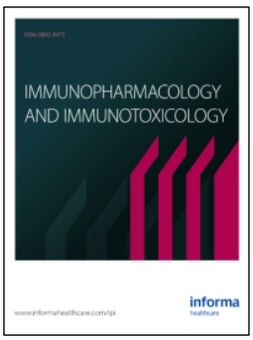

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RESEARCH ARTICLE

Heat shock protein 90 inhibitors induce functional inhibition of human natural killer cells in a dose-dependent manner

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Abstract

Heat shock protein 90 (Hsp90) is a ubiquitously expressed ATP-dependent molecular chaperone across all species that helps to the correct the folding of many proteins related to important signaling pathways. Tumor cells expressing Hsp90 have more ATP-binding affinity than normal cells. Many correlative inhibitors have been developed to promising anti-tumor strategies and have been evaluated in clinical trials. However, the effect of Hsp90 inhibitors on immunocytes cannot be ignored. Natural killer (NK) cells are key components of the innate immune system that play a pivotal role in tumor surveillance. The present study has investigated the potential effect of four Hsp90 inhibitors (NVP-AUY922, BIIB021, 17-DMAG, and SNX-2112) on human primary NK cells. The viability, cytotoxicity, apoptosis, phenotype, and cytokine secretion of NK cells after inhibitor treatment were assessed. The results of this study demonstrated that the inhibitors had negative effects on NK cell activity in a dose-dependent manner. The four inhibitors significantly reduced the cytotoxicity of the NK cells by decreasing viability, inducing apoptosis and down-regulating the expression of cytokines and functional receptors. These findings suggest that more attention should be given to the effect of Hsp90 inhibitors on NK cell function during clinical trials and also represent a potential immunosuppressant strategy.

Keywords

Apoptosis, cytotoxicity, Hsp90 inhibitors, human natural killer cells, immunotherapy

History

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Introduction

Heat shock protein 90 (Hsp90) is an ATP-dependent molecular chaperone that is ubiquitously expressed across all species and considered as an essential factor for multiple cell signaling pathways and oncogenic transcription[1,2]. Signaling pathways related to Hsp90 include AKT (PI3K/AKT pathway), IL-6R (JAK/STAT pathway), FAK (integrin pathway), Bcr-Abl (RAS/ERK pathway), Cdk 4, 6, and 9 (cell cycling), IkB kinases (NFkB pathway), and Apaf-1 (apoptosis)[2–5], which are important for the survival and proliferation of tumor cells by maintaining the conformation, stability, and the activity of several key oncogenic client proteins[6]. Therefore, many Hsp90 inhibitors have been developed to the promising anti-tumor strategies and have been evaluated in clinical trials.

17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) is the first Hsp90 inhibitor clinically evaluated in patients with advanced malignancies[7]. However, the drawbacks of 17-AAG, including weak target potency, low aqueous solubility, low bioavailability, and considerable toxicity, limit its clinical application[8]. NVP-AUY922 (AU), BIIB021 (BI), 17-DMAG (DM), and SNX-2112 (SN) are a group of novel Hsp90 inhibitors that show significant anti-tumor efficacy in *in vivo* or *in vitro* experiments and will be (or have been) evaluated in clinical trials[9–14].

In addition, Hsp90 is also important for the conformation of the MHC complex, receptors and other important immune cell functional proteins influencing client proteins that are involved in human malignancies[15–17]. Geldanamycin, a type of Hsp90 inhibitor has been first widely reported and described to inhibit immunocyte (dendritic cell, T cell, and NK cell) phenotype and functions[3,4].

Human natural killer (NK) cells, which are large granular lymphocytes defined by the presence of CD56 and absence of CD3 on their cell surface ($CD56^+CD3^-$)[18]. Without requiring prior immunization, NK cells are able to specifically and efficiently lyse virally infected oncogenically transformed cells and trigger a subsequent immunological reaction by secreting many types of cytokines. That plays a vital role in tumor identification and surveillance. Depressed NK cell activity compromise immune surveillance, which is responsible for the inadequate host anti-tumor immune response. That function has been recognized as a possible cause of the high incidence of a poor overall survival outcome in carcinoma[19].

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In recent years, stimulated and expanded NK cells *in vitro* have been increasingly used in immunotherapy[20]. Hsp90 inhibitors are prevalent anti-tumor drugs in in clinical trials. Considering the inhibited effects of geladanamycin on immunocyte, more attention should be paid to the potential effects of Hsp90 inhibitors on NK cells in drugs and immunocytes combination therapy.

The anti-tumor effects of Hsp90 inhibitors (NVP-AUY922 (AU), BIIB021 (BI), 17-DMAG (DM) and SNX-2112 (SN)) have been well studied in various carcinoma cells. However, their effects on NK cells have not been well characterized. In this study, the potential effect of Hsp90 inhibitors on the phenotype, activity, and function of NK cells and how NK cells respond to the inhibitors have been investigated. These data provide some evidence on the immune modulation effects of these Hsp90 inhibitors and guide their application in clinical trials.

Materials and methods

Preparation of primary human NK cells and cell lines

Primary human NK cells were prepared following the method described in Huang et al.[21] The blood sample collection methods conformed to the informed consent guidelines of the Ethics Committees of Northwestern Polytechnical University (Supplementary material). Peripheral venous blood (10 mL) was collected from healthy donors ($n = 8$) in heparinized tubes. Blood samples were diluted 2-fold using sterile 1 × phosphate-buffered saline (PBS). Lymphocyte Separation Liquid (Haoyang TBD, Tianjin, China) was used to separate the peripheral blood mononuclear cells (PBMCs) following the instructions. The obtained PBMCs were washed with PBS and cultured in RPMI-1640 media (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD), 100 U/mL IL-2 (Peprotech, Rocky Hill, NJ), and 100 μg/mL of penicillin and streptomycin (Genview, Carlsbad, CA). The PBMCs were counted and co-cultured with equal numbers of stimulating cells (irradiated genetically modified K562 cells), which were prepared following the literature[22]. After 3 weeks of cultivation, *ex vivo*-expanded cells (1×10^5) were collected and washed with PBS, and the percentage of NK cells was determined by flow cytometry (BD FACS Calibur, San Jose, CA) by staining with CD56-PE and CD3-FITC monoclonal antibodies (mAbs) and their isotype-matched controls (IgG1-FITC/IgG2-PE) (QuantoBio, Beijing. China). K562 cells, MCF-7 cells (purchased from American Type Culture Collection, ATCC, Manassas, VA) and stimulating cells were cultured in RPMI-1640 cell media supplemented with 10% FBS containing 100 μg/mL of penicillin and streptomycin and routinely cultured at 37 °C in a 5% CO_2 incubator.

