Comparative effects of SNX-7081 and SNX-2112 on cell cycle, apoptosis and Hsp90 client proteins in human cancer cells

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Abstract. SNX-2112, a novel 2-aminobenzamide inhibitor of Hsp90, previously showed a broad spectrum of anticancer activity. However, subsequent development has been discontinued due to ocular toxicity as identified in a phase I study. SNX-7081, another closely related Hsp90 inhibitor with a side chain of indole instead of indazole, has recently attracted attention. The aim of the present study was to investigate the anticancer effects of SNX-7081 in eleven cell lines, as well as the mechanisms involved, with SNX-2112 serving as a reference. The cytotoxic effects were determined using an MTT assay and apoptosis was measured using flow cytometry. The results showed that SNX-7081 exerted better inhibitory effects than SNX-2112 in six eighths of the human cancer cell lines, with an average IC_{50} of 1 μM. The two inhibitors exerted low cytotoxicity in L-02, HDF and MRC5 normal human cells (IC_{50} >50 μM), and arrested cancer cells at the G2/M phase in a similar manner to normal cells. Compared with SNX-2112, SNX-7081 exhibited more potent effects on cell apoptosis in four sixths of the human cancer cell lines, and was more active in the downregulation of Hsp90 client proteins. In addition, SNX-7081 exhibited a stronger binding affinity to Hsp90 than SNX-2112 in molecular docking experiments. Considering the superior effects against Hsp90 affinity, cell growth, apoptosis, and Hsp90 client proteins in a majority of human cancer cells, the novel SNX-7081 may be a promising alternative to SNX-2112, which merits further evaluation.

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Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone with a key role in the preservation of the conformation, stability and function of its client proteins (1). The largest groups of client proteins associated with Hsp90 (e.g., IKK, GSK3, CHK1 and Raf-1) are protein kinases and transcription factors that are important in cellular carcinogenesis (2). Due to the critical roles of Hsp90 client proteins in cancer cell growth arrest, apoptosis and other cancer hallmarks, the inhibition of Hsp90 has received considerable attention as a potential target for cancer treatment (2-4). 17-AAG is the first Hsp90 inhibitor to undergo clinical research, but has several issues related to poor solubility, unacceptable hepatotoxicity and limited bioavailability (5-7). These drawbacks have catalyzed efforts to explore novel scaffolds with improved pharmacological properties for clinical applications.

SNX-2112, a novel Hsp90 inhibitor with the 2-aminobenzamide scaffold, has been widely used in cancer research (8-12). SNX-2112 exerts its growth inhibitory and apoptosis-inducing activity on many human cancer cells (1,8,11,13). However, subsequent development has been discontinued due to ocular toxicity observed in animal models and in a separate phase I study (14). Developing alternatives of SNX-2112 based on the 2-aminobenzamide scaffold may be a feasible method for meeting clinical needs. SNX-7081 is another Hsp90 inhibitor derived from 2-aminobenzamide and optimized by SAR explorations (15), with a side-chain of indole instead of indazole at SNX-2112. To the best of our knowledge, SNX-7081 has not attracted enough attention in cancer therapy, and is only limited to investigations on its potential in chronic lymphocytic leukemia and inflammatory diseases (16-19).

In the present study, we tested the anticancer effects and examined the mechanisms of SNX-7081 using several human cancer cells *in vitro*, with SNX-2112 as a reference.

Table I. IC_{50} values of SNX-7081 and SNX-2112.

Cell lines	Drug: IC_{50} (μM) SNX-7081	SNX-2112
Human cancer cells		
K562 (leukemia)	<0.01	<0.01
A375 (melanoma)	0.26±0.12[a]	0.55±0.22
MCF-7 (breast cancer)	1.09±0.74	2.58±1.30
Hep-2 (laryngeal cancer)	1.13±0.64[a]	3.58±0.95
HepG2 (liver cancer)	1.29±0.51	2.52±1.35
A549 (lung cancer)	3.61±1.50[a]	5.86±2.24
SW-620 (colon carcinoma)	0.05±0.03	<0.01
HeLa (cervical cancer)	9.16±4.50	6.31±3.34
Normal human cells		
L-02 (liver cells)	>100.00	>100.00
HDF (dermal fibroblasts)	>100.00	>100.00
MRC5 (fetal lung fibroblasts)	70.16±27.85	>100.00

Cells were cultured with various concentrations (0-10 μM in cancer cells, 0-50 μM in normal cells) of compounds for 72 h. IC_{50} values of SNX-7081 and SNX-2112 towards eight cancer cell lines (K562, A375, MCF-7, Hep-2, HepG2, A549, SW620 and HeLa) and three human normal cells (L-02, MRC5 and HDF) at 72 h as determined by using MTT assay. Data are the means ± SD of three replicates ([a]$P<0.05$).

To demonstrate more details involved in the antitumor effects of the two compounds, we investigated Hsp90 affinity, cell growth, cell cycle, apoptosis and the expression of Hsp90 clients in various cancer cells.

