Complex Crystal Structure Determination and *in vitro* Anti–non–small Cell Lung Cancer Activity of Hsp90N Inhibitor SNX-2112

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SNX-2112, as a promising anticancer lead compound targeting heat shock protein 90 (Hsp90), absence of complex crystal structure of Hsp90N-SNX-2112 hindered further structural optimization and understanding on molecular interaction mechanism. Herein, a high-resolution complex crystal structure of Hsp90N-SNX-2112 was successfully determined by X-ray diffraction, resolution limit, 2.14 Å, PDB ID 6LTK, and their molecular interaction was analyzed in detail, which suggested that SNX-2112 was well accommodated in the ATP-binding pocket to disable molecular chaperone activity of Hsp90, therefore exhibiting favorable inhibiting activity on three non–small cell lung cancer (NSCLC) cell lines (IC50, 0.50 ± 0.01 µM for A549, 1.14 ± 1.11 µM for H1299, 2.36 ± 0.82 µM for H1975) by inhibited proliferation, induced cell cycle arrest, and aggravated cell apoptosis. SNX-2112 exhibited high affinity and beneficial thermodynamic changes during the binding process with its target Hsp90N confirmed by thermal shift assay (TSA, ΔTm, and −9.51 ± 1.00°C) and isothermal titration calorimetry (Kd, 14.10 ± 1.60 nM). Based on the complex crystal structure and molecular interaction analysis, 32 novel SNX-2112 derivatives were designed, and 25 new ones displayed increased binding force with the target Hsp90N verified by molecular docking evaluation. The results would provide new references and guides for anti-NSCLC new drug development based on the lead compound SNX-2112.

**Keywords:** heat shock protein 90N, non–small-cell lung cancer, drug development, SNX-2112, complex crystal structure, molecular interaction

INTRODUCTION

Lung cancer, as the leading cause of cancer death worldwide, accounts for 18% of total cancer deaths each year (Petrek and Yu, 2019). According to histological types and therapeutic aims, lung cancer can be traditionally classified into small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC); NSCLC accounts for 80–85% (Edinger et al., 2017). NSCLC is increasingly thought as an...
oncogene addicted tumor (Molina et al., 2008); therefore, targeted drugs may be an effective way for NSCLC treatment (Tomasini et al., 2016; Xiong et al., 2019).

Heat shock protein 90 (Hsp90), as an evolutionarily conserved chaperone in eukaryotes, plays a vital role in cell differentiation, proliferation, and apoptosis by assisting more than 300 client protein proper folding or conformational maturation (Li et al., 2012a; Vahid et al., 2017). Among the client proteins, 48 proteins play an important, even decisive role in tumorigenesis and development, such as transmembrane tyrosine kinase, steroid receptors, and cell cycle regulators, etc. (Jhaveri et al., 2014; Hyun et al., 2018). Hsp90N inhibitors lead to multiple misfolded or immature client proteins to be degraded by the ubiquitin protease complex pathway (Chen et al., 2014), which involves simultaneous intervention of multiple potential antitumor target proteins and a “multipoint attack” on the tumor to achieve favorable therapeutic efficacy (Chehab et al., 2015; Dutta Gupta et al., 2019). Hsp90 inhibitor development, therefore, has become a promising strategy in cancer therapy (Barrott and Haystead, 2013; Soga et al., 2013; Hu et al., 2019).

Heat shock protein 90 contains four isoforms (Hsp90α, Hsp90β, Trap1, and Grp94), and in cytosol Hsp90 exists in α-β or β-β homologous dimer form, and every monomer consists of three domains (Hsp90N, Hsp90M, and Hsp90C; Donnelly and Blagg, 2008; Mayer et al., 2012). Hsp90 inhibitors accordingly are classified as Hsp90N inhibitors, Hsp90M inhibitors, and Hsp90C inhibitors (Sidera and Patsavoudi, 2014). Hsp90N inhibitor development targeting to ATP-binding pocket of Hsp90N remains a research hot spot (Prodromou et al., 2000; Li et al., 2012b; Sung et al., 2016). There are four groups for Hsp90N inhibitors based on chemical structures including ansamycin groups, resorcinol groups, benzamide groups, and purine groups (Mollapour et al., 2011). On behalf of benzamide groups, SNX-2112 (IC50, 3 nM) and its oral form SNX-2112. Herein, the complex crystal structure of Hsp90N-SNX-2112 was determined by X-ray diffraction (XRD); molecular interaction analysis based on complex crystal structure, thermal shift assay (TSA), and isothermal titration calorimetry (ITC) was performed. Meanwhile, in vitro anti-NSCLC activity of SNX-2112 was evaluated, which would provide new references for anti-NSCLC new drug development based on the lead compound SNX-2112.