Inhibitors treatment

The NVP-AUY922 (AU), BIIB021 (BI), 17-DMAG (DM), and SNX-2112 (SN) (Selleck Chemicals, Houston, TX) inhibitors were prepared in storage solutions (100 × working concentration, including 100 nM, 1 μM, 10 μM, and 100 μM) in dimethyl sulfoxide (DMSO), Invitrogen, Carlsbad, CA, USA. NK cells (1.2×10^6) were suspended in 2 mL of RPMI-1640 with 20 μL of inhibitor storage solution, then added to the well of a 24 well-plate for 48 h. The working concentrations of the inhibitors were 1 nM, 10 nM, 100 nM, and 1 μM, respectively. Twenty microliters of DMSO were added to the control group to exclude its effect on the cells.

Cell viability and cytotoxicity assay

After treatment with each inhibitor, the NK cells were centrifuged ($1000 \times g$, 5 min), washed with PBS, and re-suspended in 600 μL of RPMI-1640 media (IL-2 free). Twenty microliters of CCK-8 (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) were added to each well of a 96-well plate containing 200 μL cell suspensions in triplicate for each group. The plate was then incubated for 2 h at 37 °C in a 5% CO_2 incubator. A microplate reader (BioTek Instruments Inc., Winooski, VT) was used to record the optical density (OD) values of each well at 450 nm. The viability was calculated using the following equation:

$$\text{Viability}\,(\%) = (\text{OD}_\text{i}/\text{OD}_\text{c}) \times 100\%$$

OD_i, average OD_{450} of NK cell treated by each inhibitor; OD_c, average OD_{450} of control NK cells. The half maximal inhibitory concentration (IC_{50}) was calculated by SPSS 16.0 statistical software (IBM, New York, NY).

The NK cell cytotoxicity assay was performed by the following the procedure that was described in the literature[23]. Briefly, NK cells (8×10^5) were collected from each inhibitor-treated group, washed with PBS, and re-suspended in 400 μL of RPMI-1640 (IL-2 free). 2×10^5 NK cells were mixed with 8×10^4 K562 cells and plated into a well of a 96-well plate with the total medium volume is 200 μL. Each test well was repeated 3 times. The effector control wells contained NK cells (2×10^5 cells) in 200 μL of medium. The target control wells contained K562 cells (4×10^4 cells) in 200 μL of medium. The effector-to-target ratio (E:T) was 5:2. Plate was incubated at 37 °C in a 5% CO_2 incubator. After 4 h co-culture, 20 μL of CCK-8 was added to each well and the plate was then incubated for another 2 h and OD values was recorded at 450 nm. Cytotoxicity was determined by evaluating the rate of NK cell-mediated killing of K562 cells. The killing rate was calculated by using the following equation[24]:

$$\text{Killing rate}\,(\%) = [1 - (\text{OD}_{\text{e+t}} - \text{OD}_\text{e})/\text{OD}_\text{t}] \times 100\%$$

OD_e, average OD_{450} of triplicate wells for NK cell control; OD_t, average OD_{450} of triplicate wells for K562 cell control; OD_{e+t}, average OD_{450} of triplicate wells for NK cells plus K562 cells.

Apoptosis and NK receptors expression using flow cytometry

NK cells in each inhibitor-treated group were centrifuged and washed with PBS, then labeled with AnnexinV-FITC and PI (AnnexinV-FITC Apoptosis Detection Kit, Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) following the corresponding instructions. Then, additional NK cells were divided into four groups (1×10^5 cells each) and labeled with PE-conjugated mouse anti-human NKG2A and isotype control mAbs (R&D Systems, Minneapolis, MN), NKG2D, NKp30, NKp44, and isotype control mAbs (BD Bioscience,

San Jose, CA). All of the cells were analyzed by using flow cytometry.

Real-time quantitative PCR analysis

The total RNA from the NK cells (1×10^7) treated with inhibitors was isolated by using TRIzol reagent (Invitrogen, Carlsbad, CA). The mRNA levels of cytokines (IFN-γ, perforin, granzyme B), and receptors related to NK cell cytotoxicity (NKG2A, NKG2D, NKp30, and NKp44) were evaluated by real-time quantitative PCR (RT-qPCR) and the SYBR Green random mixing method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The relative changes in gene expression were calculated according to the $2^{-\triangle\triangle Ct}$ method[25]. Table 1 shows the primers used in this study. RT-qPCR was performed by the following the instructions of the TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China). The reaction mixtures were incubated for 30 min at 48 °C, followed by 40 cycles of PCR at 94 °C for 5 s, 55 °C for 15 s, and 72 °C for 10 s. At the end of 40 cycles, a melting curve analysis was performed to confirm the presence of an amplified product of the expected size.

Enzyme-linked immunosorbent assay

ELISA kits (BD Bioscience, San Jose, CA) were used to detect the secretion of cytokines (IFN-γ and perforin) by NK cells after inhibitor treatment. The NK cells (8×10^5) in each group were centrifuged and washed with PBS, re-suspended in 400 μL RPMI-1640 (IL-2 free), mixed with 1.6×10^5 K562 cells (target cells, were used to activate the cytokine secretion of NK cells according the literature[26] in 100 μL RPMI-1640 (IL-2 free), then incubated at 37 °C in 5% CO_2 incubator for 4 h. The cell-free supernatants were harvested and assessed for IFN-γ and perforin using specific ELISA kits. Each condition was performed twice in the ELISA assay, and the OD values were recorded at 450 nm. Curve Expert 13.0 software was used to create the standard curve according to the OD values of the standards, and the standard curves were used to calculate the quantities of these cytokines that were secreted by NK cells.

Statistics analysis

Statistical analysis and IC_{50} calculation was performed by using SPSS 16.0 statistical software. The data were presented as the mean ± SD ($\bar{x} \pm s$). The results were analyzed by using the analysis of variance (ANOVA). Multiple comparisons used LSD test in order to evaluate the significance of differences between groups. Statistical significance was defined as $p < 0.05$.

Results

NK cells ex vivo expansion

Primary human NK cells were harvested after 21 d *ex vivo* expansion. The percentage of NK cells ($CD56^+ CD3^-$) within the PBMCs was determined by flow cytometry using staining with CD56-PE and CD3-FITC mAbs and their isotype-matched controls. From one donor, the percentage of NK cells after expansion was 92.31% (Figure 1), and the mean percentage of NK cells from 10 donors was 90.12% ± 4.23% ($n = 10$).

Hsp90 inhibitors have negative effect on viability of NK cells and MCF-7 cells

The resistance of NK cells to the inhibitors was better than that of MCF-7 cells, with the exception of DM. The NK cells viability was 89.76 ± 1.48% in the 1 nM AU-treated group. When the concentration of AU was increased to 10 nM, the viability of the NK cells did not change significantly; it was approximately 70% (70.28 ± 1.18%, 71.28 ± 2.2%, and 73.89 ± 1.95%) in the 10 nM, 100 nM, and 1 μM treated groups. However, the viability of the MCF-7 cells was reduced even further with increases in the AU concentration; the viability values were 84.76 ± 2.22%, 58.45 ± 3.17%, 50.30 ± 4.10%, and 48.87 ± 3.21%, respectively, from 1 nM to 1 μM (Figure 2A). After BI treatment, both NK cells and MCF-7 cells viability were decreased with the increase of the inhibitor concentration. The MCF-7 cells were more sensitive to BI than NK cells; the viability of NK cells was 97.20 ± 2.48%, 91.37 ± 3.18%, 85.94 ± 2.8%, and 71.29 ± 3.1%, compared with 82.36 ± 3.32%, 81.12 ± 2.17%,

Table 1. Primer pairs used in real-time qPCR analysis.