Materials and methods

Reagents and antibodies and cells. SNX-7081 and SNX-2112 were prepared in our laboratory with purities of >98.0% according to a previously described procedure (20,21), dissolved in 10 mM dimethyl sulfoxide (DMSO) as a stock solution and stored at -20˚C. Antibodies against IKKα, Raf-1, CHK1 and GSK3β were purchased from Epitomics (Burlingame, CA, USA), and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Millipore (Billerica, MA, USA).

Cell culture. Eleven cell lines, including eight human cancer cell lines and three human normal cell lines, were used in the present study (Table I). Primary HDF cells were isolated from the foreskins of newborns using a previously reported procedure (22,23). All other cell lines were purchased from the Cell Bank of the China Science Academy (Shanghai, China). CML K562 cells, A375 melanoma cells, the normal human diploid fibroblast (HDF) and the normal human liver L-02 cells were cultured in RPMI-1640 medium. MCF-7 breast cancer cells, Hep-2 laryngeal epidermoid carcinoma, HepG2 liver carcinoma, A549 lung adenocarcinoma, HeLa cervical carcinoma and SW-620 colon carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The normal human fibroblastic cell line (MRC-5) was cultured in Minimum Essential Medium (MEM). Cells were maintained at 37˚C in a 5% CO_2 atmosphere. All the media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) supplemented with 50 U/ml penicillin and streptomycin.

MTT assay. Exponentially growing cells were seeded in 96-well culture plates (5-10x10^3 cells/well) and allowed to adhere overnight. Cancer cells were incubated with SNX-7081 or SNX-2112 at various concentrations (0-10 μM) for 72 h. HDF, MRC5 and L-02 normal human cells were incubated with drugs at various concentrations (0-50 μM) for 72 h, along with an equal volume of DMSO as the solvent control. MTT solution (10 μl) was added to each well (0.5 mg/ml) for an additional 4-h incubation (37˚C, 5% CO_2). The precipitated formazan was dissolved in 100 μl of DMSO. A 96-well multi-scanner autoreader (M450; Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the absorbance of each well at 570 nm, with a reference wavelength of 630 nm. The IC_{50} values, defined as the drug concentration that caused 50% inhibition of absorbance compared with the control cells treated with DMSO only, were calculated using the PrismPad program.

Cell cycle analysis. Cell cycle distribution was determined by DNA staining with propidium iodide (PI) (13). Briefly, cancer cell lines were cultured and treated in 6-well culture plates (6x10^5 cells/well) with SNX-7081 or SNX-2112 (1.0 μM) for 48 h. The cells were then washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight. The cells were collected and resuspended in PBS containing 50 μg/ml PI, 0.1 mg/ml RNase, and 5% Triton X-100 and incubated at 37˚C for 30 min. Subsequently, the cells were analyzed on a flow cytometer (Becton-Dickinson, San Jose, CA, USA), and the percentages of cells present in different phases of the cell cycle were analyzed using the CellQuest software (Becton-Dickinson).

Apoptosis assay. Apoptosis was measured by flow cytometry after staining with Annexin V-FITC and PI according to the instructions of the Annexin V-FITC/PI staining kit (Nanjing KeyGen Biotech., Co., Ltd., Nanjing, China). Briefly, cancer cells were cultured in the presence of the indicated concentrations of SNX-7081 or SNX-2112 (1.0 μM) for 48 h, harvested, washed twice and resuspended in 500 μl of 1X binding buffer containing Annexin V-FITC and PI. Samples were incubated at room temperature for 10 min and analyzed by FACS.

Immunoblotting. K562 cells were incubated with 1.0 μM SNX-7081 or SNX-2112 for 0, 6, 12, 24 or 48 h. Whole-cell lysates were prepared by washing the cells with PBS and subjecting them to lysis with RIPA buffer for 30 min on ice. Total protein concentrations of whole-cell lysates were determined using the BCA protein assay kit. Equal amounts of protein samples were loaded onto 8-12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore), examined with primary antibodies and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Specific protein bands were visualized using the chemiluminescence method and imaged by autoradiography. Any differences in