MATERIALS AND METHODS

Materials and Reagents
A549, H1299, and H1975 NSCLC cell lines were obtained from the AOLUKEJI (Shanghai, China). Fetal bovine serum and RPMI 1640 medium were purchased from Gibco-BRL (Gaithersburg, MD, United States).

SNX-2112 was acquired from Target Molecule Corp. (Boston, MA, United States). Dimethyl sulfoxide (DMSO), magnesium chloride (MgCl2), PEG4000, sodium acetate (NaAc), Tris, and Tris–HCl and were purchased from Sigma–Aldrich Corp. (St. Louis, MO, United States). Glycerol, ethyl alcohol, sodium hydroxide, hydrochloric acid (HCl), and sodium chloride (NaCl) were obtained from Xi’an Chemical Reagent Company (Xi’an, China). SNX-2112 was dissolved in DMSO, and other reagents were dissolved in double distilled water (ddH2O).

Methods

Anticancer Activity in vitro

Cell viability assay
Non–small cell lung cancer cell lines A549, H1299, and H1975 were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in a humidified 5% CO2 atmosphere incubator (Thermo Fisher Scientific Inc., Waltham, MA, United States). Cell lines were placed in 96-well plates at 4 × 103 per well in triplicates and SNX-2112 (100, 10, 1, 0.1, 0.01 μM) treated cell lines for 72 h (quantitative IC50 value). Cell lines were placed in triplicate at 4 × 103 per well in 96-well plates and then treated by SNX-2112 (4 × 0.50 μM for A549, 4 × 1.14 μM for H1299, and 4 × 2.36 μM for H1975) or DMSO for 24, 48, or 72 h (cell viability assay); 2.5 h after adding CCK-8, OD550 was detected by CCK-8 assay (7Sea, Shanghai, China) using an ELISA plate reader (BioTek Instruments, United States; Xue et al., 2016; Wei et al., 2018).

Cell cycle assay
Non–small cell lung cancer cell lines A549, H1299, and H1975 were seeded in a 6-well culture plate at density 3 × 105 cells per well, which were treated with different concentrations of SNX-2112 or DMSO for 72 h (4 × 0.50 μM for A549, 4 × 1.14 μM for H1299, and 4 × 2.36 μM for H1975). Cells were then gathered and washed with cold phosphate-buffered saline (PBS). And then at 4°C, 1 mL PBS and 2 mL 100% ethanol were added. After centrifugation at 1,000 g for 5 min, they were washed with 2 mL PBS and resuspended with 400 μL PBS. In the end, 50 μL propidium iodide (PI) and 50 μL RNase were added, and cells were darkly cultivated for 30 min at 37°C. The effects of SNX-2112 on cell cycle distribution were analyzed with flow cytometry (Becton Dickinson, Franklin Lakes, NJ, United States; Olszewska et al., 2020).

Cell apoptosis assay
Based on instructions from the manufacturer, the apoptosis rate was detected by the annexin V–fluorescein isothiocyanate
(FITC)/PI Apoptosis Detection kit (556547, Becton Dickinson, United States). Cells were treated identically with mentioned as Cell Cycle Analysis. And then, cells were gathered and washed with 2 mL PBS and resuspended in 100 μL binding buffer. Lastly, 5 μL annexin V-FITC and 10 μL PI were added to the buffer and darkly cultivated at 4°C for 30 min. Effects of SNX-2112 on cell apoptosis were determined by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, United States; Ye et al., 2017).

Molecular Interaction Analysis

Thermal shift assay

Thermal shift assay was applied for evaluating molecular interaction between target Hsp90N and its ligand SNX-2112 using real-time PCR (7500, ABI Corp., United States). A 20-μL reaction system consisted of Hsp90N (1 mg/mL) 2 μL, ligand SNX-2112 (dissolved in DMSO, 100 mM) 0.5 μL, buffer (pH 7.5, 20 mM Tris–HCl and 150 mM NaCl, and 10% glycerol) 10 μL, Protein Thermal Shift Buffer (Applied Biosystems, United States) 5 μL, and TSA dye (Applied Biosystems, United States) 2.5 μL, eight replicates. The samples were centrifuged at 1,000 revolutions/min (rpm) for 1 min. The samples were run from 25 to 95°C with a ramp rate of 1°C/min. The protein unfolds when it is heated and exposes hydrophobic regions to bind the environmentally sensitive TSA dye and fluoresces. The melting temperature (Tm) and melting temperature differences (ΔTm) were derived from the melting curve, which was related to the binding affinity of Hsp90N-SNX-2112 (Andreotti et al., 2015).

