The effect of small molecule inhibitor NSC348884 on nucleophosmin 1-mutated acute myeloid leukemia cells

J.-F. ZHU, L.-L. HAN, Y. MA, Y.-X. WANG, F. ZHANG

Department of Hematology, The First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui Province, China

Abstract. – OBJECTIVE: Nucleophosmin 1 (NPM1) is a common shuttling protein. Mutation in the NPM1 gene is the most frequent gene alteration in acute myeloid leukemia (AML). This study aims to explore the inhibitory effects of small molecule NSC348884 on wild-type and NPM1-mutated AML cells.

MATERIALS AND METHODS: Immunofluorescence was used to determine the intracellular localization of NPM1 protein in wild-type (OCI-AML2) and NPM1-mutated (OCI-AML3) AML cell lines. The oligomerization state of NPM1 was assessed by Western blot analysis, and the inhibitory effect of NSC348884 on the proliferation of AML cells was evaluated by Cell-counting kit-8 (CCK-8). Flow cytometry was used to detect the proapoptotic effect of NSC348884 on AML cells.

RESULTS: Western blot results showed a significant reduction in the levels of the oligomeric NPM1 protein after the treatment with NSC348884. NSC348884 had an inhibitory effect on the proliferation of both wild-type and NPM1-mutant AML cells. The inhibitory effect on OCI-AML3 cells was stronger, compared to OCI-AML2 cells. Flow cytometry showed that NSC348884 could significantly induce AML cell apoptosis and had a stronger proapoptotic effect on OCI-AML3 cells.

CONCLUSIONS: NSC348884 had inhibitory and proapoptotic effects on both wild-type and NPM1-mutated AML cells. The effect of NSC348884 on AML cells, carrying NPM1 mutation was significantly stronger.

Key Words: NSC348884, NPM1, AML, Proliferation, Apoptosis.

Introduction

Nucleophosmin 1 (NPM1) is a widely expressed nuclear phosphoprotein that is mainly located in the nucleolus and shuttles between the nucleoplasm and the cytoplasm. Its main functions include ribosome generation, mRNA processing, chromatin remodeling, apoptosis regulation and DNA repair. Recent studies found that the expression of NPM1 is increased in solid tumors such as liver, ovarian, and colon cancers, and overexpression of NPM1 is closely related to tumor proliferation, invasion, and patient prognosis. NPM1 is also the most common mutated gene in acute myeloid leukemia (AML). Approximately 35% of AML patients have frameshift mutations in the 12th exon of the NPM1 gene.

The mutation changes the nucleolar localization signal (NoLS) of NPM1 into the nuclear export signal (NES), resulting in the cytoplasmic localization of the NPM1-mutated protein NPMc+. In 2016, WHO updated the identification of unique biomarkers associated with some myeloid neoplasms and acute leukemias and classified myeloid tumors as a subtype with reproducible genetic abnormalities. While NPM1 mutation is an important cause of AML, there is still a lack of drugs specifically targeting NPM1. Small molecule inhibitor NSC348884 can act on NPM1, affecting its function by impacting the oligomerization state of the protein. Multiple studies have shown that NSC348884 has anti-tumor effects. The aim of this study is to compare the inhibitory effects of NSC348884 on wild-type and NPM1-mutated AML cells, providing some reference for developing more effective clinical treatment of leukemia.
protein 2 (Bcl-2) and Bcl-2-associated X (Bax) antibodies were purchased from Proteintech Group Inc. (Rosemont, IL, USA). RPMI-1640 culture medium, and penicillin/streptomycin solution were purchased from Invitrogen Company (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Sichuan Sijiqing Company (Sichuan, China). The CCK-8 proliferation kit was purchased from Vazyme Company (Nanjing, China), and the Annexin V-FITC/PI cell apoptosis detection kit was purchased from ServiceBio Company (Wuhan, China). The study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College with No. BY2022026, date: 2022-March-05th.