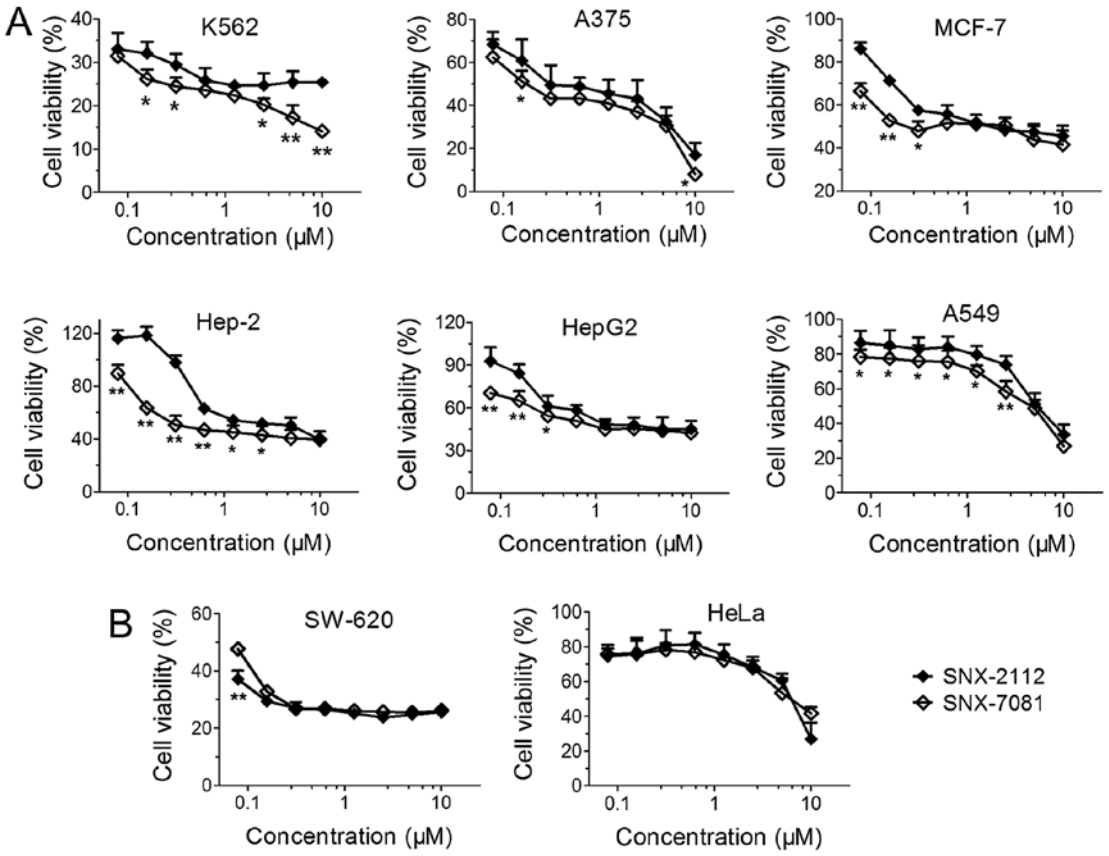

Figure 1. Cytotoxic effects of SNX-7081 and SNX-2112 on human cancer cell lines were determined by the MTT assay. (A) K562, A375, MCF-7, Hep-2, HepG2, A549 and (B) SW-620 and HeLa cells were treated with various concentrations of SNX-7081 and SNX-2112 for 72 h. Percentages of viable cells were calculated by comparing treated and solvent control cells. Data are the means ± SD of three replicates (*P<0.05; **P<0.01).

protein loading were normalized to the corresponding levels of the GAPDH control.

Docking assay. The affinity of SNX-7081 and SNX-2112 against Hsp90 was determined by the MOE docking assay. The crystal structure of Hsp90 was taken from the Protein Data Bank (PDB code: 3R92). The two compounds were converted to 3D structures, and energy was minimized in MOE. The binding site of Hsp90 was minimized using the AMBER 99 force field in MOE with the default parameter. The two compounds were docked, employing Triangle Matcher as the placement method and the function London dG as the first scoring function. The refinement was set to force field (AMBER 99), and the docked poses were energy minimized in the receptor pocket (24). The conformations of lowest energy were given, and a lower scoring value indicated a more favorable binding.

Statistical analysis. Data were evaluated by the Welch t-test when only two value sets were compared. One-way ANOVA followed by the Dunnett's test was used for more group comparisons if ≥3 experiments were involved. Results were expressed as means ± SD with a significance at *P<0.05 or **P<0.01.

Results

Anti-proliferative effects of SNX-7081 and SNX-2112 on hunman cancer cells. The growth inhibitory effects of SNX-7081 and SNX-2112 on human cancer lines originating from bone marrow, colon, skin, larynx, breast, liver, cervix and lung were comopared. Cells were exposed to various concentrations (0-10 μM) for 72 h and cell viability was quantified by the MTT assay. SNX-7081 and SNX-2112 significantly inhibited the growth of eight human cancer cells in a dose-dependent manner. The cell viability of SNX-7081 was significantly lower than that of SNX-2112 at the low concentration (<1 μM) in K562, A375, MCF-7, Hep-2, HepG2 and A549 cells (Fig. 1A). In addition, SNX-7081 exhibited similar or weaker effects to SNX-2112 in SW-620 and HeLa cells (Fig. 1B). The IC_{50} values of SNX-7081 and SNX-2112 for these cells were always ~1 μM (Table I). For the remaining experiments, 1 μM was applied as the optimal concentration.

Cytotoxic effects of SNX-7081 and SNX-2112 on human normal cells. The cytotoxic effects of two agents on three human normal cell lines (L-02, HDF and MRC5 cells) were determined by the MTT assay. The cells were exposed to SNX-7081 or SNX-2112 at concentrations of 3.13-50.0 μM for 72 h. The IC_{50} value could not be determined even at the high concentration of 50.0 μM (Table I and Fig. 2), suggesting that SNX-7081 cytotoxicity was lower as compared to SNX-2112 in L-02, MRC5 and HDF cells. Therefore, SNX-7081 and SNX-2112 were provided with acceptable cytotoxicity toward normal cells and high selectivity in cancer cells.