ITC

Isothermal titration calorimetry was used for further detecting Hsp90N-SNX-2112 molecular interactions using ITC (ITC-200, Malvern Instrument Ltd., United Kingdom). SNX-2112 in DMSO was diluted with buffer for protein purification (pH 7.5, 20 mM Tris–HCl and 150 mM NaCl) to 500 μM. Fresh-purified Hsp90 was extensively dialyzed against the same buffer and concentrated to 50 μM. After being centrifuged and degassed, 2 μL aliquots of SNX-2112 were注入 into Hsp90N solution in the cell with an interval of 200 s and 750-rpm stirring speed. With Microlab Origin software, the experimental data were fitted with a bimolecular binding model with stoichiometry (ν), enthalpy (ΔH°), and association constant (Kd) as adjustable parameters. The thermodynamic parameters ΔG° (free energy) and ΔS° (entropy) were derived from the equation -RT ln Kd = ΔG° = ΔH° - TΔS (He et al., 2014b).

Complex Crystal Structure Determination

Homo sapiens Hsp90N gene containing residues 9 to 236 was artificially synthesized and cloned into plasmid pET-28a, which was transformed into Escherichia coli BL21 (DE3; TIANGEN Biotech Corp., Beijing, China) to express and purify Hsp90N as reported by Cao (Cao et al., 2017).

With 5:1 molar ratio, SNX-2112 was added into Hsp90N to incubate for 30 min at 4°C. Then, the mixture was centrifuged for 10 min at 3,000 g, and the supernatant was taken to mix with the same amount of a crystal precipitant [pH 8.5, 100 mM Tris–HCl, 200 mM MgCl2, 25–30% PEG4000 (Stebbins et al., 1997)]. With the hanging drop vapor diffusion method, cocrystallization was carried out at 4°C for 3–7 days in an incubator controlled by a bath circulator (PolyScience 9712, PolyScience, United States). Complex crystal image of Hsp90N-SNX-2112 was captured by a stereomicroscope (M165, Leica Microsystems, Germany).

Complex crystals were mounted with cryo-loop (Hampton Research Corp., Aliso Viejo, CA, United States), and then quickly soaked in the cryoprotectant solution containing 20–25% glycerol and crystal reagent mentioned previously (Prodomou et al., 2000). Then, crystals were flash-frozen in liquid nitrogen for XRD. With an ADSC Quantum 315r CCD detector (Wang et al., 2015), all data sets were collected at 100 K on Macromolecular Crystallography Beamline17U1 (BL17U1) at Shanghai Synchrotron Radiation Facility (SSRF, Shanghai, China; Wang et al., 2018).

Aquarium pipeline was applied for automatic processing of diffraction data (Yu et al., 2019b). Using the crystal structure of Hsp90N-FS23 (PDB ID 5CF0) as the research model (Li et al., 2015), the complex crystal structure was determined and refined by molecular replacement with PHENIX software (Adams et al., 2010; Yu et al., 2019a). The initial model was rebuilt by Coot software (Emsley et al., 2010), and CCP4MG software was applied for graphing and molecular interaction analysis (McNicholas et al., 2011; Winn et al., 2011).

Design of New SNX-2112 Derivatives and Molecular Docking Evaluation

A series of new SNX-2112 derivatives were designed based on the complex crystal structure and molecular interaction analysis, which were evaluated by molecular docking with software SYBYL-X 2.0 (Tripos Associates Inc., St. Louis, MO, United States). The crystal structure of Hsp90N-FS23 (PDB ID 5CF0) was as the docking model. First, a compound library of newly designed SNX-2112 derivatives was built, and new derivatives were conducted with geometric and force field optimization for 10,000–100,000 times. Second, the target Hsp90N was optimized by adding hydrogen and charges. Last, molecular docking was carried out using the Surflex-Dock module of SYBYL software. Total score and Cscore were chosen as parameters to evaluate the binding affinity between the target Hsp90 and new ligands. CCP4MG software was applied for simulated complex three-dimensional (3D) structure reconstruction and molecular interaction analysis.

Statistical Analysis

Statistical analysis was conducted with SPSS 13.0 software (International Business Machines Corporation, United States) and GraphPad Prism 5.01 software (GraphPad Software, San Diego, United States). Data were presented as mean ± standard deviation (SD). Differences between two groups were performed according to unpaired Student t test. P < 0.05 was deemed as statistically significant.
FIGURE 1 | SNX-2112 inhibited NSCLC cell proliferation. (A) A549 cells, (B) H1299 cells, and (C) H1975 cells. CCK-8 assay was performed to detect cell viability after treatment with SNX-2112 or DMSO for 24, 48, and 72 h. *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significant differences vs. control. SNX-2112 significantly suppressed cell viability against A549, H1299, and H1975 cells, and the inhibition activity increased with treatment time extension. Annotations: Control, cells treated with DMSO; SNX-2112, cells treated with SNX-2112.

