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# Direct potentiation of NK cell cytotoxicity by 8-azaguanine with potential antineoplastic activity

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ABSTRACT

This study identified 8-azaguanine (8-AG) as a novel immunomodulatory drug (IMiD) through a high-throughput screen of the Preswick Chemical Library in a model of human NK cell cytotoxicity against blood cancer cells. 8-AG, originally developed as an antineoplastic agent, significantly increased the cytotoxicity of NK cells and was superior in this activity to previously known IMiDs, such as fluoxetine and amphotericin B, identified from the same library. IFN-γ expression was also slightly increased by 8-AG. Mechanistically, 8-AG increased conjugate formation between NK and target cells and subsequent cytolytic granule polarization, but not calcium mobilization, regulation of activating receptors, or expression of perforin or granzyme B. Thus, the antineoplastic activity of 8-AG should be re-evaluated in light of this novel potentiating effect on NK cells.

## 1. Introduction

8-Azaguanine (8-AG) is a triazolo analog of guanine, which is a known inhibitor of purine nucleotide biosynthesis. It interferes with the modification of tRNA by competing with guanine for incorporation into tRNA, which is catalyzed by tRNA-guanine ribotransferase. It also inhibits the formation of 43S and 80S initiation complexes, thereby interfering with initiation of translation and inhibiting protein synthesis. It inhibits tumor growth and stimulates cell differentiation. The anticancer effect of 8-AG was much explored *in vivo* and *in vitro* in the 1950s [1,2]; however, disappointing clinical results in leukemia patients and reports of chemoresistance [3–6] have diminished interest in its development as an anticancer agent.

In the context of treatment-resistant tumors, where limited choices of chemotherapy are available, recent application of high-throughput screening (HTS) with chemical libraries has shown possible repositioning/repurposing opportunities for 8-AG. 8-AG was identified as a lead compound for treatment of high-ploidy breast cancer [7]. It inhibits the accumulation of unspliced and singly spliced HIV-1 RNAs, Gag and Env expression, and Rev. localization, and alters splice site usage [8]. Among the four lead compounds with potential activity against *Mycobacterium tuberculosis*, 8-AG showed superior growth inhibition of wild-type, Δ*mshA*, Δ*egtA*, and Δ*egtAc* strains [9]. It was also one of the lead compounds stabilizing mutated von Hippel Lindau protein [10]. Furthermore, certain drugs, such as thalidomide, lenalidomide, and pomalidomide, were re-classified as immunomodulatory drugs (IMiDs) upon repurposing efforts [11]. Natural killer (NK) cells play an important role in anticancer and antiviral immunity. Thalidomide and its derivatives stimulate NK cells, thereby increasing cytotoxicity against cancer cells through cytokines produced by stimulated T cells and dendritic cells (DCs) [12,13]. They are a good example of drug repositioning/repurposing. Thalidomide had initially been developed as a sedative, but is currently administered as a therapeutic for multiple myeloma and myelodysplastic syndrome [14,15].

In our previous study, amphotericin B (AMP-B), an anti-fungal agent, was found in a Preswick Chemical Library HTS to increase the cytotoxicity of human NK cells [16]. Herein, we report that 8-AG, identified from the same library as AMP-B, increases the cytotoxicity of NK cells against leukemic cell lines to a greater degree than AMP-B, suggesting 8-AG as an efficient but previously unappreciated IMiD.

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## 2. Materials and methods

### 2.1. Cells

Human blood samples from healthy donors were drawn for research purposes using a protocol approved by the Asan Medical Center Institutional Review Board with informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (MP Biomedicals). The human NK cell line NKL was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and 200 U/mL recombinant IL-2 (Roche). K562, a human chronic myelogenous leukemic cell line (ATCC CCL-243), and 721.221, a human lymphoblastoid cell line (hereafter referred to as 221), were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 2 mM L-glutamine. PBMCs and NK cells were activated with 200 U/mL IL-2 for 24 h. The K562-mb15-41BBL cell line (a gift from D. Campana) for NK cell expansion was cultured in RPMI 1640 supplemented with 10% FBS. All chemicals were from Sigma unless indicated otherwise.

### 2.2. Antibodies and reagents

The following fluorochrome-conjugated antibodies were used to determine NK cell function by flow cytometry: anti-CD3-PerCP (SK7), anti-CD56-PE (NCAM16.2), anti-CD107a-FITC (H4A3), and anti-IFN-γ-FITC (25,723.11) (BD Biosciences). The following antibodies were used for phenotypic analysis of NK cells: anti-CD3-PerCP (SK7), anti-CD56-FITC (NCAM16.2), anti-2B4-PE (C1.7), anti-NKp46 (9E2), anti-DNAM-1 (DX11), and anti-CD16 (3G8) (BD Biosciences), and anti-NKG2D-PE (149810) and anti-NKG2C-PE (134591) (R&D Systems). For confocal microscopy analysis, CFSE, CellTracker orange CMTMR, and Alexa Fluor 488-phalloidin were obtained from Invitrogen, and Alexa Fluor 647-anti-perforin (dG9) was obtained from Biolegend. All chemicals were from Sigma unless indicated otherwise.