Induction of cell cycle arrest at G2/M phase by SNX-7081 and SNX-2112. To explore the anti-proliferative mechanism

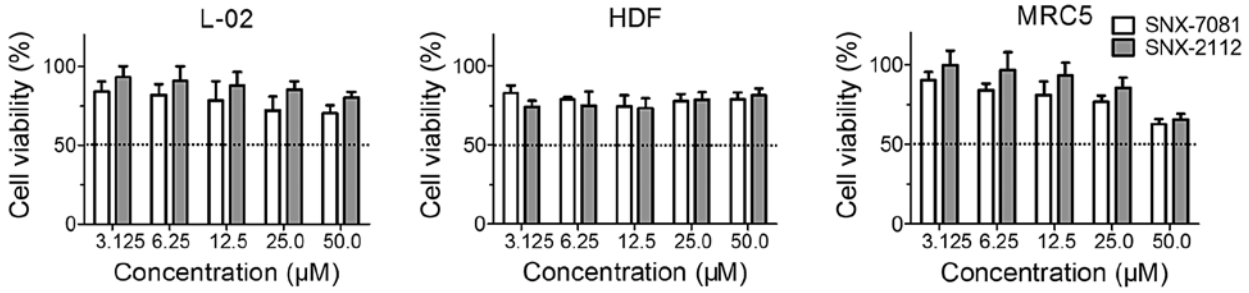

Figure 2. Cytotoxic effects of SNX-7081 and SNX-2112 on normal human cell lines were determined by the MTT assay. Cells were treated with various concentrations of SNX-7081 and SNX-2112 for 72 h. Percentages of viable cells were calculated by comparing treated and solvent control cells. Data are the means ± SD of three replicates.