FIGURE 2 | SNX-2112 induced NSCLC cell cycle arrest. Cell cycle distribution for A549 cells (A), H1299 cells (C), and H1975 cells (E) treated with SNX-2112 or DMSO for 72 h detected by DNA flow cytometry assay. The DNA content in different phase of A549 cells (B), H1299 cells (D), and H1975 cells (F) treated with SNX-2112 or DMSO for 72 h. SNX-2112 induced A549, H1975, and H1299 cells cycle arrest at S and G2 phase or G2 phase. Annotations: Control, cells treated with DMSO for 72 h; SNX-2112, cells treated with SNX-2112 for 72 h.
RESULTS

Anticancer Activity in vitro

SNX-2112 Inhibited NSCLC Cell Proliferation

SNX-2112 exhibited a credible anti-NSCLC activity in vitro against A549 cells (IC_{50} 0.50 ± 0.01 µM), H1299 cells (IC_{50} 1.14 ± 1.11 µM), and H1975 (IC_{50} 2.36 ± 0.82 µM).

As shown in Figure 1, SNX-2112 significantly suppressed cell proliferation of A549, H1299, and H1975 at 24, 48, and 72 h (p < 0.05), especially for A549 at 24 and 72 h (p < 0.001), H1299 at 72 h (p < 0.001), and H1975 at 48 h (p < 0.001). The results suggested the cell viability of A549, H1299, and H1975 was inhibited by SNX-2112, and the inhibition activity increased with treatment time extension.

SNX-2112 Induced NSCLC Cell Cycle Arrest

Flow cytometry was used to explore effects of SNX-2112 on NSCLC cell cycle distribution by calculating cellular DNA content. As can be seen from Figure 2, with treatment with SNX-2112 for 72 h, cell percentage in G1, S, and G2 phase exhibited obvious changes compared with the control treated with DMSO for A549 cells (G1 ↓14.48%; S ↑7.62%; and G2, ↑7.23% vs. control), for H1299 (G1 ↓5.29%; S ↑0.05%; and G2, ↑7.24% vs. control), and for H1975 cells (G1 ↓5.29%; S ↑1.80%; and G2, ↑3.50% vs. control). It was suggested that Hsp90\textsuperscript{N} inhibitor SNX-2112 induced cell cycle arrest for A549 and H1975 at G2 and S phase and for H1299 at G2 phase.

SNX-2112 Aggravated NSCLC Cell Apoptosis

During early apoptosis stage, annexin V combined with phosphatidylserine on the external leaflet of plasma membrane, which excluded PI and stained negatively. However, cells of late apoptosis stage and necrosis stained positively. Figure 3 showed the percentage of specific cell population at different apoptosis stages (B3 for normal cells, B4 for early apoptosis cells, B2 for late apoptosis cells, and B1 for necrotic cells). Compared with the control, SNX-2112 increased apoptotic cell percentage of the three NSCLC cell for A549 and H1975 at G2 and S phase and for H1299 at G2 phase.

FIGURE 3 | SNX-2112 aggravated NSCLC cell apoptosis. SNX-2112 aggravated apoptosis of A549 cells (A), H1299 cells (C), and H1975 cells (E). Apoptosis determined by flow cytometry method for cells treated with SNX-2112 or DMSO. Distribution difference of A549 cells (B), H1299 cells (D), and H1975 cells (F) in four quadrants. The four quadrants represent as B3, Normal cell; B4, early apoptosis; B2, late apoptosis; and B1, necrotic cells. It was revealed that SNX-2112 aggravated NSCLC cell apoptosis. Annotations: Control, treated with DMSO for 72 h; SNX-2112, treated with SNX-2112 for 72 h.
late apoptotic, ↑17.2% vs. control), and H1975 (normal cells, ↓2.6%; early apoptosis, ↑5.5%; late apoptosis, ↓3.3% vs. control).

It was indicated that SNX-2112 aggravated cell apoptosis of A549, H1299, and H1975 cells.

To sum up, SNX-2112 disclosed favorable in vitro anti-NSCLC activity (IC\textsubscript{50}, 0.50 ± 0.01 µM for A549; 1.14 ± 1.11 µM for H1299; and 2.36 ± 0.82 µM for H1975), which was achieved by inhibiting cell viability, inducing cell cycle arrest, and aggravating cell apoptosis. Therefore, SNX-2112 would be a potential lead compound for anti-NSCLC new drug development.

