 µl medium) were plated in 96-well culture plates, cultured overnight, and incubated with a series of concentrations of SNX-2112 (0-2 µM) or 5-FU (0-100 µg/ml) for 48 h. After adding 10 µl MTT solution per well, the plates were incubated at 37˚C for 4 h, the medium removed, formazan crystals solubilized in 100 µl DMSO/well, and the absorbance values read at 570 nm. The inhibition ratio was calculated as follows:

\[ \frac{A_{\text{control}} - A_{\text{treated}}}{A_{\text{control}}} \times 100\% \]

where \( A_{\text{treated}} \) and \( A_{\text{control}} \) are the absorbance of the treated and control groups after 48-h incubation, respectively.

Calculation of the combination effect index. We determined the inhibitory effects of SNX-2112 and 5-FU using the MTT assay. We used the combination index (CI) described by Chou and Talalay for analysis (19), performed using CalcuSyn software (BioSoft, Oxford, UK). CI<1 indicates synergism; CI=1 indicates summation; CI>1 indicates antagonism (20).

Cell cycle analysis. Cells were exposed to 0.125 µM SNX-2112 or 25 µg/ml 5-FU alone or in combination for 48 h, harvested in cold phosphate-buffered saline, fixed in 70% ethanol, stored overnight at 4°C, and resuspended in 50 µg/ml PI staining reagent containing 100 µg/ml RNase and 0.1% Triton X-100 for 30 min in the dark. Cells were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

Quantitative real-time RT-PCR (Q-PCR). Total cellular RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was carried out using a Chromo4 instrument (Bio-Rad, Richmond, CA, USA) and SYBR® Premix Ex Taq™ kit (Takara Bio, Otsu, Japan) to detect the mRNA level of cyclin D1, Cdk2, Cdk4, p53, Chk1 and Chk2, with GAPDH as a normalizing control. The specific PCR primer sequences of these genes were designed by Primer premier 5.0 software (Table I). Cycling conditions were: 95°C for 20 sec to activate DNA polymerase, followed by 40 cycles of 95°C for 10 sec, 55°C for 20 sec, and 65°C for 30 sec. The relative changes in gene expression were calculated with the 2^{-ΔΔCt} method, where \( ΔΔCt = ΔCt \text{ (drugs treated) - } ΔCt \text{ (control)} \) for RNA samples.

Annexin V-FITC/PI analysis. Cells were exposed to the indicated concentrations of SNX-2112 or 5-FU alone or in combination for 48 h, resuspended in 500 µl incubation buffer containing Annexin V-FITC and PI, incubated in the dark for 15 min, and analyzed by flow cytometry. Data acquisition and analysis were performed using a FACSCalibur flow cytometer with CellQuest software (Becton-Dickinson). Mitochondrial membrane potential assay. Cells were cultured on glass cover slips and treated with SNX-2112 and 5-FU for 48 h, then incubated in complete medium containing 10 µg fluorescent lipophilic cationic JC-1 dye for 20 min at 37°C in the dark. The stained cells were washed twice with JC-1 buffer solution and examined by laser scanning confocal microscopy. We also analyzed the cells by flow cytometry. Data acquisition and analysis were performed in a FACSCalibur flow cytometer using CellQuest software. The loss of mitochondrial membrane potential (MMP) was quantified as the percentage of cells expressing JC-1 monomer fluorescence.

Western blotting. Cell were treated with SNX-2112 and 5-FU for 48 h, harvested, and lysed in sodium dodecyl sulfate (SDS) lysis buffer (SDS:phenylmethylsulfonyl fluoride = 50:1) at 100°C for 20 min. Lysates were clarified by centrifugation (12,000 rpm) at 4°C for 15 min and the supernatant was collected. Equal amount of lysate (20-30 µg) was denatured in 5X SDS lysis buffer (SDS:phenylmethylsulfonyl fluoride = 50:1) at 100°C for 20 min. Lysates were clarified by centrifugation (12,000 rpm) at 4°C for 15 min and the supernatant was collected. Equal amount of lysate (20-30 µg) was denatured in 5X SDS lysis buffer, resolved with 6-15% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h, and probed with primary antibody (1:1,000-1:5,000) overnight at 4°C. The membranes incubated with secondary antibody (1:5,000) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence kit (Beyotime, Shanghai, China) and imaged by autoradiography. GAPDH was used as the loading control.