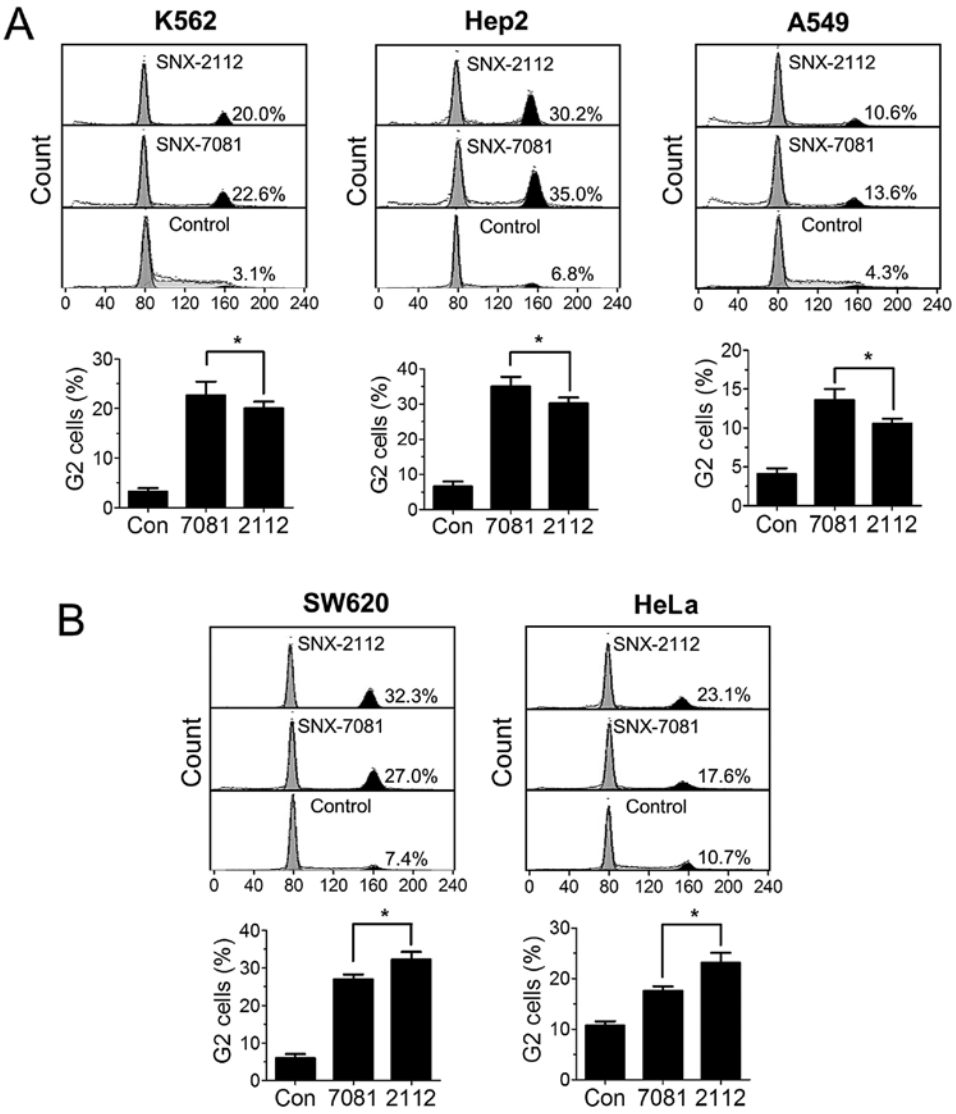

Figure 3. Cell cycle analysis of cancer cells after treatment with SNX-7081 and SNX-2112 in five cancer cells lines. (A) K562, Hep-2, A549 and (B) SW-620 and HeLa cells were cultured with SNX-7081 and SNX-2112 at 1 μM, collected after 48 h and stained with PI. The DNA content and cell cycle distribution were analyzed by flow cytometry. Results are the means ± SD of three replicates. Representative images from three experiments are shown, and percentages of cells in cycle arrest are indicated. Data are the means ± SD of three replicates ($^{*}P<0.05$).

of SNX-7081 and SNX-2112, we assessed their effects on the cell cycle distribution in the K562, Hep-2, A549, SW620 and HeLa cancer cell lines using flow cytometry. Cells treated with SNX-7081 and SNX-2112 (1 μM) for 48 h, were subjected to flow cytometric analysis after PI staining. SNX-7081 more effectively increase the cell amount of G2/M phase in K562, Hep-2 and A549 cells (Fig. 3A), while SNX-2112 induces a more significant G2/M arrest in SW-620 and HeLa cells

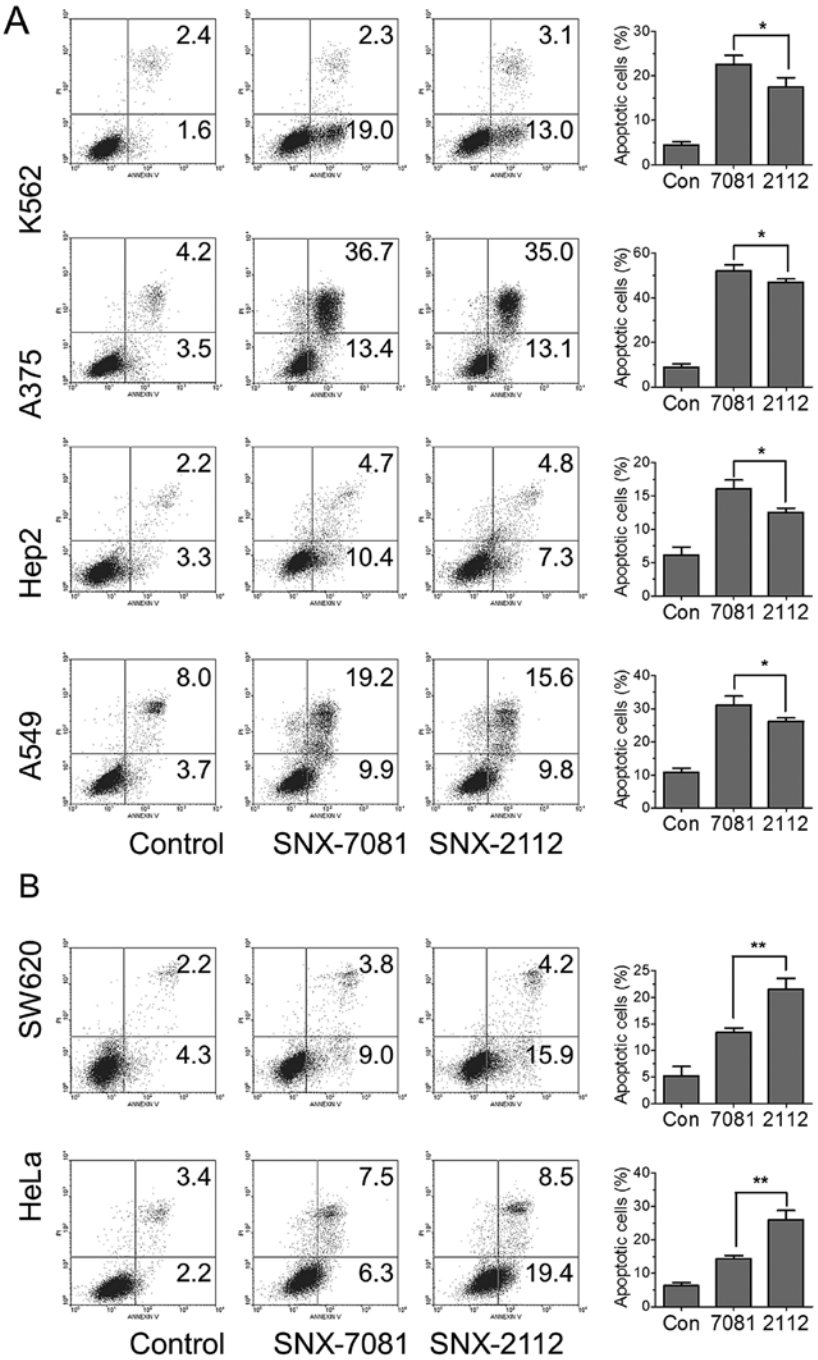

Figure 4. Effects of SNX-7081 and SNX-2112 on apoptosis induction in human cancer cells. (A) K562, A375, Hep-2, A549 and (B) SW-620 and HeLa cells were treated with control (vehicle), SNX-7081 and SNX-2112 at 1 μM for 48 h. Apoptosis was detected by flow cytometric analysis based on PI (y-axis) and Annexin V (x-axis) staining. Representative images from three experiments are shown, and the percentages of apoptotic cells (lower and upper right-quadrants) are indicated. Data are the means ± SD of three replicates (*P<0.05; **P<0.01).