**Molecular Interaction Analysis**

**TSA**

Usually, every protein has a specific Tm value under certain conditions, which would change when binding a ligand. Greater ∆Tm means higher affinity, and absolute ∆Tm value greater than three is deemed as a favorable ligand (Lo et al., 2004; Andreotti et al., 2015). As can be seen from Figure 4, Tm value of Hsp90\textsuperscript{N} was 48.36 ± 0.82°C (Figure 4A), and Tm was shifted to 38.84 ± 1.35°C after Hsp90\textsuperscript{N} combining with SNX-2112 (Figure 4B). As a result, ∆Tm value was -9.51 ± 1.00°C (**∗∗∗p < 0.001 vs. control, Hsp90\textsuperscript{N}, n = 8; Figure 4C). It suggested that an intense binding force existed between the ligand SNX-2112 and its target Hsp90\textsuperscript{N}.

**ITC**

As shown in Figure 4D, the upper panel manifested the raw titration data. The power was supplied to the system to maintain a constant temperature against time, and the area of each peak meant interaction heat for injection. The lower panel exhibited bimolecular fit of normalized interaction heats plotted against molar concentration. Hsp90\textsuperscript{N} binding with SNX-2112 was exothermic at 25°C from the negative peaks in the raw ITC titration data. A fine S-shaped curve was obtained after nonlinear data fitting. The stoichiometry was determined to be 0.83 ± 0.01, indicating a 1:1 binding mode between target Hsp90\textsuperscript{N} and ligand SNX-2112. As shown in Table 1, the K\textsubscript{d} values here were 14.10 ± 1.60 nM, which indicated an intense binding force between the ligand SNX-2112 and its target Hsp90\textsuperscript{N}.

The thermodynamic signature was dominated by both favorable enthalpy change (ΔH\textsubscript{a}, −6.80 ± 0.20 kcal/mol) and entropy change (TΔS\textsubscript{a}, 3.90 kcal/mol), indicating the establishment of favorable interactions during the Hsp90\textsuperscript{N}-SNX-2112 binding process (He et al., 2011, 2014a).

**Complex Crystal Structure Determination**

With a molecular weight of 25 kDa, Hsp90\textsuperscript{N} was expressed in E. coli BL21 (DE3) strain. Purified Hsp90\textsuperscript{N}...
protein was obtained by immobilized Ni²⁺ affinity chromatography and molecular sieve chromatography. Complex crystals of Hsp90⁹⁻SNX-2112 were obtained by the hanging-drop method at 4°C for 3–5 days. As shown in Supplementary Image 1, the average dimension of rhombus crystals was approximately 230 μm × 130 μm × 50 μm.

Crystal structure of Hsp90⁹⁻FS23 (PDB ID 5CF0) was reported as the research model; a complex crystal structure of Hsp90⁹⁻SNX-2112 was successfully determined by molecular replacement. The complete data collection and refinement statistics are shown in Table 2. The coordinates and structure factors have been deposited in PDB (PDB ID 6LTK). Diffraction data were collected up to 2.14 Å resolution limit with R factors have been deposited in PDB (PDB ID 6LTK). Diffraction source BL17U1, SSRF.

As shown in Figure 5, the asymmetric unit of the refined model included a protein monomer (residues 17–224) and 47 α = a cell parameter, R work = 0.24 and R work = 0.21. The space groups showed I222 (unit cell parameter, a = 69.70 Å, b = 88.87 Å, c = 96.55 Å; α = β = γ = 90.00°).

As shown in Figure 5, various interactions, including hydrogen bonds, π-π interactions, and hydrophobic interactions, contributed to the intense binding force between the ligand SNX-2112 and its target Hsp90⁹⁻. SNX-2112 formed three hydrogen bonds with residues Y139 (2.7 Å), residue D93 (2.9 Å), and residue K58 (3.0 Å) of Hsp90⁹⁻. Meanwhile, SNX-2112 also arranged a network of water-mediated hydrogen bonds with residue K58 (3.0 Å) of Hsp90⁹⁻ and its target Hsp90⁹⁻, which was responsible for the intense binding force between the ligand SNX-2112 and its target Hsp90⁹⁻.

New SNX-2112 Derivatives Design and Molecular Docking Evaluation

New SNX-2112 Derivatives Design
Based on the complex crystal structure and molecular interaction analysis, 32 new SNX-2112 derivatives were designed, as shown in Figure 6A. It was important to maintain the special molecular configuration to achieve further structural optimization of SNX-2112. Hydrogen bonds were favorable binding force, and thus, hydrophilic groups were adopted for structural modification of SNX-2112. For instance, the hydroxy group on cyclohexanol moiety was varied to an amino group or sulfhydryl group. The amino group on benzamide moiety was replaced by a hydroxy group. The carbonyl group of 6,6-dimethyl-1,5,6,7-tetrahydro-indazol-4-one moiety was substituted by a nitro group. Hydrophobic interactions also contributed more, and introduction of hydrophobic functional groups would be a