Docking assay. The affinity of 5-FU and SNX-2112 for Hsp90 was determined by molecular operating environment docking. We obtained the crystal structure of Hsp90 from the Protein Data Bank (PDB code: 3R92). The two drugs were docked to
the binding pocket of Hsp90 to check the fitness according to our previous methods. A lower score indicated more favorable binding.

**Statistical analysis.** Data are expressed as the means ± SD. Differences between two groups were analyzed using the Student's t-test; groups of three or more were analyzed using one-way analysis of variance (multiple comparisons). P<0.05 and P<0.01 were considered statistically significant. We performed statistical analyses using SPSS 17.0 software (IBM, Armonk, NY, USA).

**Results**

**Effects of SNX-2112 and 5-FU on Eca-109 cells.** Initially, we examined the effects of SNX-2112 and 5-FU on Eca-109 and EC-9706 cell growth by MTT assay. Cells were cultured in a range of concentrations of SNX-2112 or 5-FU for 48 h. As expected, both SNX-2112 and 5-FU inhibited Eca-109 and EC-9706 cell growth in a dose-dependent manner. The median inhibitory concentration (IC50) of SNX-2112 and 5-FU was 0.12±0.01 µM and 48±0.2 µg/ml in Eca-109 cells, respectively (Fig. 1A). Similarly, the IC50 of SNX-2112 and 5-FU was 0.13±0.02 µM and 3±0.2 µg/ml in EC-9706 cells, respectively (Fig. 1B). Unexpectedly, there was an antagonistic effect in most cases in the combination group (CI>1, Fig. 1C). The occurrences of strong antagonistic effect (CI>2) were 62.5% in Eca-109 cells and 18.75% in EC-9706 cells, respectively, suggesting that the antagonistic effect in Eca-109 cells was more obvious (Tables II and III). The effect was most obvious when 0.125 µM SNX-2112 was combined with 25 µg/ml 5-FU in Eca-109 cells (CI=3.7, Table II and Fig. 1D). Therefore, we

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<th>Table I. Q-PCR primers.</th>
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<th>Table II. Combination effects of SNX-2112 and 5-FU on Eca-109 cells.</th>
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<td><strong>SNX-2112 (µM)</strong></td>
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Cells were cultured with various concentrations with SNX-2112 (0.03125-0.25 µM) and 5-FU (6.25-50 µg/ml) for 48 h. The combination index (CI) and fraction affected (Fa) values were determined using the pre-described method (19). When CI<1 indicates synergism; CI=1 indicates summation; CI>1 indicates antagonism. Values are mean ± SD (three independent experiments).

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<th>Table III. Combination effects of SNX-2112 and 5-FU on EC-9706 cells.</th>
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<td><strong>SNX-2112 (µM)</strong></td>
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Cells were cultured with various concentrations with SNX-2112 (0.03125-0.25 µM) and 5-FU (6.25-50 µg/ml) for 48 h. The combination index (CI) and fraction affected (Fa) values were determined using the pre-described method. When CI<1 indicates synergism; CI=1 indicates summation; CI>1 indicates antagonism. Values are mean ± SD (three independent experiments).
used 0.125 µM SNX-2112 and 25 µg/ml 5-FU as the optimal concentrations in Eca-109 cells for the remaining experiments.

**5-FU blocked SNX-2112-induced G2/M arrest.** Cell cycle arrest is the basic mechanism of cancer cell growth inhibition by Hsp90 inhibitors (16). To investigate the mechanism of 5-FU antagonism of SNX-2112 further, we examined cell cycle distribution with flow cytometry. There was 45.8% G2/M arrest and only 14.1% G0/G1 arrest following SNX-2112 treatment, but there was only 1.5% G2/M accumulation and the G0/G1 arrest increased to 48.2% in the combination group (Fig. 2A). This indicated that 5-FU completely recovered SNX-2112-induced G2/M arrest and partly increased the G0/G1 arrest of SNX-2112. Further, we found that the mRNA levels of cyclin D1, Cdk4 and Chk2 were decreased in the combination group compared to treatment with SNX-2112 alone (Fig. 2B), while the mRNA levels of p53, Chk1 and Cdk2 did not change significantly (Fig. 2C).