**Cell Culture**

OCI-AM2 and OCI-AML3 cells were cultured in RPMI-1640 complete medium containing 15% FBS, 1% penicillin and streptomycin, and incubated at 37°C, 5% CO₂ with saturated humidity.

**Immunofluorescence**

OCI-AML2 and OCI-AML3 cells were centrifuged at 800 rpm for five minutes, and the pellets were washed with phosphate-buffered saline (PBS). Centrifugation and washing with PBS were repeated twice. One hundred microliters of cell suspension were put onto a glass slide, fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.5% Triton x-100 for 15 minutes, and blocked with 5% BSA for one hour. Cells were incubated with the NPM1 primary antibody overnight at 4°C, followed by one hour incubation with the fluorescent secondary antibody (in the dark). Nuclear DNA was labeled by 4’,6-diamidino-2-phenylindole (DAPI) staining for 15 minutes and confocal fluorescence microscopy was carried out using FV-1000 (Olympus, Tokyo, Japan).

**Cell Apoptosis Detection**

OCI-AML2 and OCI-AML3 cells were cultured on a six-well plate and treated with different concentrations (0 μM, 1 μM, 2 μM, 4 μM) of NSC348884 for 24 hours. Cells were then transferred into a 5 ml centrifuge tube, pelleted by centrifugation, and washed twice with ice-cold PBS. Five microliters of Annexin V-FITC and Propidium Iodide (PI) staining solutions were added to each well while protecting from light, and cells were incubated at room temperature for 15 minutes in the dark. Nuclear DNA was labeled by 4’,6-diamidino-2-phenylindole (DAPI) staining for 15 minutes and confocal fluorescence microscopy was carried out using FV-1000 (Olympus, Tokyo, Japan).

**Western Blot**

After 12 hours of treatment with 2 μM NSC348884, cells were collected by centrifugation, washed twice with PBS, lysed using RIPA buffer [50 mMTris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, Beyotime Institute of Biotechnology, Jiangsu, China], and the protein concentration was measured by bicinchoninic acid assay method. Protein samples (50 μg) were separated by polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane, and blocked with 5% skim milk for one hour. Membranes were then incubated with the appropriate primary antibody overnight at 4°C, followed by the incubation with the secondary antibody labeled with horseradish peroxidase (Proteintech, Rosemont, IL, USA) at room temperature for two hours. Proteins were visualized using ECL chemiluminescence kit (Thermo Scientific, Waltham, MA, USA). GAPDH was used as a loading control.

**Statistical Analysis**

Categorical variables were compared via Fisher’s exact test or Chi-squared tests. Continuous variables are expressed as means ± SD (standard deviation) and were compared via Student’s t-tests or Mann-Whitney’s U tests. p<0.05 was the significance threshold. SPSS v26.0 (IBM Corp., Armonk, NY, USA) was used for all statistical testing.

**Results**

**Localization of NPM1 Protein in AML Cells and the Effect of NSC348884 on NPM1 Oligomerization**

Localization of the NPM1 in OCI-AML2 (expressing unmaturated NPM1 protein) and OCI-AML3 (carrying an NPM1 mutation) cells was first detected by immunofluorescence. As shown in Figure 1,
The effect of small molecule inhibitor NSC348884 on nucleophosmin

in OCI-AML2 cells, NPM1 was mainly localized in the nucleus and nucleolus. In contrast, the localization of NPM1 protein in OCI-AML3 cells changed from nuclear to cytoplasmic (Figure 1A).

To investigate whether the inhibitory effect of NSC348884 on AML cells is exerted by affecting NPM1 oligomerization, cells were incubated with 2 μM of NSC348884 for 12 hours. Western blot analysis showed that after 12 hours of NSC348884 treatment, levels of oligomeric NPM1 protein in OCI-AML3 cells were significantly reduced, as shown in Figure 1B.