(Fig. 3B). The finding suggested that SNX-7081-induced inhibitory effects may be mediated by cell cycle arrest.

Effects of SNX-7081 and SNX-2112 on cancer cell apoptosis. To examine whether apoptosis is involved in the inhibition of cancer cell proliferation by SNX-2112 or SNX-7081, cancer cells were treated with 1 μM SNX-2112 or SNX-7081 for 48 h and subjected to Annexin V- and PI-double staining for flow cytometric analysis. The degree of apoptosis was calculated as the sum of the percentages of cells in the lower right and upper right quadrants. SNX-7081 and SNX-2112 clearly induced apoptosis in all six cell lines. SNX-7081 induced more apoptosis in K562, Hep-2 and A549 cells (Fig. 4A), but less apoptosis in SW-620 and HeLa cells as compared to that of SNX-2112 (Fig. 4B). The data indicated that SNX-7801-induced apoptosis in some cancer cells was more potent than SNX-2112.

Effects of SNX-7081 and SNX-2112 on the expression of Hsp90 client proteins. The basic mechanism of Hsp90 inhibitors involves binding to the ATP pocket and suppressing the ATPase activity of Hsp90, resulting in the degradation of

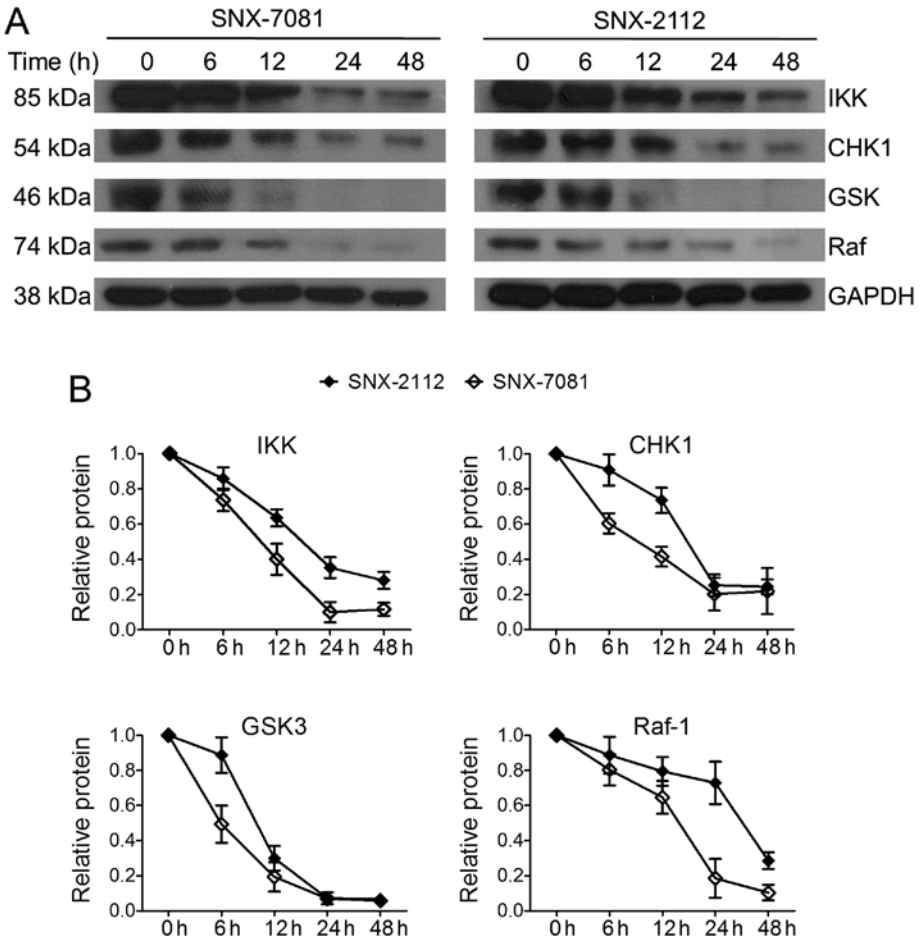

Figure 5. Inhibition of Hsp90 client proteins by SNX-7081 and SNX-2112. (A) K562 cells were cultured with control (vehicle), SNX-2112 and SNX-7081 at 1 μM for the indicated times. Equal amounts of whole-cell lysates (25-50 μg) were analyzed by western blot analysis to detect the expression of Hsp90 client proteins. (B) In the accompanying bar graphs, protein levels were normalized to GAPDH, which was used as a loading control. Results are shown as means ± SD of three independent experiments.