### 2.3. NK cell expansion

Primary human NK cells were expanded as previously described [17] with slight modifications. PBMCs ($1.5 \times 10^6$) were incubated in a 24-well tissue culture plate with 100 Gy-irradiated K562-mb15-41BBL cells ($1 \times 10^6$) in Stem Cell Growth Medium (SCGM; CellGenix) supplemented with 10% FBS and 10 U/mL IL-2. The medium was exchanged every 2 days with fresh medium containing IL-2. After 1 week of incubation, residual T cells were depleted with a CD3 positive selection kit (STEMCELL Technologies). Purified NK cells were incubated in SCGM supplemented with 10% FBS, 100 U/mL IL-2, and 5 ng/mL IL-15 for 2 extra weeks with medium exchange every 2 days. The expanded cell populations were 96–99% CD3-CD56+ as determined by flow cytometry.

### 2.4. Compound screening

Compounds that increase NK cell cytotoxicity were from the Prestwick Chemical Library (Prestwick-1200™), which comprises 1200 marketed drugs. The screening assay was the Europium-based cytotoxicity assay [18]. An automated liquid handler (Perkin Elmer model AJM8M01) was used to dispense 20 μL of a single active compound (50 μM) into the wells of columns 2–11 of 96-well V-bottom plates (total, 15 assay plates). Next, 80 μL of NKL cells ($2.5 \times 10^4$) in IMDM medium was dispensed into wells containing 20 μL of 50 μM compound solutions. The cells were then incubated with compounds for 1 h at 37 °C. Then, 100 μL of BATDA (Perkin Elmer)-labeled 221 cells ($5 \times 10^3$) in IMDM medium containing sulfinpyrazone was added, mixed briefly, centrifuged at 30 ×*g* for 3 min, and incubated for 2 h at 37 °C to achieve an effector:target (E:T) ratio of 5:1 and final assay concentrations of 5 μM per compound. After the incubation, the cells were pelleted by centrifugation, and the supernatant (20 μL) was assayed for Europium release to determine the effect of each compound on NK cell cytotoxicity. The percentage of specific cytotoxicity was calculated as [experimental release (counts) − spontaneous release (counts)]/[maximum release (counts) − spontaneous release (counts)] × 100. Spontaneous release was determined by incubating the targets in the absence of effector cells, and maximum release was determined by incubating the targets with 0.5% Triton-X. Wells in columns 1 and 12 were used to determine spontaneous release, maximum release, and experimental release for vehicle only (no compound). Raw values were transferred to Excel software to evaluate relative NK cell cytotoxicity. The quality of each assay plate was assessed by calculating the Z′-factor [19]. The Z′-factor for the total screen was 0.755, indicating that the screening was performed with high resolution. To identify compounds capable of enhancing NK cell cytotoxicity, a standard score (*Z*-score) for each tested compound was calculated using the following equation: σ = (raw value of well - mean of total tested wells in a plate)/(standard deviation of total tested wells).

### 2.5. Cytotoxicity assay

For the Europium-based cytotoxicity assay, K562 or 221 cells were loaded with 40 μM BATDA (Perkin Elmer) for 30 min at 37 °C. Cells were then washed in medium with 1 mM sulfinpyrazone (Sigma), resuspended at $1 \times 10^6$ cells/mL in the medium, and incubated for 30 min at room temperature (RT). Cells were washed and incubated with effector cells in the presence of sulfinpyrazone for 1 h (primary expanded NK cells) or 2 h (NKL cells) at 37 °C. Plates were mixed briefly and centrifuged at 30 ×*g* for 3 min. The supernatant (20 μL) was incubated with 200 μL of 20% Europium solution (Perkin Elmer) in 0.3 M acetic acid for 5 min and detected with a VICTOR X4 multi-label plate reader (Perkin Elmer).

### 2.6. Assay of NK cell degranulation

NK cell degranulation was determined by the cell surface expression of CD107a as previously described [20,21]. Briefly, IL-2-activated PBMCs ($2 \times 10^5$ cells) were mixed with an equal number of K562 cells and incubated for 2 h at 37 °C. The cell pellets were resuspended in FACS buffer [phosphate-buffered saline (PBS) with 2% FBS] and stained with anti-CD3-PerCP, anti-CD56-PE, and anti-CD107a-FITC antibodies for 30 min in the dark at 4 °C. To determine the degranulation of primary expanded NK cells, NK cells were incubated with K562 cells in the presence of anti-CD107a-FITC antibody. Lymphocytes were gated on FSC and SSC characteristics, and the CD107a expression on CD3-CD56+ NK cells was analyzed using a flow cytometer (FACScanto II, BD) and FlowJo software.

### 2.7. Conjugation assay

The conjugation assay was performed according to a previously described protocol [22]. NKL cells loaded with CFSE and 221 cells labeled with CellTracker orange CMTMR were separately chilled on ice and then mixed at an E:T ratio of 1:1. Cells were spun down at 30 ×*g* for 3 min and then incubated at 30 °C for the indicated times. Thereafter, cells were moved to ice, fixed in PBS containing 4% paraformaldehyde, and washed twice with FACS buffer. Conjugates (CFSE +CMTMR+) were detected by flow cytometry.