Primer	Sequence (5′→3′)	Amplicon size (bp)
IFNγ	F: TGC-AGG-TCA-TTC-AGA-TGT-AGC R: GGA-CAT-TCA-AGT-CAG-TTA-CCG	248
Perforin	F: GGG-ACA-ATA-ACA-ACC-CCA-TCT R:GGA-ATT-TTA-GGT-GGC-CAT-GAT	174
Granzyme-B	F: AGA-TCG-AAA-GTG-CGA-ATC-TGA R: TTC-GTC-CAT-AGG-AGA-CAA-TGC	138
NKG2A	F: TCA-TTG-TGG-CCA-TTG-TCC-TGA-GG R: AGC-ACT-GCA-CAG-TTA-AGT-TCA-GC	291
NKG2D	F: ATC-GCT-GTA-GCC-ATG-GGA-ATC-CG R: AGA-CAT-ACA-AGA-GAC-CTG-GCT-CTC	213
NKp30	F: TGA-GAT-TCG-TAC-CCT-GGA-AGG R: CAC-TCT-GCA-CAC-GTA-GAT-GCT	235
NKp44	F: TCC-AAG-GCT-CAG-GTA-CTT-CAA R: GAT-TGT-GAA-TCG-AGA-GGT-CCA′	163
GAPDH	F: TCC-TGC-ACC-ACC-AAC-TGC-TTA-GC R: ACA-CGG-AAG-GCC-ATG-CCA-GTG-AGC	254

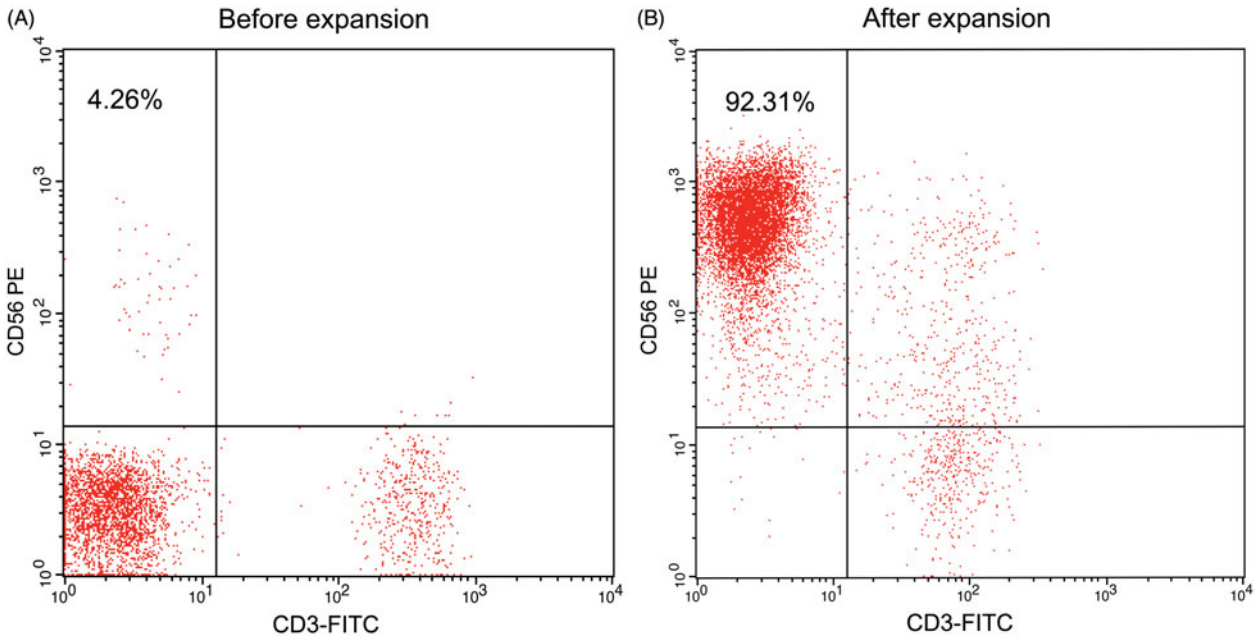


Figure 1. Pre- and post-expansion PBMCs were analyzed using flow cytometry. PBMCs were co-cultured with stimulating cells and harvested after 21-day of *ex vivo* expansion, all pellets were stained with CD56-PE and CD3-FITC mAbs and analyzed by flow cytometry. The percentage of NK cells ($CD56^+CD3^-$) in the PBMC population was tested. (A) Pre-expansion PBMCs were analyzed by flow cytometry. The percentage of NK cells ($CD56^+CD3^-$) in the PBMCs was 4.26%; (B) Post-expansion PBMCs were analyzed by flow cytometry. The percentage of NK cells in the PBMCs was 92.31%.