its client proteins (25). It was previously demonstrated that SNX-2112 led to downregulation of the Hsp90 client proteins Bcr-abl and Akt in K562 cells (13). In the present study, we compared the effect of SNX-7081 and SNX-2112 on the expression of other essential Hsp90 client proteins, including IKKα, GSK3, CHK1 and Raf-1, which are crucial for the growth of cancer cells. Western blot analysis showed that the levels of these proteins were reduced in a time-dependent manner following exposure to the compounds (Fig. 5). After treatment with 1 μM of SNX-7081 for 48 h, the expression of IKKα, CHK1, GSK3 and Raf-1 was significantly decreased to 11.7, 21.8, 5.8 and 10.4%, respectively. SNX-7081 exerted stronger inhibitory effects on the expression levels of Hsp90 client proteins than SNX-7081. These data support the principle that SNX-7081 and SNX-2112 act via the inhibition of Hsp90 chaperone function, and the SNX-7081 was more potent in suppressing Hsp90 client proteins.

Binding affinity of SNX-7081 and SNX-2112 to Hsp90. To determine the binding affinity of SNX-7081 and SNX-2112 to Hsp90, the inhibitors were docked into the ATP site of Hsp90, using MOE. A lower scoring value suggests a more favorable binding. The ligand-receptor interaction maps for SNX-7081 and SNX-2112 are shown in Fig. 6. We found two hydrogen bond residues (Phe-138 and Trp-163), two side chain donor molecules (Lys-58 and Thr-184), and one side chain acceptor molecule (Asp-93) that bound to SNX-7081. SNX-7081 interacted with five residues in the pocket, while SNX-2112 only interacted with three residues. The scoring value of SNX-7081 was -33.53 kcal/mol, which was lower than the -30.94 kcal/mol on SNX-2112. It was evident that SNX-7081 was the highly-scored compound, suggesting that SNX-7081 had a stronger binding affinity to Hsp90 than SNX-2112.

Discussion

The discontinuity in clinical trials of SNX-2112 results in opportunities and challenges for other 2-aminobenzamide inhibitors of Hsp90. In the present study, we found that SNX-7081, an analogue of SNX-2112, exhibited higher affinity for Hsp90 than SNX-2112 in molecular docking experiments. The structure of SNX-7081 is different from SNX-2112, with a side chain of indole instead of indazole. In addition, the methyl substituent in the indole ring instead the of 5-fluoro substituent in indazole may also contribute to the higher affinity of SNX-7081. A similar binding model suggested that SNX-7081 may be a feasible alternative of SNX-2112, leading to a comparison of the anticancer activity of the two compounds.