### 2.8. Granule polarization assay

Polarization of perforin to target cells was examined as described previously with some modifications [23]. To discriminate NKL cells from 221 target cells, 221 cells were first stained with CellTracker orange CMTMR according to the manufacturer's instruction. The CMTMR-labeled 221 cells were mixed with unlabeled NKL cells at a 1:1 ratio in

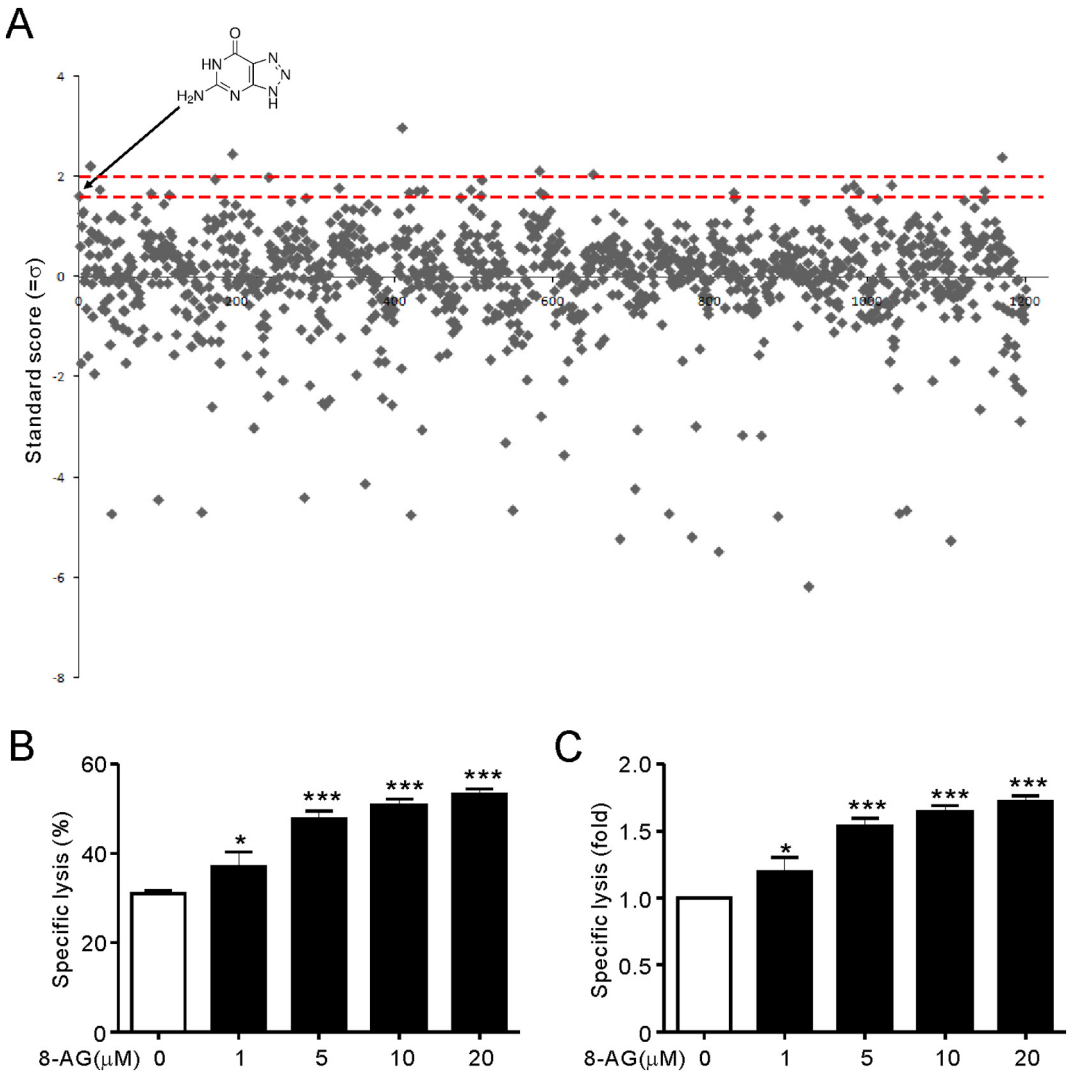


**Fig. 1.** 8-Azaguanine (8-AG) increases NK cell cytotoxicity.
NKL cells were seeded into 96-well plates and pretreated with each compound (5 μM) from the Prestwick Chemical Library at 37 °C for 1 h. NKL cells were then incubated with 221 target cells [effector (E):target (T) ratio 5:1] for 2 h. The cytotoxicity by NKL cells was assessed using the Europium assay. (**A**) Normalized standard score distribution for the 1200 small molecule screening. The $+1.6\sigma$–$+2\sigma$ cut-off value was used for hit definition and is indicated by a dotted line. The arrow indicates 8-AG. (**B**) Lysis (%) of 221 cells by NKL cells (10:1 E:T ratio) pretreated with the indicated concentrations of 8-AG. (**C**) The relative lysis of 221 cells by 8-AG-treated NKL cells is presented as fold change. Data represent the mean ± SD of three independent experiments. *$P < 0.05$ and ***$P < 0.001$.