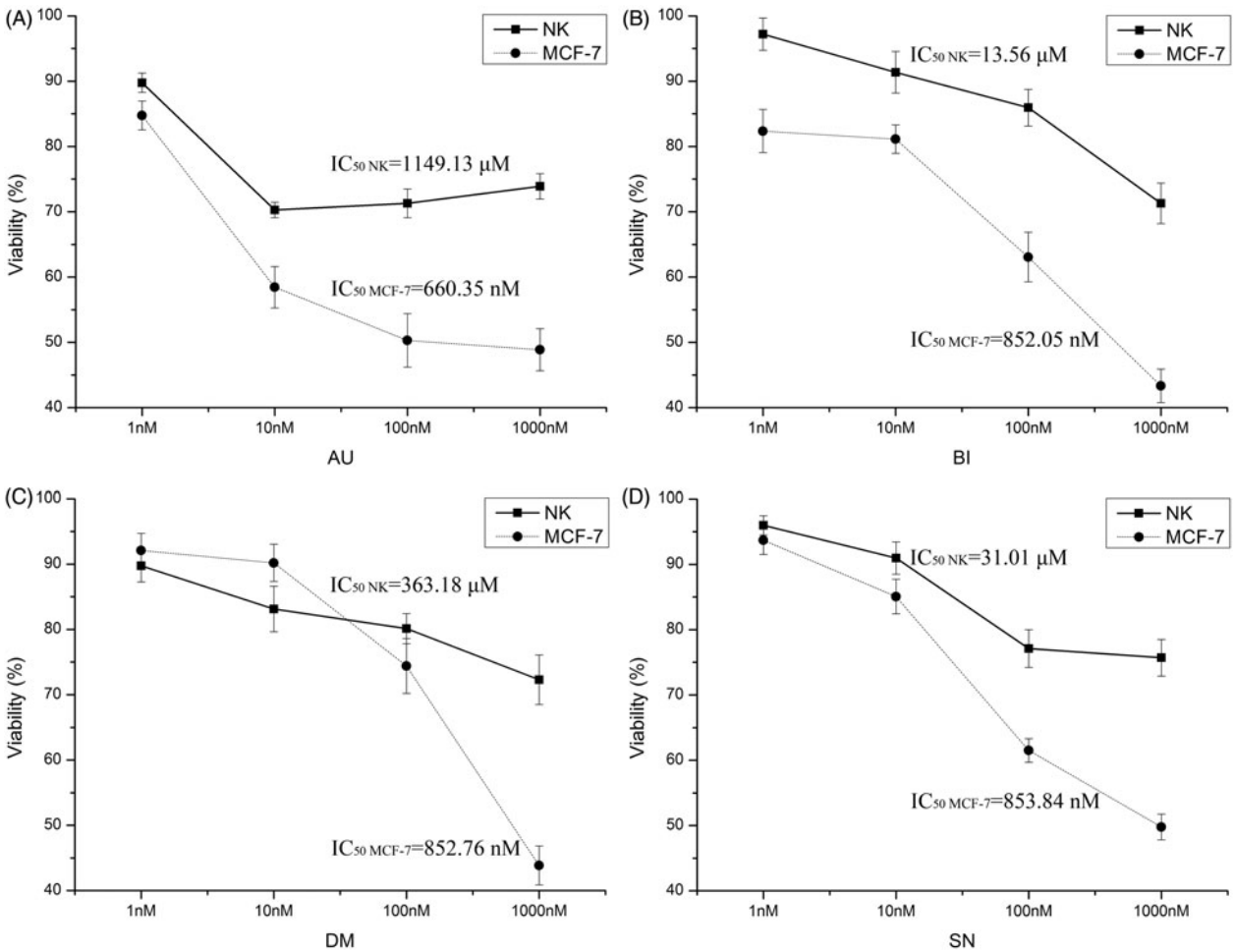


Figure 2. NK cell viability after Hsp90 inhibitors treatment in different concentration. CCK-8 reagent was added to each group of NK cells, after co-culture for 2 h, the OD values were recorded. The data represent the mean ± SD from four independent experiments. One-way ANOVA and LSD test, $*p<0.05$ compared with control ($n=4$). IC_{50} were calculated by SPSS 16.0 software.

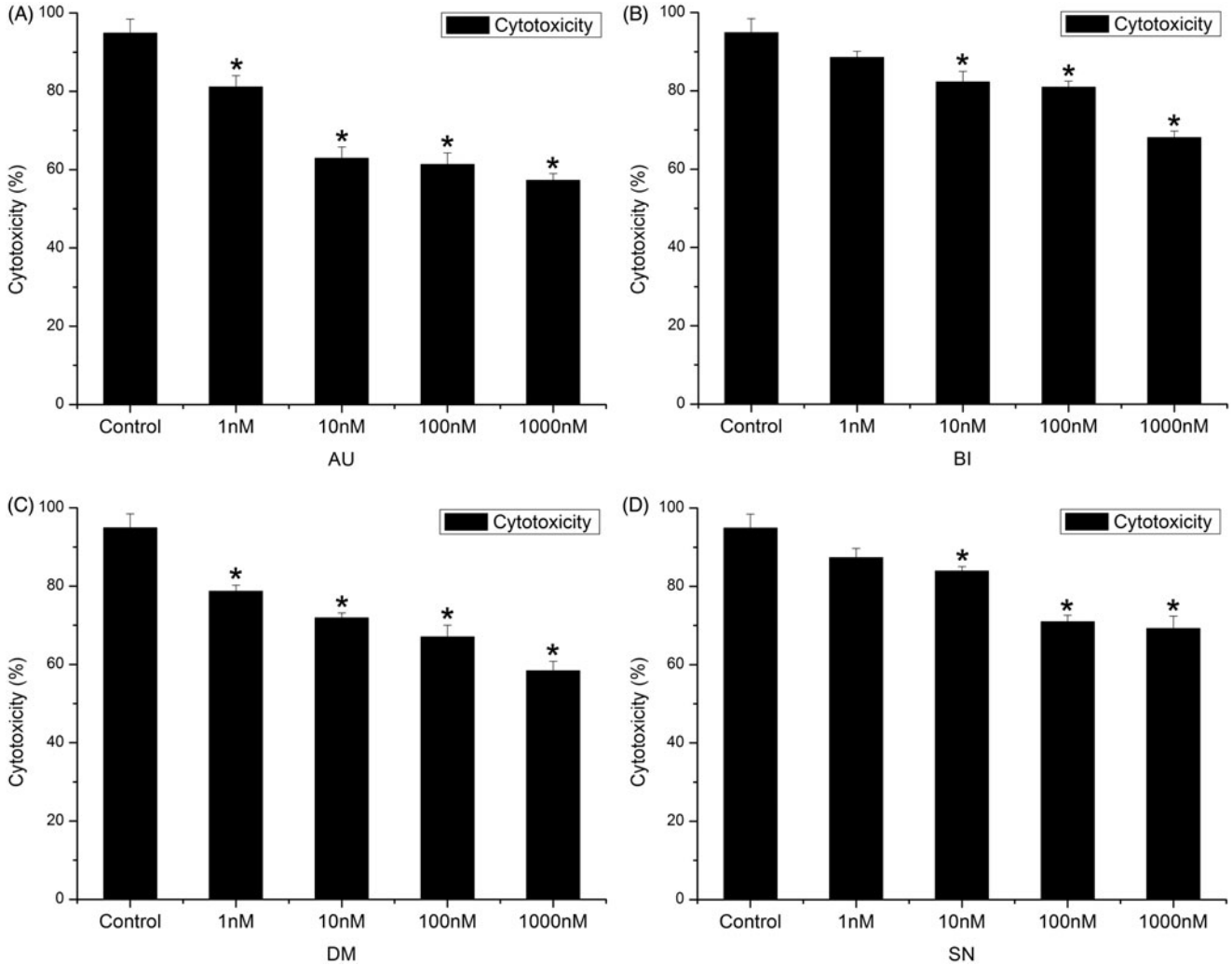


Figure 3. NK cell cytotoxicity after Hsp90 inhibitors treatment in different concentration. Each group of NK cells was mixed with K562 cells at an E:T ratio of 5:1. After co-culture for 4 h, CCK-8 reagent was used to detect remaining viable cells. NK cell cytotoxicity was determined by calculating the rate of NK cells kill K562 cells. The killing rates (%) were calculated as follows: $[1-(OD_e+t-OD_e)/OD_t]\times 100\%$. The data represent the mean ± SD from four independent experiments. One-way ANOVA and LSD test, $*p<0.05$ compared with control ($n=4$).