**5-FU suppressed SNX-2112-induced caspase-dependent apoptosis.** To determine the effect of SNX-2112 plus 5-FU on apoptosis, we examined the ratio of apoptosis using flow cytometry. We found that SNX-2112 and 5-FU alone induced 47.02 and 20.71% apoptosis, respectively; SNX-2112 plus 5-FU led to apoptosis decreasing to 16.98% (Fig. 3A). We then examined caspase-3 and PARP expression (indicators of apoptosis). 5-FU suppressed the caspase-3 downregulation and PARP cleavage induced by SNX-2112 (Fig. 3B). Taken together, our results indicate that 5-FU inhibits SNX-2112-induced apoptosis and caspase activation in Eca-109 cells.

**5-FU prevents the initial decrease in MMP during SNX-2112-induced apoptosis.** Mitochondria play a central role in determining cell survival or response to diverse stimuli (21). The SNX-2112 and 5-FU combination greatly suppressed the caspase-9 activity (a downstream indicator of the mitochondrial apoptotic pathway) induced by SNX-2112,
while caspase-8 (a downstream indicator of the death receptor apoptotic pathway) was inhibited slightly (Fig. 4A). To determine whether the mitochondria mediated the 5-FU antagonism of SNX-2112-induced apoptosis, we evaluated MMP by JC-1 staining. Following SNX-2112 treatment, the red/green fluorescence ratio was significantly decreased to 0.97, which 5-FU recovered to 1.45 (Fig. 4B). Flow cytometry showed 39.1% JC-1 fluorescence following SNX-2112 treatment; that of the combination group was 21.8% (Fig. 4C). These results show that 5-FU partly reversed the MMP decline caused by SNX-2112, indicating that 5-FU antagonism of SNX-2112-induced apoptosis might occur through mitochondrial repair.

5-FU inhibits SNX-2112-induced downregulation of Hsp90 client proteins. Previously, we found that SNX-2112 caused depletion of Hsp90 client proteins such as Akt, p-Akt, IKK, ERK1/2, and GSK-3β (18). To determine whether 5-FU antagonized the anticancer effect of SNX-2112 on Hsp90 client proteins, we investigated the expression of these proteins following SNX-2112 and 5-FU treatment. The SNX-2112 and 5-FU combination partly inhibited Akt, p-Akt, ERK1/2, and GSK-3β depletion and recovered the SNX-2112-induced IKKα downregulation completely (Fig. 5A). These results suggest that 5-FU attenuates SNX-2112-induced downregulation of Hsp90 client proteins in Eca-109 cells.

5-FU and SNX-2112 do not bind competitively to the Hsp90 N-terminal ATP pocket. To study whether 5-FU binding to the N-terminal ATP pocket of Hsp90 competed with that of SNX-2112, the fit was examine by docking studies. A hydrogen bond residue (Phe-138) and a side chain donor molecule (Lys-58) were in contact with SNX-2112; 5-FU did not interact with Hsp90, and a major portion of Hsp90 was exposed to the solvent. The scoring value of SNX-2112 and 5-FU was -30.94 and -12.28 kcal/mol, respectively, indicating that 5-FU could...
Figure 3. Caspase-dependent apoptosis after 5-FU and SNX-2112 treatment in Eca-109 cells. (A) Representative image of assessment of apoptosis by Annexin V/PI staining. Statistical histograms indicate the percentage of apoptotic cells. (B) Western blot assessment of apoptosis-associated proteins PARP and caspase-3. GAPDH was used as the protein loading control. Values are the mean ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the SNX-2112 group. Con, untreated control; SNX, SNX-2112; Comb, combination; C-PARP, cleaved PARP.

Figure 4. Effect of 5-FU and SNX-2112 on MMP in Eca-109 cells. (A) Western blot assessment of caspase-9 and -8. GAPDH was used as the protein loading control. (B) JC-1 fluorescence images of cells treated with SNX-2112, 5-FU, or SNX-2112 and 5-FU. Red fluorescence indicates high membrane potential; green fluorescence represents low membrane potential. (C) Representative flow cytometric analyses of MMP in cells treated with SNX-2112, 5-FU, or SNX-2112 and 5-FU; increased JC-1 expression indicates reduced MMP. Values are the mean ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the SNX-2112 group. Con, untreated control; SNX, SNX-2112; Comb, combination; C-caspase-8, cleaved caspase-8; C-caspase-9, cleaved caspase-9.
Discussion