serum-free IMDM medium and incubated for 30 min at 37 °C to allow conjugate formation. The cell suspension was subsequently transferred to coverslips coated with Cell-Tak (Corning) and incubated for an additional 15 min at 37 °C for attachment. Thereafter, cells were fixed in PBS containing 4% paraformaldehyde, washed twice with PBS, permeabilized in PBS containing 1% BSA, 0.2% Triton-X 100, and 0.1% sodium citrate, and blocked for 30 min at RT in PBS supplemented with 1% BSA and 1% goat serum. After washing, cells were stained for 90 min at RT with Alexa Fluor 488-phalloidin (Invitrogen) and Alexa Fluor 647-anti-perforin (Biolegend). After an additional wash, coverslips were mounted over glass slides using ProLong Gold antifade reagent (Molecular Probes). Data were acquired using a laser-scanning microscope LSM 710 (Carl Zeiss). Only conjugates where one NKL cell was conjugated with single 221 cells were analyzed. At least 100 different conjugates were analyzed for each condition. Conjugate stages were defined as follows: 0, conjugates lacking actin polymerization and granule polarization; 1, conjugates with polymerized actin without granule polarization; 2, conjugates in which perforin-containing granules partially polarized toward the lytic synapse; and 3, conjugates in which granules fully polarized toward the synapse.

### 2.9. Perforin and granzyme B staining of NK cells

NKL cells ($2 \times 10^5$ cells) were incubated with 10 μM 8-AG for 3 h at 37 °C, followed by incubation in BD Cytofix/Cytoperm solution. Before and after intracellular staining with anti-perforin-Alexa 647 and anti-granzyme B-Alexa 647, the cells were washed twice with BD Perm/Wash buffer and then analyzed by flow cytometry.

### 2.10. Statistical analysis

All the experiments were independently repeated at least two times. Statistical analyses of cytotoxicity and degranulation after treatment with different concentrations of 8-AG were performed by one-way ANOVA. Dunnett's tests were performed for multiple comparison post-tests. For statistical analysis of conjugate formation, data for different groups were compared by two-way ANOVA. All statistical analyses were performed using GraphPad Prism 5.0 software, and *P*-values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. 8-AG increases NK cytotoxicity

As previously described [16], the Prestwick Chemical Library was screened to discover small molecules that increase the cytotoxicity of NKL cells, a human NK cell line, against 221 cells. The activity of each molecule in terms of increasing cytotoxicity was assessed by calculating the *Z*-score (standard score). Twenty-one molecules with Z-scores of 1.6–2.0 were selected for further cherry-pick tests (Fig. 1A), given previous success at validating six molecules with Z-scores > 2.0 [16]. Digoxin, fluoxetine, azelastine, and 8-AG were further selected as they do not elicit severe side-effects and improved NK cell cytotoxicity consistently in the first and second rounds of Europium-based cytotoxicity assay (Supplemental Table 1 and Supplemental Fig. 1). Then, the selected molecules were obtained from a commercial source and assessed for their potential to increase NK cell cytotoxicity. Interestingly, 8-AG had the strongest effect on NK cell cytotoxicity, although the *Z*-score of AMP-B (2.97) was higher than that of 8-AG (1.61). Digoxin failed to show any significant increase in the cytotoxicity of NKL cells. Therefore, 8-AG was selected for further experiments. The cytotoxicity of NKL cells was significantly increased by 8-AG in a concentration-dependent manner (Fig. 1B–C). At 5 μM, 8-AG increased specific lysis by approximately 50%. In summary, 8-AG was a potential IMiD candidate among the molecules tested.

### 3.2. 8-AG increases the functionality of primary NK cells

It is a prerequisite for translational application to confirm these results using human primary cells, since an established cell line often has changes in functional properties compared with its parental cells. Thus, the results obtained with the NKL cell line were confirmed using human primary NK cells. PBMCs were activated with IL-2 for 24 h and then pretreated with 10 μM 8-AG for 1 h. The cytotoxicity of NK cells against target cells correlates with the degranulation efficiency of NK cells. As expected, degranulation, which is assessed by CD107a expression on NK cells, was increased by 8-AG in a concentration-dependent fashion in response to K562 cells (Fig. 2A–B). The increase was statistically significant at 10–20 μM 8-AG. Furthermore, pretreatment with 8-AG promoted the efficient killing of K562 cells and 221 cells by a highly pure primary NK cells after expansion with IL-2 and IL-15, a protocol previously tested in clinical trials for hematological malignancies (Fig. 2C–D). At 10 μM, 8-AG showed the best cytotoxicity against K562 cells, as specific lysis reached a plateau of approximately 70%. In addition, 8-AG increased the IFN-γ production of primary NK cells in response to K562 and 221 cells by about 21% and 16%, respectively (Fig. 3), although the increase in IFN-γ production was not statistically significant and less prominent than that in NK cell cytotoxicity. Taken together, 8-AG significantly increased the functionality of primary NK cells, mainly by increasing degranulation and cytotoxicity.

### 3.3. 8-AG increases NK-target cell conjugate formation and subsequent granule polarization

To understand the underlying mechanism of 8-AG on NK cytotoxicity, the sequential steps required for triggering NK cytotoxicity were investigated. Firstly, a cell adhesion assay was performed since cytotoxicity can be promoted by increased cell-cell interaction. Conjugate formation between fluorescence-labeled NKL cells and 221 cells was measured by flow cytometry. As seen in Fig. 4, conjugate formation between NKL and 221 cells was significantly increased by 8-AG in a time-dependent manner at least for 5 min.