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**Table 1** Dissociation constant and thermodynamic data during the Hsp90⁹⁻SNX-2112 binding process at 25°C.

| Items | Thermodynamic data (mean ± SD, n = 4) |
|-------|-------------------------------------|
| n     | 0.83 ± 0.01                         |
| K_d   | 14.10 ± 1.60                        |
| ΔG_d  | −10.70                              |
| ΔH_d  | −6.80 ± 0.20                        |
| TΔS_d | 3.90                                |

**Table 2** Data collection and refinement statistics of Hsp90⁹⁻SNX-2112 complex crystal.

**Diffraction data**
- Resolution (Å): 50.00–2.14 (2.18–2.14)
- Space group: I222
- Unit cell parameters: a, b, c (Å) = 69.700, 88.868, 96.550
- Wavelength (Å): 0.97890
- Total reflections: 217137
- Uniqueness reflections: 16764
- Redundancy: 13.0 (11.4)
- Mean I/σ(I): 90.0/2.6 (2.3/1.8)
- Completeness (%): 99.1 (99.1)
- Rsym or Rmerge: 0.074 (1.267)

**Refinement data**
- Resolution range (Å): 36.24–2.14
- Reflections in test set 1674
- Reflections in working set 15070
- Reflections in test set 1674
- R_work: 0.21 / 0.24
- R_free: 0.707
- Mean temperature factor (Å²): 0.587
- Wilson B-factor (Å²): 51.5
- Total number of atoms: 1715
- R.m.s. deviations: 0.004
- Bond lengths (Å): 0.002
- Bond angles (°): 0.587
- Wilson B-factor (Å²): 51.5
- R_free: 0.074 (1.267)
- R_mol: 0.074, calculated the same as R_work, but from a test set containing 5% of data excluded from the refinement calculation.

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*a The numbers in parentheses represent the values for the highest-resolution shells.
*b R_work = ∑|Fobs| − |Fcalc| / ∑|Fobs|, where Fobs and Fcalc are observed and calculated structure factors, respectively.
*c R_free, calculated the same as R_work, but from a test set containing 5% of data excluded from the refinement calculation.

**New SNX-2112 Derivatives Design**
Based on the complex crystal structure and molecular interaction analysis, 32 new SNX-2112 derivatives were designed, as shown in Figure 6A. It was important to maintain the special molecular configuration to achieve further structural optimization of SNX-2112. Hydrogen bonds were favorable binding force, and thus, hydrophilic groups were adopted for structural modification of SNX-2112. For instance, the hydroxy group on cyclohexanol moiety was varied to an amino group or sulfhydryl group. The amino group on benzamide moiety was replaced by a hydroxy group. The carbonyl group of 6,6-dimethyl-1,5,6,7-tetrahydro-indazol-4-one moiety was substituted by a nitro group. Hydrophobic interactions also contributed more, and introduction of hydrophobic functional groups would be a
suitable scheme for structural modification. To be specific, the trifluoromethyl part was modified to a dimethylamino or tert-butyl group. Considering the π–π interactions formed, the 1H-pyrazole ring of SNX-2112 was replaced by a 1H-pyrrole ring.

Molecular Docking Evaluation

Based on the design scheme, 32 new SNX-2112 derivatives were designed, and the binding capacity with the target Hsp90\(^N\) was evaluated by molecular docking from theoretical calculation aspect using the software SYBYL-X 2.0. Total score and CScore were chosen as key evaluation parameters, which were positively related to their binding force. Their chemical structures, docking results, and total score increment compared with SNX-2112 are shown in Supplementary Table 1. Among them, a total score of 25 new derivatives increased compared with SNX-2112, which suggested a favorable design scheme. The top 10 new derivatives with high total score increment compared with SNX-2112 and chemical structures were shown in Figure 6B. The total score of the optimal one S15 was as high as 9.5 (increment, 3.5 vs. SNX-2112), which suggested that there was an intense banding force between S15 and its target Hsp90\(^N\). There were obvious differences in structures between S15 and SNX-2112. First, compared to SNX-2112, the hydroxy group on cyclohexanol moiety of SNX-2112 was varied between S15 and SNX-2112. First, compared to SNX-2112, the trifluoromethyl part of SNX-2112 was modified to a dimethylamino group in S15. The changes in the structure improved the Hsp90\(^N\)-S15 binding affinity.