This study marks the first demonstration that the combination of SNX-2112 with 5-FU exhibits antagonistic effects in esophageal cancer cells. The antagonist effects were related to: i) growth inhibition; ii) G2/M arrest; iii) caspase-dependent and mitochondrial-mediated apoptosis; iv) Hsp90 client protein expression. In most cases, however, the combination of Hsp90 inhibitors with chemotherapy drugs (such as SNX-2112 with paclitaxel and cisplatin in head and neck SCC, ganetespib with the taxanes paclitaxel and docetaxel in non-small cell lung cancer, 17-AAG with cisplatin in glioma) is synergistic (17,22,23). From this point of view, the combination effect of SNX-2112 plus 5-FU is quite different from that of other combination therapies based on Hsp90 inhibit-
tors and chemotherapy drugs, and the antagonistic effect in this exception catalyzed our efforts to uncover the underlying mechanism.

SNX-2112 arrests the cell cycle at the G2/M phase in various cancers (16,18). It has been established that cyclins, cell cycle kinases [such as checkpoint proteins, cyclin-dependent kinases (Cdks), and non-Cdk kinases], and other cell cycle-related protein are responsible for cell cycle control. For instance, Cdk4 is inactive in their monomeric form and activated by cyclin D1, and the formation of Cdk4/cyclin D1 complexes plays an important role on the change of cell cycle (24). Stepanova et al reported that the inactivation of Hsp90 increased the complexes of Cdk4/cyclin D1, but has no effect on Cdk2/cyclin E1 (25). These findings are consistent with our study that the mRNA level of cyclin D1 and Cdk4 were decreased in combination group compared to SNX-2112 treatment alone, with no significant change of Cdk2. In addition, we found that 5-FU completely reversed SNX-2112-induced G2/M arrest and partly increased the G0/G1 arrest of SNX-2112. However, this contrasts with the 5-FU enhancement (but not decrease) of G2/M cell percentage that may antagonize the effect of celecoxib in breast cancer (26). It remains unclear why 5-FU has bidirectional effects in cell cycle arrest when combined with different anticancer drugs.

We demonstrated that 5-FU suppressed caspase-dependent and mitochondria-mediated apoptosis induced by SNX-2112. Consistent with our previous studies, SNX-2112 activated caspase-3, -8 and -9 and decreased MMP (14-16,18), which 5-FU neutralized in this study. Mitochondria are involved in many cellular processes, such as metabolism, signaling, cell growth, and apoptosis (21). Caspase-9 activation is significantly associated with mitochondrial dysfunction, while caspase-8 activation is related to the death receptor pathways (27). In our protein assay, caspase-9 appeared more sensitive than caspase-8 to 5-FU treatment, suggesting that 5-FU antagonism of SNX-2112 is mainly regulated by mitochondrial-dependent pathways.

The exact manner in which 5-FU decreases the anticancer effect of the Hsp90 inhibitor SNX-2112 remains to be determined. However, based on the literature and our findings, we believe that there are at least two probabilities: i) 5-FU might bind competitively to the N-terminal ATP pocket or another site of Hsp90, altering Hsp90 conformation and function. However, it should be noted that our molecular docking study only ruled out the possibility of 5-FU indirect binding to the N-terminal ATP pocket of Hsp90; ii) 5-FU recovers the downstream signaling pathways of Hsp90 downregulated by SNX-2112. Our report proves that SNX-2112 suppresses the phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor kB (NF-kB) pathways (16,28). It has been reported that 5-FU alone upregulates p-Akt and IKK expression in cancer cells (29,30). Therefore, we speculate that the recovery of the PI3K/Akt and NF-kB pathways might be a possible mechanism of 5-FU antagonism of the anticancer effect of SNX-2112.

In conclusion, the combination of SNX-2112 with 5-FU exhibits antagonistic effects by reversing G2/M arrest, suppressing caspase-dependent apoptosis, preventing the initial MMP decrease, and decreasing the downregulation of Hsp90 client proteins (Fig. 5C). Although further demonstration of a more precise mechanism of 5-FU antagonism of SNX-2112 is required in other cancer types, we suggest that the SNX-2112 plus 5-FU combination should be used with special care in clinical application.

Acknowledgements

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