63.06 ± 3.8%, and 43.33 ± 2.57% in MCF-7 cells from 1 nM to 1 μM (Figure 2B). The effect of SN on the viability of NK cells and MCF-7 cells was similar to that of BI. The viability of both cells was decreased with increased SN concentration. The viability values were 95.98 ± 1.48%, 90.96 ± 2.48%, 77.11 ± 2.9%, and 75.7 ± 2.8% in NK cells and 93.7 ± 2.19%, 85.08 ± 2.65%, 61.51 ± 1.81%, and 49.78 ± 1.98% in MCF-7 cells from 1 nM to 1 μM (Figure 2D). For DM, when the concentration dropped under 100 nM, it had a greater inhibiting effect on NK cells than on MCF-7 cells. The viability of NK cells was 89.76 ± 2.48%, 83.13 ± 3.58%, and 80.12 ± 2.3% from 1 nM to 100 nM, and these data in MCF-7 cells were 92.1 ± 2.62%, 90.2 ± 2.87%, and 74.41 ± 4.2%. When the concentration of DM was increased to 1 μM, the MCF-7 cell viability reduced sharply, falling to 43.85 ± 2.97%, but in NK cells the viability was only reduced marginally, to 72.29 ± 3.8% (Figure 2C). The IC_{50} of these inhibitors (AU, BI, DM, and SN) on NK cells were 1149 μM, 13.56 μM, 363.1 μM, and 31.01 μM respectively, and which on MCF-7 were 660.34 nM, 852.05 nM, 852.75 nM, and 853.84 nM.

Hsp90 inhibitors decrease the cytotoxicity of NK cells

Figure 3 shows the NK cells cytotoxicity after treatment with the four inhibitors. Among the inhibitors, the inhibiting effects of AU and DM on NK cells cytotoxicity were stronger than those of BI and SN. When the concentrations of AU and DM increased over 1 nM, the cytotoxicity of the NK cells decreased significantly compared with the control. When the AU concentration increased over 10 nM, the NK cells cytotoxicity decreased slowly. However, when the DM concentration increased over 10 nM, the NK cells cytotoxicity decreased markedly. Compared with 94.84 ± 1.23% in the control, the NK cells cytotoxicity values were 80.26 ± 2.91%, 63.43 ± 2.94%, 61.16 ± 2.92%, and 57.07 ± 1.75% in the AU-treated group and 78.82 ± 1.59%, 71.54 ± 1.31%, 66.82 ± 3.01%, and 58.64 ± 2.42% in the DM-treated group from 1 nM to 1 μM (Figure 3A–C). The inhibiting effects of BI and SN on the NK cells cytotoxicity were not so intense as those of AU and DM. When their concentrations increased over 10 nM, BI and SM had a significant inhibitory effect on NK cells cytotoxicity. The NK cells cytotoxicity values were

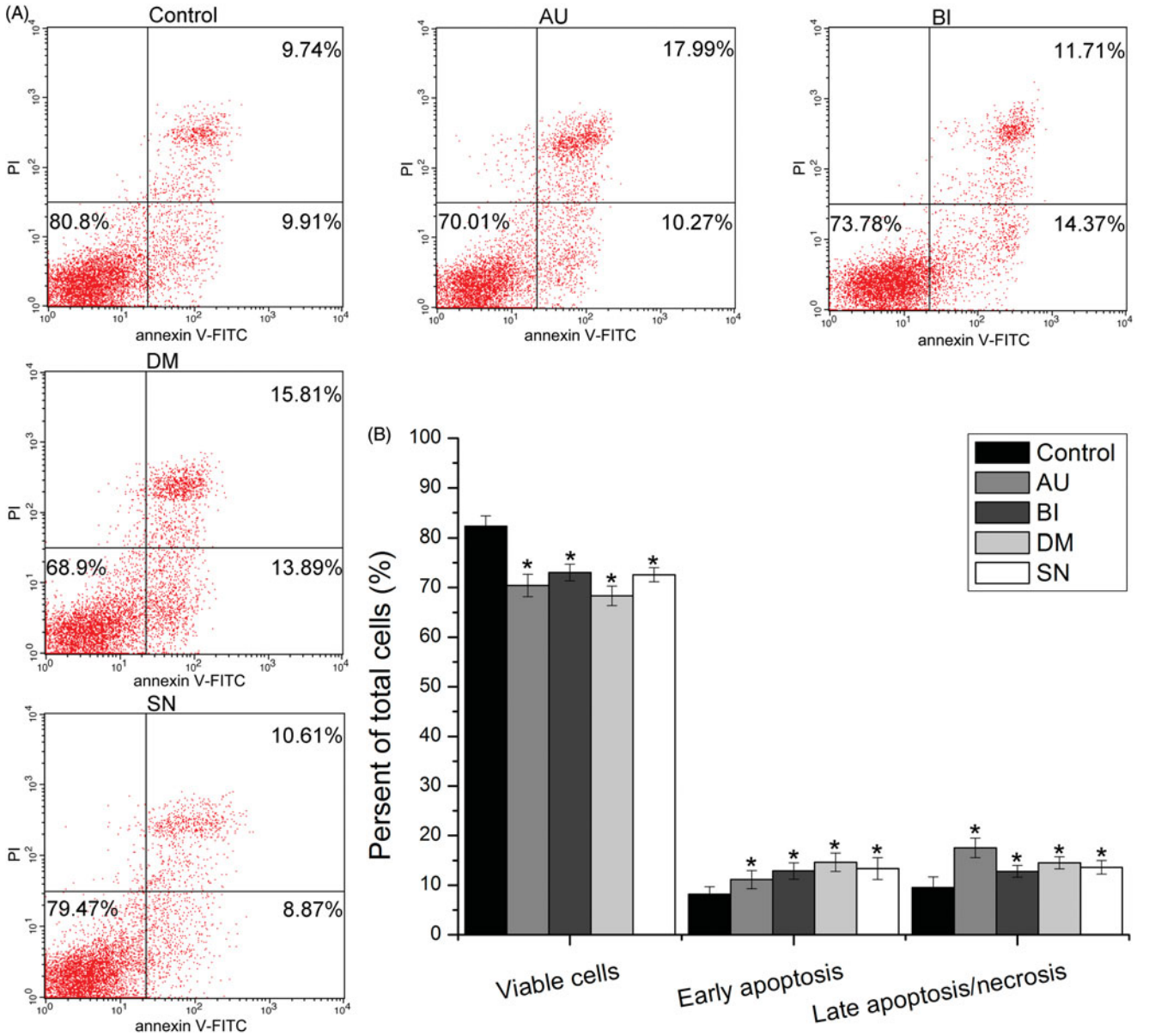


Figure 4. NK cell apoptosis and necrosis after Hsp90 inhibitors treatment (100 nM). After Hsp90 inhibitors treatment (100 nM) for 48 h, NK cells were stained with FITC-conjugated Annexin V and PI and analyzed by flow cytometry. (A) A representative flow cytometry assay of NK cells from one donor. The percentage of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), and late apoptotic and necrotic (Annexin V^+/PI^+) NK cells are shown. (B) The percentage of apoptosis in these cells is summarized in the bar graph. Each column represents the mean ± SD from four independent experiments. One-way ANOVA and LSD test, $*p < 0.05$ compared with the control ($n = 4$).