In the optimal derivative S15 as a representative (Figure 6F), simulated complex 3D structure reconstruction and molecular interaction analysis of Hsp90\(^N\)-S15 were performed by SYBYL software and CCP4MG software, as shown in Figures 6C-E. S15 was suitably located in the ATP-binding pocket of Hsp90\(^N\) (Figures 6C,E). Hydrogen bonds were responsible for their intense binding force. S15 formed three hydrogen bonds with residues N51 (2.9 Å), F138 (2.7 Å), and T184 (3.0 Å) of Hsp90\(^N\). Moreover, the hydrophobic effects apparently also played a vital role for binding of S15 to Hsp90\(^N\).

Specifically, the hydrophobic interactions appeared between the 4,5,6,7-tetrahydro-1H-indazole moiety and residues L107, G135, and Y139 of Hsp90\(^N\). In addition, the benzamide moiety together with cyclohexylamine moiety formed the hydrophobic interactions with residues A55, K58, and M98 (Figure 6D).

In short, hydrogen bonds and hydrophobic interactions of Hsp90\(^N\)-S15 were responsible for their intense binding force, which would provide a potential lead compound for anti-NSCLC new drug development.

DISCUSSION

Non–small cell lung cancer accounts for more than 80% of lung cancer cases, which is the leading cause of cancer death worldwide (Petrek and Yu, 2019). Targeted drugs, for NSCLC as an oncogene addicted tumor, could be an effective way of treatment. However, drug resistance and toxicity confined their clinical applications (Tomasini et al., 2016; Xiong et al., 2019). Hsp90, as a chaperone, plays a vital and even decisive role in tumorigenesis and development by assisting 300 client proteins to obtain their correct folding and mature conformation (Jhaveri et al., 2014; Hyun et al., 2019). Hsp90 exists with an α-α or β-β homodimer in the cytoplasm, and each monomer consists of three domains. The C-terminal domain (Hsp90\(^C\)) plays a key role for dimerization of Hsp90, whereas the central domain (Hsp90\(^M\))...
FIGURE 6 | Design and molecular docking evaluation results of new SNX-2112 derivatives. (A) Design of new SNX-2112 derivatives based on complex crystal structure and molecular interaction analysis of Hsp90\textsuperscript{N}-SNX-2112. (B) Top 10 optimal new SNX-2112 derivatives with high total score increment evaluated by molecular docking using software SYBYL-X 2.0. (C) Overall fold of complex crystal of Hsp90\textsuperscript{N}-S15. Hsp90\textsuperscript{N} and S15 are shown as cartoon and stick, respectively. (D) Molecular interaction between Hsp90\textsuperscript{N} and S15. The carbon, nitrogen, and oxygen atoms of S15 are colored in green, blue, and red, respectively. In addition, the carbon, nitrogen, oxygen, and sulfur atoms of Hsp90\textsuperscript{N} residues are shown in cyan, blue, red, and yellow, respectively. The hydrogen bonds are represented as red dashed lines. (E) Connolly surface of ATP-binding site of S15 in Hsp90\textsuperscript{N}. (F) Molecular structure of S15.

possesses a large hydrophobic surface to bind client proteins and facilitate their folding. The N-terminal domain (Hsp90\textsuperscript{N}), with ATP-hydrolase activity, contains an ATP-binding pocket that is responsible for ATP binding and hydrolysis to provide energy for client protein correct folding or conformation maturation, which is indispensable for the chaperone function of Hsp90 (Donnelly and Blagg, 2008; Mayer et al., 2012).

Accordingly, Hsp90 inhibitors were divided as Hsp90\textsuperscript{N} inhibitors, Hsp90\textsuperscript{M} inhibitors and Hsp90\textsuperscript{C} inhibitors. Hsp90\textsuperscript{N} inhibitors could bind in the ATP-binding pocket to prevent ATP binding and then disable chaperone function of Hsp90 (Prodromou et al., 2000; Li et al., 2012b). Hsp90\textsuperscript{N} inhibitor SNX-2112 was perfectly sitting at the ATP-binding pocket of Hsp90\textsuperscript{N}, which prevented ATP binding to disable chaperone function of Hsp90. Therefore, immature or misfolding client proteins would be captured and degraded by proteasomes (Chen et al., 2014). With degraded proteins accumulated, multiple signaling pathways can be derailed simultaneously, which can hinder proliferation, and progression of cancer (Chehab et al., 2015; Dutta Gupta et al., 2019). Hsp90, therefore, was paid more attention to as a promising anticancer drug target (Barrott and Haystead, 2013; Soga et al., 2013; Hu et al., 2019).