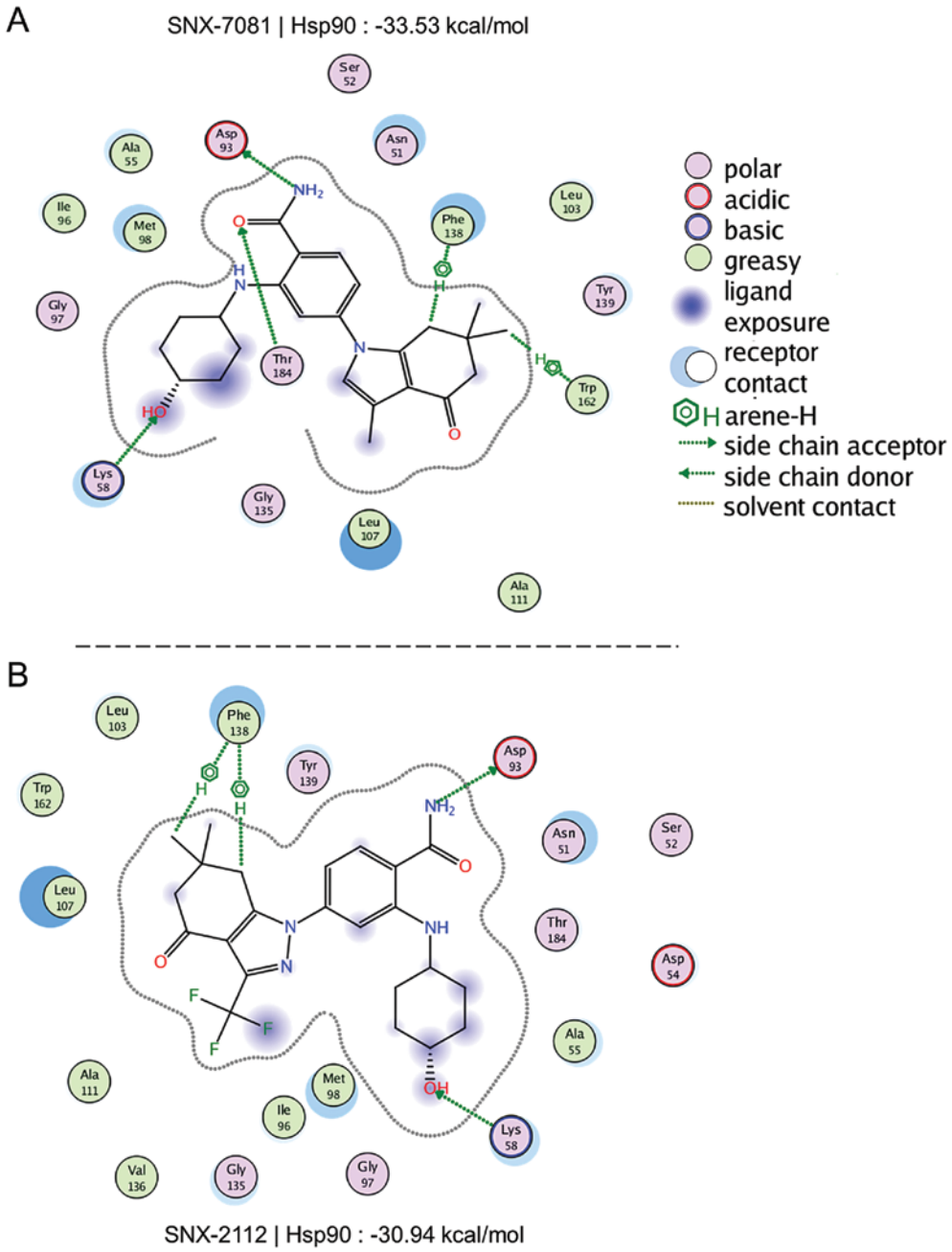

Figure 6. Molecular modeling of SNX-7081 and SNX-2112 binding with Hsp90. Docking of (A) SNX-7081 and (B) SNX-2112 into the N-terminal domain of Hsp90 (protein structure with PDB code 3R92). The conformations of lowest energy are shown, and a lower scoring value indicates a more favorable binding.

SNX-7081 had better activity than SNX-2112 in most cancer cells. One interpretation of this finding is that the expressions and functions of various Hsp90 isoforms are not uniform in different cell types. These Hsp90 isoforms include Hsp90α and Hsp90β in the cytoplasm and nucleus, GRP94 in the endoplasmic reticulum (ER) and TRAP1 in the mitochondria (25). The functions of Hsp90α include stress-induced cytoprotection and cell cycle regulation, whereas Hsp90β plays an important role in early embryonic development, signal transduction and long-term cell adaptation (26,27). GRP94 is specialized for protein folding and plays a role in the degradation of misfolded proteins through the ER-associated degradation pathway (28). Additionally, TRAP1 is involved in signal transduction, protein folding, protein degradation and morphologic evolution (29). Thus, the development of Hsp90 inhibitors against specific Hsp90 isoforms is a promising strategy for cancer therapy.

In addition, SNX-7081 and SNX-2112 exhibited strong selectivity in cell growth inhibition between cancer cells and normal cells. This is probably because the expression and molecular conformation of Hsp90 are greatly different in cancer and normal cells. In tumor cells, Hsp90 may be exclusively complexed with co-chaperones, forming a super-chaperone complex in a state of high affinity for ATP/ADP or ligands (e.g., Hsp90 inhibitors) of this regulatory pocket. However, in normal cells, Hsp90 is predominantly in a

latent, uncomplexed and low affinity state (30,31). Therefore, compared with Hsp90 in normal cells, tumor Hsp90 is hypersensitive to Hsp90 inhibitors.

Results of this study show that SNX-7081 exhibits an antitumor profile of the natural product Hsp90 inhibitors: growth inhibition, G2 cell cycle arrest, cell apoptosis induction and degradation of Hsp90 clients (32). Previously, it was found that SNX-2112 can induce the degradation of Bcr-abl and Akt in K562 cells (21). Although there are many other Hsp90 client proteins, the focus of the present study was on the levels of IKKα, GSK3, CHK1 and Raf-1, due to their critical roles in the proliferation of human leukemia cells. For instance, IKKα is an NF-κB inhibitory protein that contributes to the de-regulated expression of various cellular genes in T-cell leukemia cells (33,34). GSK3 controls cell survival, and its inactivation induces cell apoptosis in leukemia cells (35,36). Inhibition of CHK1 kinase has been shown to induce cell cycle arrest in K562 cells (37), while targeting Raf-1 gene expression by a DNA enzyme inhibits the growth of leukemia cells (38). Therefore, the downregulation of Hsp90 client proteins in the upstream pathway may be the mechanism of the induction of growth inhibition, cell cycle arrest and apoptosis pertaining to SNX-7081 in cancer cells.

In conclusion, we have demonstrated that SNX-7081 was significantly more potent than SNX-2112 in a majority of human cancer cells. Consequently, this superiority effect merits further confirmation in xenograft experiments *in vivo*. The mode of action of antitumor activity may be associated with the induction of cell cycle arrest, apoptosis and Hsp90 client proteins degradation. Our results suggest that the novel Hsp90 inhibitor SNX-7081 is a promising alternative to SNX-2112, although more fundamental investigations are required to confirm the results.

Acknowledgements

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