88.79 ± 1.53%, 82.77 ± 2.67%, 80.96 ± 1.54%, and 68.04 ± 1.69% in the BI-treated group and 87.26 ± 2.37%, 84.27 ± 1.18%, 71.02 ± 1.69%, and 69.07 ± 3.18% in the SN-treated group from 1 nM to 1 μM (Figure 3B–D).

Hsp90 inhibitors can promote apoptosis of NK cells

After inhibitor treatment (100 nM), NK cells apoptosis was evaluated using the AnnexinV/PI double dye method, as shown in Figure 4. The highest total apoptosis rate of the NK cells was in the DM-treated group. There was 29.14% NK cells apoptosis or necrosis (early apoptosis was 14.63 ± 1.83% and late apoptosis/necrosis was 14.51 ± 1.21%) after DM treatment compared with 17.7% (8.19 ± 1.52% early apoptosis and 9.51 ± 2.14% late apoptosis/necrosis) in the control. AU caused 28.67% NK cell death, including 11.13 ± 1.82% early apoptosis and 17.54 ± 1.96% late apoptosis/necrosis. The pro-apoptotic effects of BI and SN were not so strong as the other two inhibitors. The early apoptosis of NK cells was 12.88 ± 1.66% in the BI-treated group and 13.38 ± 2.20% in the SN-treated group, and late apoptosis/necrosis levels were 12.79 ± 1.19% in the BI-treated group and 13.61 ± 1.37% in the SN-treated group.

Hsp90 inhibitors down-regulate the functional genes expression of NK cells on the mRNA level and protein level

The expression profiles of genes involved in NK cells functions were assessed by using an RT-qPCR and flow cytometry method after inhibitor treatment (100 nM). Results shows that Hsp90 inhibitors down-regulated the cytokine

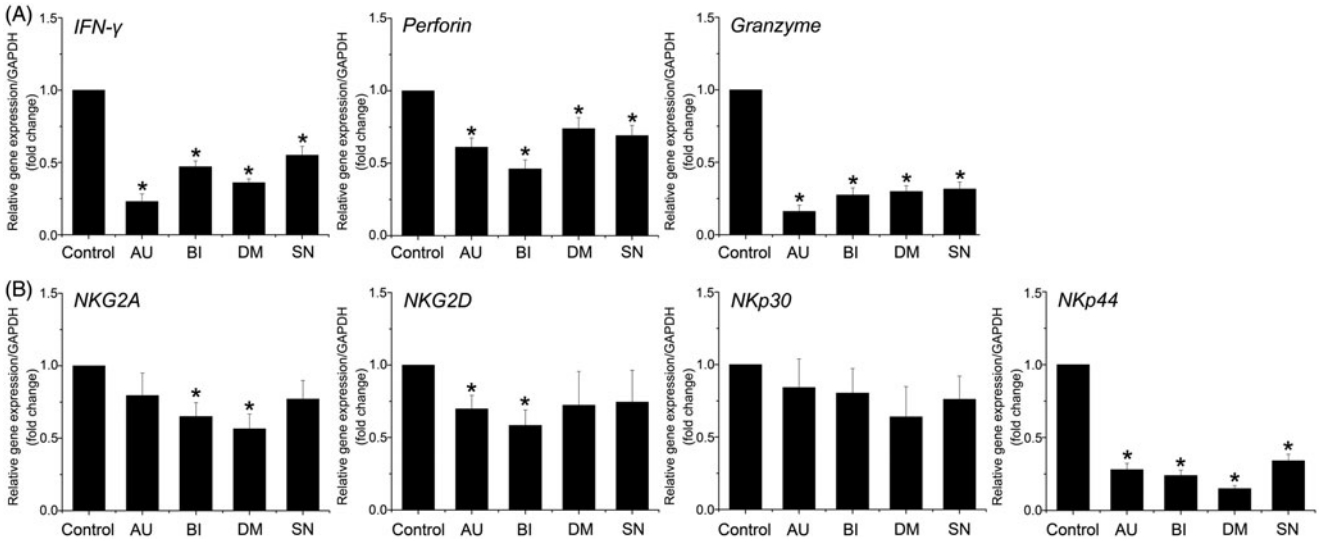
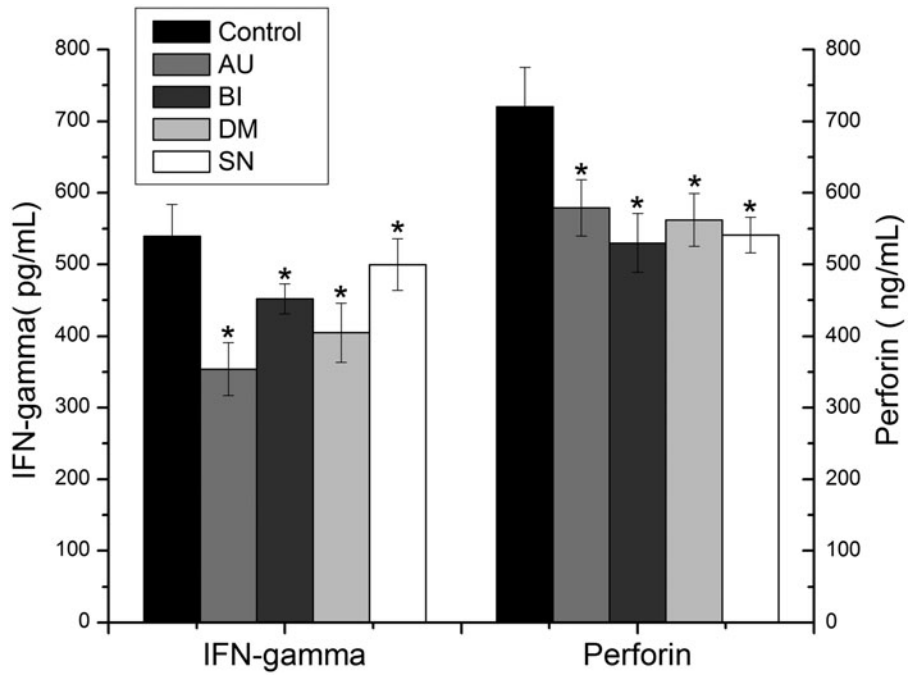


Figure 5. Gene expression profiles of IFN-γ, perforin, granzyme-B, NKG2D, NKG2A, NKp30, and NKp44 in NK cells after Hsp90 inhibitors treatment (100 nM). SYBR green-based RT q-PCR was used to examine the mRNA levels of genes that are important for NK cell functions. The data represent the mean ± SD of four independent experiments. One-way ANOVA and LSD test, $*p < 0.05$ compared with control group ($n = 4$).