Absence of the complex crystal structure of Hsp90\textsuperscript{N}-SNX-2112, however, prevented further structural optimization of SNX-2112 and understanding of binding mode and molecular interaction of Hsp90\textsuperscript{N}-SNX-2112. A high-resolution complex crystal structure of Hsp90\textsuperscript{N}-SNX-2112 was successfully determined by XRD (resolution limit, 2.10 Å, PDB ID 6LTK), and their molecular interaction was analyzed in detail. The complex crystal structure suggested that SNX-2112 was well accommodated in the ATP-binding pocket to disable molecular chaperone activity of Hsp90, therefore exhibiting favorable inhibiting activity on three NSCLC cell lines (IC\textsubscript{50}, 0.50 ± 0.01 μM for A549, 1.14 ± 1.11 μM for H1299, and 2.36 ± 0.82 μM for H1975) by inhibited proliferation, induced cell cycle arrest, and aggravated cell apoptosis.

The molecular binding mode and interaction mechanism of Hsp90\textsuperscript{N}-SNX-2112 were analyzed in detail by the complex crystal structure, which suggested that hydrogen bonds, hydrophobic interactions, and π–π interactions significantly contributed to the
intense binding force between the ligand SNX-2112 and its target Hsp90N, which was verified by TSA and ITC. TSA was a useful tool for target-ligand binding force valuation by determining melting temperature differences (ΔTm) of the target with or without binding a ligand. Every protein has a definite melting temperature under certain conditions, and it changes after binding a ligand. Greater absolute ΔTm value means stronger binding force. It is deemed as a favorable ligand with absolute ΔTm value greater than 3. The TSA result (ΔTm, -9.51 ± 1.00°C) indicated an intense binding force between Hsp90N and SNX-2112, which was verified again by ITC. ITC, another tool for binding force determination, measured heat absorption or release amount during target-ligand binding process. Smaller Kd means stronger binding force and protein thermostability. The ITC results (Kd, 14.10 ± 1.60 nM) suggested that the thermostability of Hsp90N increased binding with its ligand SNX-2112.

Based on the complex crystal structure, molecular interaction analysis from TSA and ITC, the design scheme of new SNX-2112 derivatives focused on promoting the interactions by group replacements, such as hydrogen bonds, hydrophobic interactions, and π-π interactions, contributed to the intense binding force of Hsp90N-SNX-2112. Thirty-two novel SNX-2112 derivatives were designed, and 25 exhibited increased binding force with the target Hsp90N, which confirmed a favorable design scheme evaluated by molecular docking assay. The results would provide promising lead compounds for anti-NSCLC new drug development based on the lead compound SNX-2112.

CONCLUSION

SNX-2112 is a promising compound, but the absence of the complex crystal structure of Hsp90N-SNX-2112 restricted the structural optimization of SNX-2112. Herein, a complex crystal structure of Hsp90N-SNX-2112 was determined, and anti-NSCLC activity in vitro of SNX-2112 was evaluated. The main results were as follows:

(1) SNX-2112 displayed favorable anti-NSCLC activity (IC50, 0.50 ± 0.01 μM for A549, 1.14 ± 1.11 μM for H1299, and 2.36 ± 0.82 μM for H1975) by inhibiting cell viability, inducing cell cycle arrest, and aggravating cell apoptosis.

(2) A high-resolution complex crystal structure Hsp90N-SNX-2112 was successfully determined by XRD (resolution limit, 2.14 Å, PDB ID 6LTK). It was indicated that SNX-2112 well accommodated in the ATP-binding pocket to disable molecular chaperone function of Hsp90, therefore suppressing cancer cells.

(3) The result from TSA disclosed that an intense binding force resulted in a more stable conformation of Hsp90N after binding with SNX-2112 (ΔTm, -9.51 ± 1.00°C), which was verified from favorable thermodynamic changes by ITC (Kd, 14.10 ± 1.60 nM).

(4) Based on complex crystal structure and molecular interaction analysis of Hsp90N-SNX-2112, 32 new SNX-2112 derivatives were successfully designed. Among them, 25 new derivatives exhibited increased binding force with Hsp90N, which verified a favorable design scheme by molecular docking evaluation.

(5) The results would provide new references and guides for anti-NSCLC new drug development basis on the lead compound SNX-2112.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

DZ, Y-MX, and L-QC performed the experiments, analyzed the data, interpreted the results, and wrote the manuscript. FY, HZ, and WQ collected and determined the data. H-JL, C-XH, and XJ participated in the cell study. LX, XZ, and P-QL participated in the molecular interaction analysis. YH, J-HH, and H-LC participated in the study design, result interpretation and manuscript improvement. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.650106/full#supplementary-material

Supplementary Image 1 | Complex crystals of Hsp90N-SNX-2112. Complex crystals were obtained by the hanging-drop method at 4°C for 3–5 days. The average dimension of rhombus crystals was approximately 230 μm × 130 μm × 50 μm.

Supplementary Table 1 | Results for molecular docking evaluation of SNX-2112 or its new derivatives binding with the target Hsp90N.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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