Next, subsequent cytotoxic granule polarization in NK cells was assessed by confocal microscopy. Polymerized actin accumulating in the lytic synapse was detected by phalloidin, along with cytolytic granules by anti-perforin staining (Fig. 5). Accordingly, different conjugate stages were defined as follows: stage 0, conjugates lacking both actin polymerization and granule polarization; stage 1, conjugates with polymerized actin but without granule polarization; stage 2, conjugates where perforin-containing granules partially polarized toward the lytic synapse; and stage 3, conjugates where granules fully polarized toward

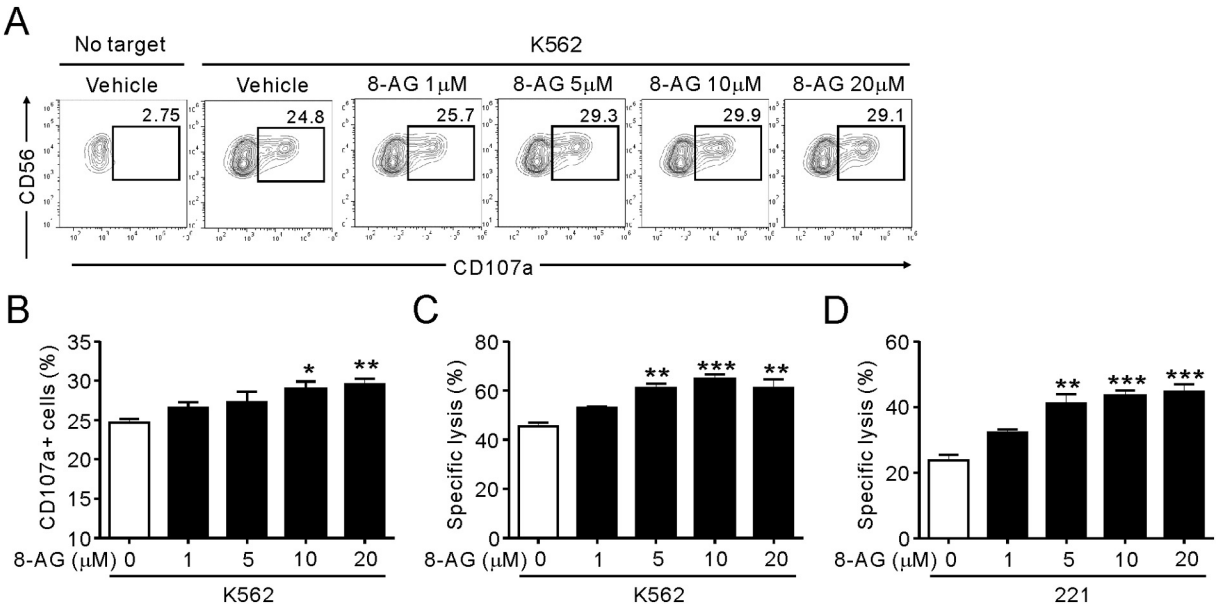


**Fig. 2.** 8-AG increases the natural cytotoxicity of primary NK cells.
(**A**, **B**) PBMCs exposed to IL-2 were pretreated for 1 h with the indicated doses of 8-AG and incubated with target cells (K562) for 2 h in the presence of 8-AG. Degranulation of NK cells was measured by cell surface expression of CD107a on CD3-CD56 + NK cells. (**A**) Representative flow cytometry profiles showing the percentages of CD107a + NK cells. (**B**) Summary graphs of statistical bar charts showing the expression of CD107a by NK cells. Mean values ± SEM of three independent experiments are shown. (**C**, **D**) Primary NK cells after expansion were pretreated for 1 h with the indicated doses of 8-AG and then incubated with K562 or 221 target cells for 1 h in the presence of 8-AG. Shown are summary graphs of statistical bar charts demonstrating lysis (%) of K562 (**C**) or 221 (**D**) target cells by primary expanded NK cells (2:1 E:T ratio). The mean values ± SD of three independent experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