**NSC348884 Inhibits the Proliferation of NPM1-Mutated AML Cells**

We next investigated whether NSC348884 inhibits AML proliferation by affecting NPM1 oligomerization. To determine the dose-dependent effect, OCI-AML2 and OCI-AML3 cell lines were incubated with varying concentrations (0 μM, 1 μM, 3 μM, 6 μM, 10 μM) of NSC348884 for 12 hours (Figure 2A). Alternatively, to test the time-dependent effect, cells were incubated with 2 μM NSC348884 for 12, 24, 36, and 48 h (Figure 2B). Cell proliferation was measured by CCK-8. NSC348884 had a significant inhibitory effect on both OCI-AML2 and OCI-AML3 cells in a concentration- (Figure 2A) and time-dependent (Figure 2B) manner. At the concentration of 2 μM, NSC348884 had a significantly stronger inhibitory effect on OCI-AML3 cells compared to OCI-AML2 cells (Figure 2) (**p<0.01).

**NSC348884 Induces Apoptosis in AML Cells**

To investigate the proapoptotic effect of NSC348884 on AML cells, cells were treated with 2 μM NSC348884 for 12 hours, and the expression of apoptosis-related proteins Bax and Bcl-2 in OCI-AML2 and OCI-AML3 cells was detected by immunoblotting. The results showed that the expression level of Bax in OCI-AML3 cells was significantly higher (Figure 3A), and the expression level of Bcl-2 was significantly lower than that of OCI-AML2 cells (Figure 3B) (**p<0.05). After NSC348884 treatment, the expression of anti-apoptotic protein Bcl-2 in OCI-AML3 cells was significantly reduced (Figure 3B) (**p<0.05). Flow cytometry analysis showed that NSC348884 had a proapoptotic effect on both OCI-AML2 and OCI-AML3 cells. As the concentration increased, cell survival decreased, and the proapoptotic effect was enhanced. The proapoptotic effect of NSC348884 on the OCI-AML3 cell line was significantly higher compared to OCI-AML3 cells (Figure 3D and E) (**p<0.05).
Figure 2. The proliferative inhibitory effect of NSC348884 on OCI-AML2 and OCI-AML3 cell lines was detected by CCK-8. (A) Different concentrations of NSC348884 inhibit the proliferation of AML cells; (B) The inhibitory effect of 2 μM NSC348884 on AML cell proliferation at different time points (all data are shown as mean ± SD. Of three replicates: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure 3. The proapoptotic effect of NSC348884 on AML cell lines. (A) Western blot analysis of the expression of Bax protein in AML cells; (B) Western blot analysis of the expression of Bcl-2 protein in AML cells; (C) Western blot analysis of the expression of Bcl-2 protein in cells, treated with 2 μM of NSC348884; (D) Flow cytometry was used to detect the apoptotic effect of NSC348884 on OCI-AML2 cells; (E) Flow cytometry was used to detect the apoptotic effect of NSC348884 on OCI-AML3 cells (statistically significant differences was set as p<0.05, *p<0.05, **p<0.01, ***p<0.001).
The effect of small molecule inhibitor NSC348884 on nucleophosmin

Discussion

This study compared the inhibitory effects of NSC348884 in AML cell lines with and without the mutation in NPMI and found that NSC348884 can inhibit the proliferation activity of AML cells in a time- and concentration-dependent manner. NSC348884 had a proapoptotic effect on both OCI-AML2 and OCI-AML3 cells, but this effect was stronger in cells carrying the NPMI mutation.

