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Transcriptomic and proteomic approach to studying SNX-2112-induced K562 cells apoptosis and anti-leukemia activity in K562-NOD/SCID mice

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ABSTRACT

SNX-2112, a novel inhibitor of Hsp90 currently used as an anti-tumor drug, induces apoptosis in multiple tumor cell lines. It destabilizes specific client proteins, but the molecular mechanism of the apoptosis effect of SNX-2112 is poorly understood. Here, we analyzed the apoptotic effect of SNX-2112 on human chronic myeloid leukemia (CML) K562 cells. Transcriptomic and proteomic approaches further revealed that caspase signals originated from mitochondria dysfunction, mediated by Akt signaling pathway inactivity. Additionally, SNX-2112 prolonged the survival time of NOD/SCID mice inoculated with K562 tumor cells. Our results demonstrated the therapeutic potential of SNX-2112 against human CML.

Structured summary:
MINT-7033976: *BAD* (uniprotkb:Q92934) *physically interacts* (MI:0218) with *Bcl2-Xl* (uniprotkb:Q07817) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

Hsp90 (heat shock protein 90) is a molecular chaperone that modulates the stability and/or transport of a diverse set of critical cellular regulatory, metabolism, organization, and signaling proteins [1]. It allows cancer cells to tolerate the many components of dysregulated pathways in a transformation specific manner by interacting with several client substrates, such as kinases, hormone receptors and transcription factors directly involved in driving multi-step malignancy, and also with mutated oncogenic proteins required for the transformed phenotype [2].

SNX-2112, a novel inhibitor of Hsp90, selectively binds to the ATP pocket of Hsp90 and is more pharmacologically effective than 17-(allylamino)-17-demethoxygeldanmycin (17-AAG) [3]. Here, we provide evidence that SNX-2112 can induce apoptosis in human chronic leukemia K562 cells. A series of assays revealed that SNX-2112 not only degraded its client protein Bcr-Abl but also impaired mitochondria function resulting in K562 cells apoptosis. Simultaneously, we found a possible mechanism related to Akt signaling pathway inactivity. Furthermore, consecutive injection of SNX-2112 prolonged the survival time of NOD/SCID mice inoculated with K562 cells.

2. Materials and methods

2.1. Reagents, antibodies and cells

SNX-2112 was made by our lab as described [4] with a purity > 98.0%. 17-AAG was from Alexis Biochemicals (San Diego,

Abbreviations: 17-AAG, 17-(allylamino)-17-demethoxygeldanmycin; DMSO, dimethylsulfoxide; CML, chronic myeloid leukemia; FBS, fetal bovine serum; IEF, isoelectric focusing; IPG, immobilized pH gradient; PRDX5, peroxiredoxin V; DLD, dihydrolipoamide dehydrogenase; ECH1, enoyl coenzyme A hydratase 1; IDH3A, isocitrate dehydrogenase alpha subunit; ALDH2, aldehyde dehydrogenase 2 family; NDUFV2, NADH dehydrogenase flavoprotein 2; TRAP1, tumor necrosis factor-associated protein 1; Crkl, v-crk sarcoma virus CT10 oncogene homolog (avian)-like; Grb2, growth factor receptor bound protein 2

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CA). SNX-2112 and 17-AAG stocks at 10 mmol/L dimethylsulfoxide (DMSO) in solution were stored at 4 °C and −20 °C, respectively. Antibodies against these proteins were purchased: caspase-3, 8, 9, PARP, p-Bad (Ser112), and Bcr-Abl protein (Cell Signaling, Beverly, MA); cytochrome *c*, p-Akt(Thr308), Akt, Bad, Bcl-xL,14-3-3 (Epitomics, Burlingame, CA); Crkl, Grb2 and β-actin (Santa Cruz, Santa Cruz, CA). Human chronic myeloid leukemia (CML) K562 cells (ATCC, Manassas, VA) were grown in RPMI-1640 with 10% heat inactivated fetal bovine serum (FBS), and 100 U/ml of penicillin and streptomycin.

2.2. MTT assay

2×10^5 K562 cells were seeded in a 96-well plate with various concentrations of SNX-2112 for 72 h.

Then 20 μl of RPMI-1640 with 10% FBS and 5 mg/ml MTT were added. The precipitated formazan was dissolved in 100 μl of DMSO. Cell viability was assessed at optical density = 570 nm.

2.3. Assessment of apoptosis

Cells were washed in PBS and resuspended in 100 μl of incubation buffer containing Annexin V-FITC and PI. Samples were incubated at RT for 10 min and analyzed by FACS.

2.4. Western blot

Cells were lysed in RIPA buffer and protein measured by Bradford assay. Cell extracts were run on SDS–PAGE, blotted on PVDF membranes, blocked with 5% non-fat milk, and probed with various antibodies. Specific protein bands were visualized with ECL chemiluminescence (Pierce, Rockford, IL) and imaged by autoradiography.