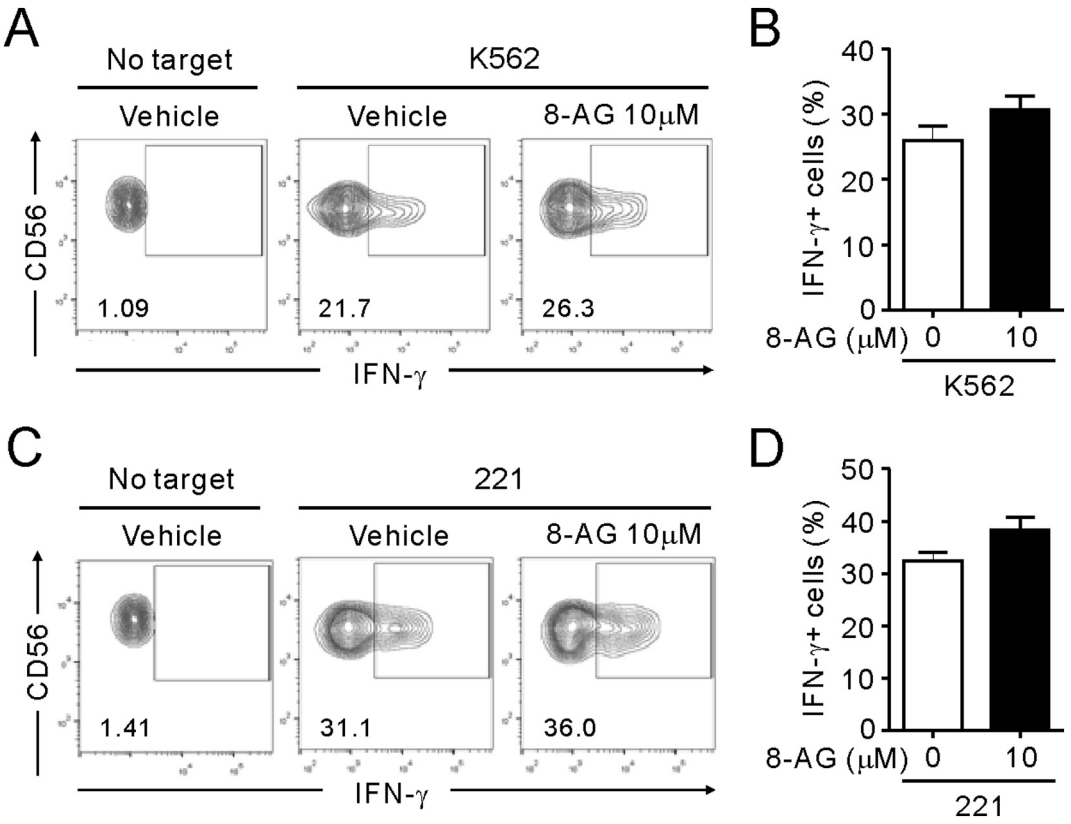


**Fig. 3.** 8-AG increases IFN-γ production by primary NK cells.
PBMCs exposed to IL-2 were pretreated with 10 μM 8-AG and then mixed with K562 or 221 cells in the presence of 8-AG for intracellular cytokine assay. After 6 h incubation at 37 °C, cells were stained with fluorochrome-conjugated anti-CD3 and anti-CD56 monoclonal antibodies for surface staining. IFN-γ production by NK cells was measured in CD3-CD56+ cells by flow cytometry after intracellular staining of IFN-γ. (**A**, **C**) Representative flow cytometry profiles showing expression of IFN-γ by NK cells against K562 (**A**) or 221 (**C**) target cells. (**B, D**) Summary graphs of statistical bar charts showing the expression of IFN-γ by NK cells against K562 (**B**) or 221 (**D**) target cells. Mean values ± SEM of three independent experiments are shown.

the lytic synapse. Actin filaments (green) are reorganized to form polymerized F-actin in stage 1, and then perforin (red)-containing granules are polarized to the synapses in stages 2 and 3 (Fig. 5A). As seen in Fig. 5B, 8-AG caused a clear increase in granule polarization in NKL cells at stages 2 and 3 relative to a decrease at stages 0 and 1. In particular, NKL cells at stage 3 were prominently increased by 8-AG, suggesting that 8-AG stimulated NK cells to facilitate conjugate formation and then granule polarization for cytotoxicity.

### *3.4. 8-AG did not affect calcium mobilization, or levels of cytolytic molecules or activating receptors*

To gain further insight into the mode of action of 8-AG on NK cells, we assessed calcium mobilization, which is crucial to NK cell function upon target cell recognition [18]. NKL cell stimulation with a combination of NKG2D and 2B4 receptors evoked a robust $Ca^{2+}$ mobilization, which was not increased by 8-AG (5–10 μM) (Supplemental Fig. 2). Moreover, the expression levels of intracellular perforin and granzyme B, critical mediators of cytotoxicity, were measured by flow cytometry. 8-AG did not affect the intracellular levels of these cytolytic molecules (Supplemental Fig. 3). 8-AG-mediated NK cell potentiation was also unrelated to the expression of NK cell activating receptors, given no significant alteration after treatment with 8-AG (Supplemental Fig. 4). Taken together, these results suggest that the increased cytotoxicity of NK cells by 8-AG is independent of $Ca^{2+}$ mobilization or of changes in cytotoxic molecule or activating receptor expression, but relies on a mechanism involving increased conjugation formation and subsequent granule polarization.

## 4. Discussion

In this study, we suggest that 8-AG could serve a dual function as an IMiD and antileukemic agent. 8-AG increased the functionality of NK cells *via* NK-target cell conjugate formation and cytolytic granule polarization. HTS and validation studies showed the superior cytotoxicity of 8-AG-treated NK cells compared with that of NK cells treated with several other selected molecules. This was intriguing since fluoxetine and AMP-B, two of the selected molecules, were previously reported as IMiDs. Fluoxetine (Prozac), a selective serotonin reuptake inhibitor, augments NK cell activity [24]. AMP-B is an anti-fungal agent that also promotes NK cell cytotoxicity by increasing cell-cell interaction and granule polarization [16]. 8-AG is an inhibitor of purine nucleotide biosynthesis, and was intensively investigated as an antileukemic agent at earlier times. It induces a decrease in cell viability in human MOLT3 T acute lymphoblastic leukemia cells, but not in CEM cells, another human T cell leukemia line [25]. 8-AG stimulates CD26 expression only in MOLT3 cells, which may be related to the apoptotic sensitivity of MOLT3 cells to 8-AG [25]. CD26 was suggested as a leukemic stem cell marker in chronic myeloid leukemia [26] and as a prognostic biomarker for B cell chronic lymphocytic leukemia [27]. Thus, CD26 expression on patient leukemic cells may be a potential marker for the selection of patients who are likely to benefit from 8-AG treatment. This warrants further investigation.