Figure 6. IFN-γ and perforin secretion level of NK cells after Hsp90 inhibitors treatment (100 nM). NK cells in each inhibitor (100 nM) treated group were stimulated with K562 cells for 4 h, supernatants were collected, and concentrations of IFN-γ and perforin were detected by the corresponding ELISA kit. Every sample was tested twice. The data represent the mean ± SD for four independent experiments. One-way ANOVA and LSD test, $*p < 0.05$ compared with control group ($n = 4$).



expression in the NK cells. In the four groups, the mRNA level of IFN-γ decreased significantly compared with the control ($p < 0.05$), with changes of 0.23 ± 0.052 (AU), 0.47 ± 0.041 (BI), 0.36 ± 0.027 (DM), and 0.55 ± 0.062 (SN)-fold. Similar to IFN-γ, the genes for perforin and granzyme B decreased significantly in each group (Figure 5A). The levels of cytokines in the NK cell supernatants were also evaluated by ELISA, as was shown in Figure 6. The IFN-γ level was significantly decreased following treatment with all of the inhibitors, with values of 351 ± 37 pg/mL (AU), 449 ± 21 pg/mL (BI), 402 ± 41 pg/mL (DM), and 497 ± 36 pg/mL (SN) compared with 539 ± 45 pg/mL in the control. The concentration of perforin also changed significantly; the values were 578 ± 39 pg/mL (AU), 529 ± 41 pg/mL (BI), 561 ± 37 pg/mL (DM), and 540 ± 25 pg/mL (SN) compared with 720 ± 55 pg/mL in the control. Granzyme B was secreted into the target cells directly and was hardly detected in the supernatants.

For the NK cells functional receptors, the gene expressions were also down-regulated significantly after inhibitor treatment. NKp44 was the most down-regulated receptor in the NK cells at the gene level, with 0.28 ± 0.043 (AU), 0.24 ± 0.037 (BI), 0.15 ± 0.021 (DM), and 0.34 ± 0.046 (SN)-fold decreases compared with the control. The other

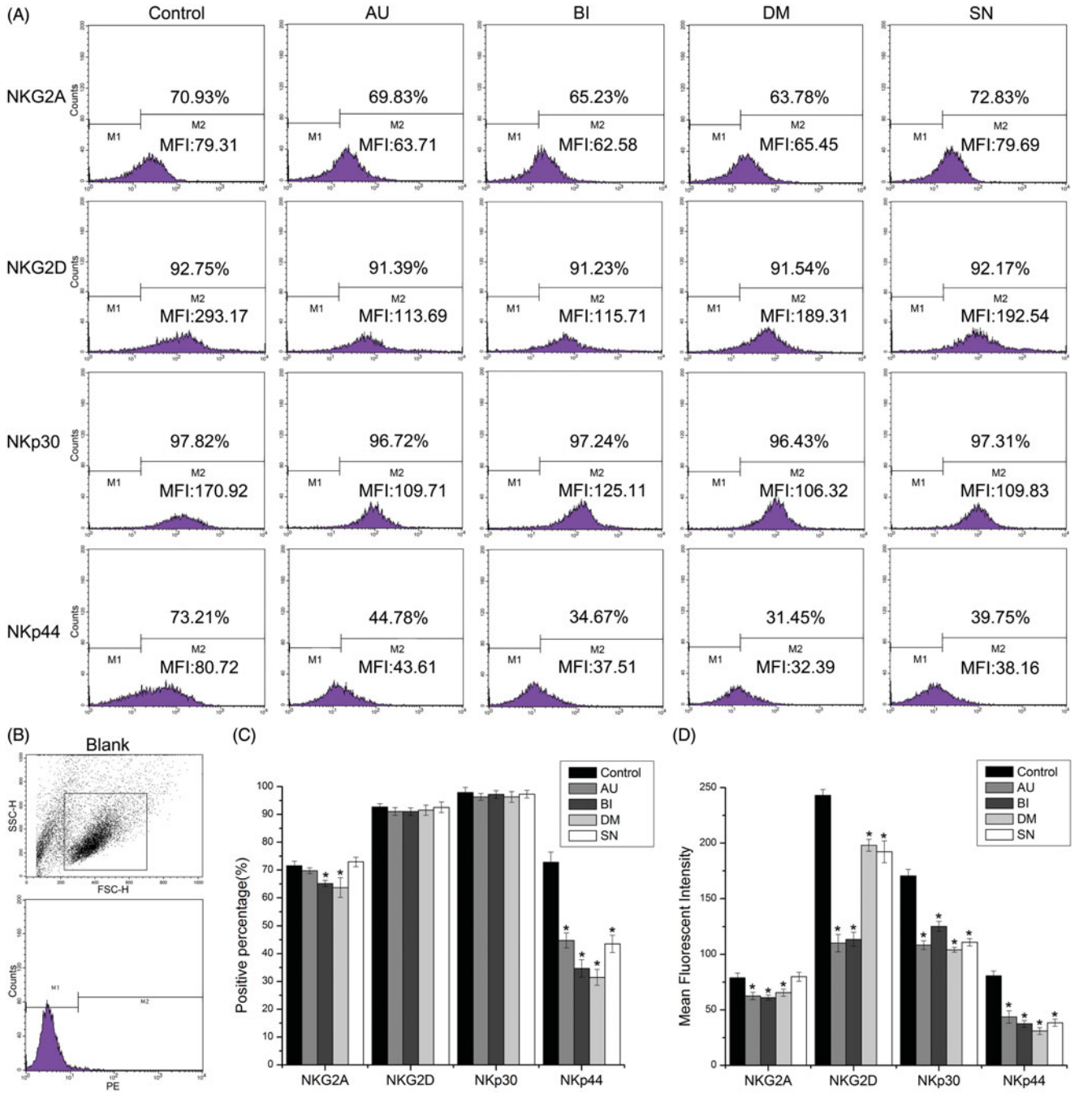


Figure 7. Protein expression profiles for the NK cell receptors NKG2A, NKG2D, NKp30, and NKp44 after Hsp90 inhibitors treatment (100 nM). (A) and (B) A representative flow cytometry assay of NK cells receptors from one donor. (C) The positive percentage of NK cells receptors expression is summarized in the bar graph. (D) The mean fluorescent intensity (MFI) of NK cells receptors expression is summarized in the bar graph. The data represent the mean ± SD from four independent experiments. One-way ANOVA and LSD test, $*p < 0.05$ compared with control ($n = 4$).