Mutations in the NPMI gene that result in the expression of the NPMc+ protein are unique to hematological tumors and occur in approximately one-third of acute myeloid leukemia cases. Recent studies suggest that the interaction between the mutated NPMI protein (NPMc+) and tumor suppressor protein alternative reading frame leads to abnormal localization of NPMc+ in the cytoplasm and its subsequent degradation. In agreement with this observation, our results demonstrated that NPMc+ localized in the cytoplasm of the AML cells, as opposed to no clearly localized wild-type NPM-1 protein. The interaction between NPMc+ and the deubiquitinating enzyme herpesvirus-associated ubiquitin-specific protease (HAUSP) of the tumor suppressor Phosphatase and TENSin homolog (PTEN) increases the degradation of PTEN. NPMc+ also interacts with the E3 ubiquitin ligase Fbwγ of c-Myc and brings it to the cytoplasm to increase its degradation, thereby upregulating c-Myc protein expression. In addition, the expression of the HOX gene, controlling the proliferation and differentiation of hematopoietic stem/progenitor cells, is upregulated in the NPMI-mutated AML cells. This leads to impaired differentiation and maturation of hematopoietic cells, which may be an important cause of leukemia. NPMI mutation is often accompanied by multiple additional mutations in various genes, such as FLT3, DNMT3A, IDH1, IDH2, RUNXI, etc. While patients with NPMI-mutated AML have a good prognosis, the presence of additional gene mutations is associated with a poor prognosis.

Numerous studies show that NPMI mutation provides an important basis for predicting the prognosis and hierarchical treatment of AML. Although NPMI-mutated AML responds well to standard chemotherapy, patients still face a risk of relapse and drug resistance after discontinuation of the treatment. So far, there is still a lack of drugs that specifically target NPMI and its exact role in the pathogenesis of AML has not been fully elucidated. Previous research established that impaired oligomerization of NPMc+ affects the occurrence and development of AML. Since oligomerization is essential for NPMI function, NPMc+ oligomers are important targets for the treatment of NPMI mutant AML. Studies have found that NSC348884 can inhibit the formation of oligomers in NPMI by disrupting the N-terminal hydrophobic pocket required for oligomerization, and can inhibit the proliferation of various tumors and induce their apoptosis. However, the mechanism of action of NSC348884 on AML cells is still poorly understood. Our results showed that NSC348884 significantly reduced levels of NPMc+ oligomers in AML cells, carrying the NPM-1 mutation, compared to AML cell lines expressing wild-type NPMI. Studies also show that NSC348884 can downregulate antioxidant Peroxiredoxin 6 (PRDX6) and increase the levels of reactive oxygen species in the body via NPMI. NPMI is the most significantly up-regulated gene in the atypical teratoma/rhabdoid tumor (AT/RT) tissues. NSC348884 treatment was able to reduce the viability of the AT/RT cell line by blocking its cell cycle in the G1 phase. NSC348884 can induce apoptosis in colorectal cancer cells by inhibiting NPMI, upregulating p53 protein, and inhibiting the AKT pathway, thereby enhancing the sensitivity of colorectal cancer cells with high expression levels of NPMI to chemotherapy. Our results demonstrated that NSC348884 treatment markedly inhibited cell proliferation and induced apoptosis of AML cells. Mutation in NPM-I was associated with a significantly lower proliferation rate and higher extent of cell death compared to cells with the wild-type NPM-I.

Conclusions

In conclusion, our results showed that NSC348884 exerts inhibitory and proapoptotic effects on AML cells in vitro. However, the effect of NSC348884 on AML cells, carrying NPMI mutation is significantly stronger. Our results may provide a basis for identifying strategies to specifically target nucleophosmin and to develop new therapeutic approaches to treating AML.

Funding

This study is supported by the Key Project of Natural Science, Bengbu Medical College (2021byzd065).

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors’ Contributions
JZ conceived and designed the study. LH, YM and YW collected the data and performed the analysis. JZ was involved in the writing of the manuscript. FZ edited the manuscript. All authors have read and approved the final manuscript.

Ethics Approval
The study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College with No. BY2022026, date: 2022-March-05th.

Informed Consent
Not applicable.

Conflict of Interest
The authors declare that they have no competing interests.

ORCID ID
Junfeng Zhu: 0000-0002-6739-6762
Lili Han: 0000-0002-6166-1502
Yue Ma: 0009-0009-7454-0871
Yuxian Wang: 0009-0002-2155-7804
Feng Zhang: 0009-0006-1057-6398

References
2) Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. Leukemia 2017; 31: 798-807.