It might be beneficial to revisit 8-AG as an anticancer drug for blood cancer patients, since blood cancer cells were efficiently eliminated by NK cells in which the treatment with 8-AG may provide additional advantages by further increasing NK cell cytotoxicity. 8-AG can be preferentially administered to leukemic patients with apoptotic

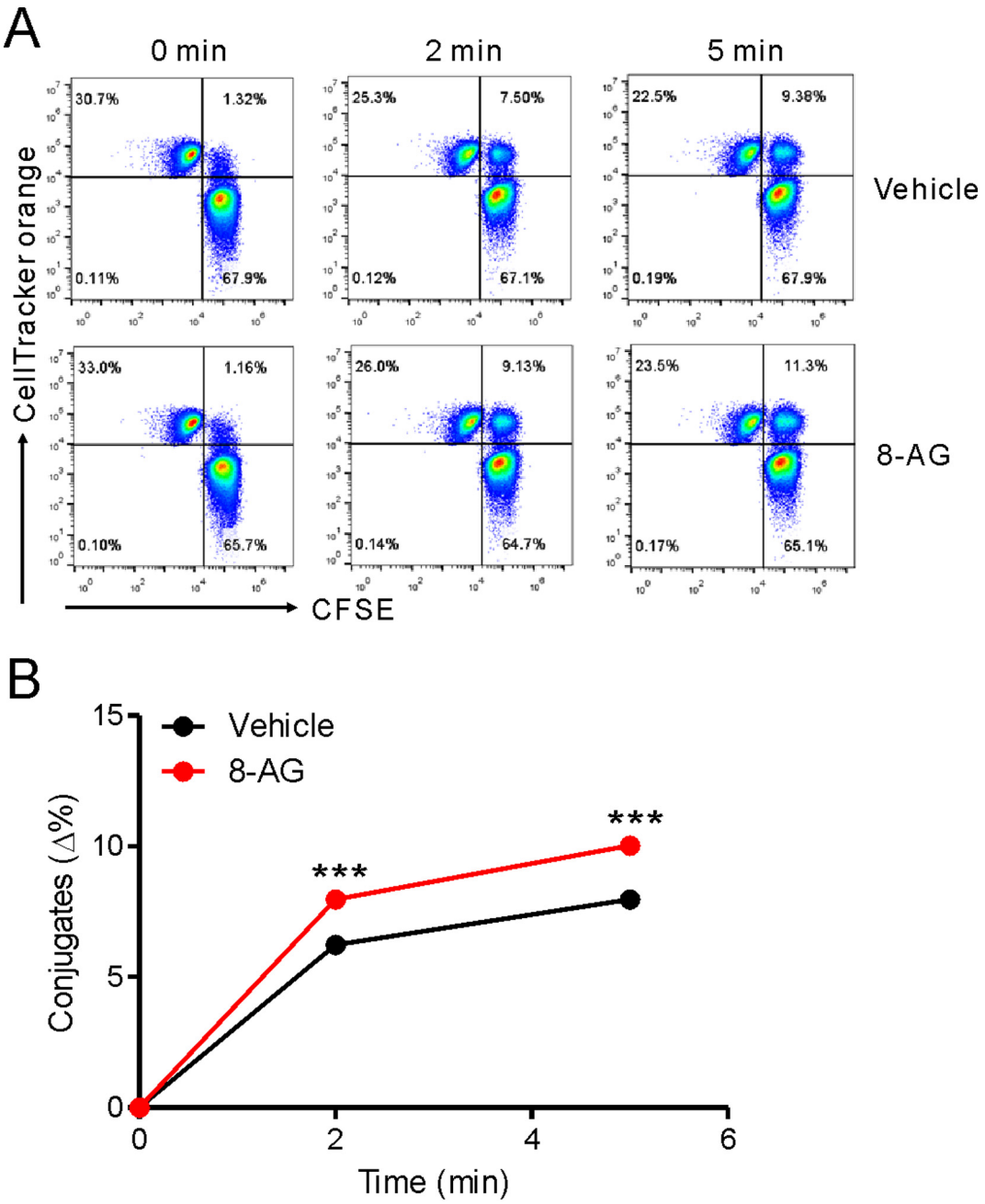


**Fig. 4.** 8-AG promotes conjugate formation between NK and target cells.
NKL cells loaded with CFSE were pretreated with 10 μM 8-AG for 30 min and then incubated with CellTracker orange CMTMR-labeled 221 target cells at an E:T ratio of 1:1 for the indicated time points. Cells were then fixed and analyzed by flow cytometry to detect conjugate formation, as shown by the double-positive population in the upper right quadrant. Shown are representative flow cytometry profiles of two independent experiments (**A**) and summary graphs of statistical line charts (**B**) demonstrating conjugate formation between NKL cells and 221 cells. ***$P < 0.001$.