three receptors were also down-regulated to different degrees after inhibitor treatment, with decreases of approximately 50–80% compared with the control ($p < 0.05$) (Figure 5B). Flow cytometry results (Figure 7) indicated that in the BI- and DM-treated groups, the percentage of NKG2A-positive cells was reduced significantly, with $65.23 \pm 1.08\%$ and $63.72 \pm 3.55\%$ positive cells, respectively, compared with $71.55 \pm 1.66\%$ in the control. The NKp44-positive cells were significantly reduced in each inhibitor-treated group compared with the control ($72.82 \pm 3.7\%$), with values of $44.73 \pm 2.7\%$ (AU), $34.67 \pm 3.13\%$ (BI), $31.45 \pm 2.85\%$ (DM), and $43.47 \pm 3.11\%$ (SN). The percentage of NKG2D- and NKp30-positive cells did not change significantly after inhibitor treatment. However, the mean fluorescent intensity (MFI) of these receptors was reduced after inhibitor treatment. For NKG2D, the MFI was 243.06 ± 5.37 in the control, but it was reduced to 110.11 ± 7.87 in the AU-treated group, 113.53 ± 6.22 in the BI-treated group, 198.17 ± 5.32 in the DM-treated group, and 192.27 ± 9.88 in the SN-treated group. The MFI values for NKp30 were 108.31 ± 3.91 (AU), 125.11 ± 4.49 (BI), 104.07 ± 2.19 (DM), and 110.74 ± 3.23 (SN), compared with 170.54 ± 6.01 in the control.

Discussion

Hsp90 is a ubiquitous and highly conserved molecular chaperone in prokaryotic and eukaryotic organisms, which helps to transport proteins, prevent aggregation. It also ensure that stress-accumulated misfolded proteins fold correctly. It has been reported that Hsp90, compared with that in normal cells, extracted from tumor cells has greater ATPase activity and 20–200 times higher ATP-binding affinity[8,27,28]. Currently, different types of cancer are treated with approximately 18 natural or synthetic Hsp90 inhibitors in *in vitro* experiments or clinical trials worldwide[3]. AU, BI, DM, and SN are four of the inhibitors that are most used among them.

AU and SN are highly potent nongeldanamycin-based Hsp90 inhibitors that have been reported to inhibit the proliferation of a broad panel of tumor cell lines at low nanomolar concentrations[29–32]. BI is a fully synthetic small-molecule Hsp90 inhibitor that has potent inhibitory effects on the growth of cell lines from a variety of tumor types at a concentration range of 3–1000 nM[11]. 17-DMAG is a synthetic derivative of geldanamycin, which has prominent anti-proliferation or apoptosis-promoting effects on many types of tumor cells in the concentration from 50 nM to over 1 μM[13].

A series of studies show that Hsp-90 inhibitors have effects on the phenotype and function of many immunocytes[3,4,16,17]. In this study, it assessed the effects of four Hsp90 inhibitors on primary activated NK cells. It demonstrate that MCF-7 cells have high sensitivity to the four Hsp90 inhibitors[13,33–36], therefore, MCF-7 cells are used for comparing the sensitivity distinction between NK cells and carcinoma cell on Hsp90 inhibitors.

All four inhibitors reduce the viability of NK cells in certain concentrations. AU has the most inhibitory effect on NK cell viability at 1 nM, but the decreased NK cell viability slows down when AU concentration increase over 10 nM, and its $IC_{50\text{-}NK}$ was 1149 μM. The most inhibitory concentrations of the other three inhibitors are 1 μM. Among them, BI has the highest IC_{50} on NK cells, which is 13.56 μM, and the $IC_{50\text{-}NK}$ of DM and SN are 363.1 μM (DM), and 31.01 μM (SN) respectively.

The IC_{50} of inhibitors on MCF-7 cells are about 600–850 nM, which are significant lower than which on NK cells. The difference of IC_{50} may induce differences in the proliferation rates of the cells and differences of the Hsp90 affinity for ATP between the carcinoma cells and the normal cells. However, at concentrations below 100 nM, DM has more inhibited efficiency on NK cells than on MCF-7 cells. This may be explained by DM, which not only affect the function of NK cells as an Hsp90 inhibitor but also has additional toxicity to them.

In addition to viability, the NK cell cytotoxicity also has an significant decrease after inhibitors treatment. Overall, AU has the highest inhibit effect on NK cells cytotoxicity in tested concentration, although BI shows the highest $IC_{50\text{-}NK}$. Increased apoptosis, decreased viability and cytokine expression and alterations in the phenotype are all responsible for the compromised cytotoxicity of the NK cells.

IFN-γ is an important cytokine secreted mainly by NK cells. It can induce target cell apoptosis and trigger subsequent immune response. Inhibitors significantly reduced the expression of IFN-γ at the gene and protein levels. Two other cytokine (perforin and granzyme B) levels were also affected by the inhibitors. Apart from cytokines, NK cell receptors play a very important role in its functions. Several studies have indicated that the changes in receptor expression on T cells and NK cells are caused by some Hsp90 inhibitors. For instance, Jooeun Bae suggests that geldanamycin down-regulates the cell surface expression of activating receptors (CD2, CD11a, CD94, NKp30, NKp44, NKp46) on NK cells[3]. In this study, these four inhibitors down-regulated the functional receptor expression on NK cells significantly. There are several possible explanations for the down-regulated expression of these receptors and cytokines. On the one hand, Hsp90 may be directly related to the correct folding of the cytokines and receptors, and depressed Hsp90 activity may disturb the formation of these proteins. On the other hand, it reports that Hsp90 inhibitors compromises T-cell proliferation and the production of IL-2 and the IL-2R[15]. IL-2 is most important cytokine that modulates NK cell activity and function. IL-2 can increase the IFN-γ, perforin and granzyme-B expression[37,38] and up-regulate the expression of NKG2D and NKp44 on NK cells[39]. IL-15 can synergistically enhance the effect of IL-2 on NK cells, sustaining the NK cells' survival and decreasing their apoptosis[40]. Inhibitors may compromise the NK cells' IL-2R/IL-15R expression, and therefore, the negative effect of the inhibitors on NK cells may also disturb the NK cells' response to interleukins.

In summary, this study has assessed the effect of four Hsp90 inhibitors on primary human NK cells. The results reveal that inhibitors can affect NK cells' activity and functions in a concentration-dependent manner. The inhibitors significantly reduced NK cell cytotoxicity by decreasing the cytokine secretion and receptor expression, which may be caused by a direct effect of the inhibitors on the protein folding or interference of the NK cell response to interleukin stimulation. Considering the differential inhibitory effects of the inhibitors on NK cells and MCF-7 cells, more attention should be given to the appropriate dose when Hsp90 inhibitors are used in future clinical trials. Moreover, it is crucial to notice that the Hsp90 inhibitors may have side effect on the cancer patients who suffer from the NK cell immunotherapy or chimeric antigen receptor NK cell immunotherapy. On the other hand, this study suggests a possible role for Hsp90 in NK cells and represents a potential immunosuppressant strategy.

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Declaration of interest

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Supplementary material available online

Supplementary Data S1–S2.