2.5. Transcriptomic analysis

cRNA was prepared and hybridized to Affymetrix Human Genome U133 Plus 2.0 Array. Each microarray dataset was normalized by Robust Multichip Averaging (RMA) method and correlation of all signals intensity > 95%. Triplicate arrays were analyzed with Welch *t*-test. Genes with $P < 0.01$ and a group mean difference $\geqslant 1$ or $\leqslant -1$ were considered significantly modulated and chosen for analysis. Gene Ontology annotation terms were generated with Pathway Studio version 5.0 software (Ariadne Genomis, Rockville, MD). The Kyoto Encyclopedia of ResNet Mammalian database was used as a search tool [5].

2.6. Proteomics analysis

Cells induced with 1 μM SNX-2112 for indicated time were harvested, rinsed, lysed for 30 min at 4 °C, then centrifuged (12 000×*g*, 4 °C) for 15 min. Proteins in the lysates were measured by the Bradford assay, then separated by isoelectric focusing (IEF) using immobilized pH gradient (IPG) drystrips with pH range 3–10 on Ettan IPGphor 3 (General Electric Company, USA) with a voltage gradient. Proteins were resolved in the 2nd dimension with SDS–PAGE and 12.5% gels were stained with silver nitrate overnight. Gel images were analyzed with Image Master 2D Platium 6.0. Peptide mass spectra were obtained on an Applied Biosystem Sciex 4800 MALDI TOF/TOF mass spectrometer.

Data were acquired in a positive MS reflector using a CalMix5 standard for calibration (ABI4700 Calibration Mixture). The MS and MS/MS spectra for each spot were combined and run on the MASCOT search engine (V2.1, Matrix Science, UK) using GPS Explorer software (V3.6, Applied Biosystems). MASCOT protein scores > 61 were statistically significant ($P < 0.05$).

2.7. Isolation of the cytosolic fraction

Isolation of the cytosolic fraction was performed at 4 °C as described [6]. Briefly, cells were lysed in ice-cold sucrose buffer. The lysate was centrifuged at 600×*g* for 10 min to remove nuclei and unbroken cells. Then, supernatant was spun at 14 000×*g* for 15 min to eliminate mitochondria. The supernatant was centrifuged again at 100 000×*g* for 1 h. The protein concentration of the supernatant, representing the cytosolic fraction, was assayed by the Bradford assay. Cytochrome *c* in the cytosolic fraction was then analyzed by Western blot.

2.8. JC-1 for mitochondrial transmembrane potential study

5×10^5 cells were incubated with 10 μg/ml JC-1 for 30 min at 37 °C, then washed with PBS and resuspended in 250 μl PBS. Highly negative membrane potential in mitochondria produces JC-1 red fluorescence. Loss of mitochondrial transmembrane potential results in green fluorescence and loss of the red fluorescence.

2.9. Co-immunoprecipitation (co-IP)

Cells seeded for indicated time were lysed with IP buffer. Clarified cell lysates were incubated with antibodies against specific proteins for 90 min at 4 °C with gentle shaking, then absorbed to protein G plus-agarose beads (Santa Cruz, Santa Cruz, CA). Beads were extensively washed; the eluted complex was resuspended in SDS sample buffer, and separated by 12.5% SDS–PAGE.

2.10. Surface antigen CD13 and CD33 analysis by flow cytometry

Cells were suspended in PBS, stained for 30 min on ice with anti-CD13-PE or CD33-PE and anti-glycophorin A-FITC antibodies (Beckman Coulter, USA). FITC- or PE-labeled mouse IgG alone were negative controls.

2.11. Animals and administration of SNX-2112

Six-eight weeks old NOD/SCID mice from Chinese Academy of Medical Sciences (Beijing, China) were given commercial food, water ad libitum, and housed at 23 ± 5 °C and 55 ± 5% RH through the experiment. Eight mice per group were inoculated by tail vein with 10^7 K562 cells. SNX-2112 was dissolved in saline with 10% DMSO; 17-AAG was dissolved in saline with 10% DMSO including 0.05% Tween-20 [7].

2.12. Statistical analysis

Data were evaluated by Welch *t*-test when only 2 value sets were compared. One-way ANOVA followed by Dunnett's test for 3 or more groups. Survival was analyzed using the Kaplan–Meier log-rank test. Results are expressed as mean ± S.D. with significance at $P < 0.05$ or $P < 0.01$.

3. Results and discussion

3.1. SNX-2112 induced apoptosis in human chronic leukemia K562 cells

We first determined the cytotoxic concentration of SNX-2112 on K562 cells using MTT assays on cells treated with various concentrations of the drug for 72 h. SNX-2112 showed a dose-dependent cytotoxic effect with IC50 = 0.92 μM (Fig. 1A). Then, we evaluated the apoptotic effect by flow cytometry of Annexin V-FITC