sensitivity to 8-AG for full therapeutic benefit. In this case, 8-AG may have a dual mechanism by directly triggering apoptosis in leukemic cells and also increasing NK cell activation. Azacytidine (5-Aza), another nucleotide synthesis inhibitor, is a standard therapeutic in acute myeloid leukemia, but the major cause of therapeutic failure in acute myeloid leukemia is acquired resistance to the treatment [28]. 5-Aza also impairs NK cell functions [29]. Thus, testing 8-AG as an anticancer drug may benefit patients with cancer cell susceptibility to NK cell cytotoxicity and/or chemoresistance. There has been research to find novel agents that improve drug sensitivity. Flavokawain B in combination with daunorubicin induces cell death in daunorubicin-resistant acute myeloid leukemic cells [30], prompting the need to explore more combination therapeutic regimens. It would be also useful to evaluate the efficacy of combination therapies with conventional chemotherapies and 8-AG, particularly for cancer patients with NK cell reactivity.

Recent developments in immunotherapy, such as cytokine-induced killer cell therapy, *ex vivo* expanded NK cell therapy, chimeric antigen receptor (CAR)-T/NK cells, and immune checkpoint inhibitors, might give 8-AG a new chance as a part of combination therapy [31]. Besides 8-AG administration, 8-AG can be added during the *in vitro* expansion of NK cells for adoptive immune cell therapy, given its direct potentiating effect on NK cells. Lenalidomide increases the antibody-dependent cellular cytotoxicity of NK cells against rituximab-treated B cell chronic

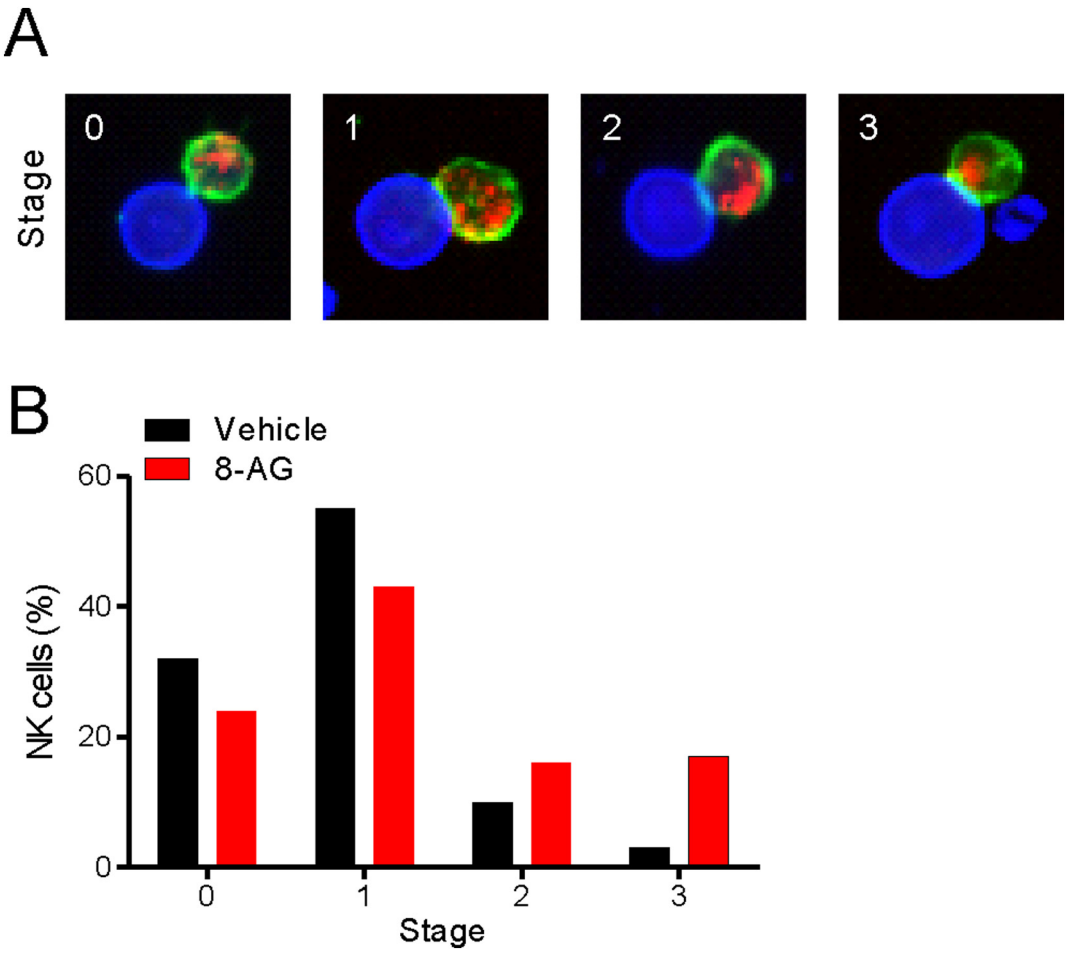


**Fig. 5.** 8-AG promotes cytolytic granule polarization toward target cells.
(**A**) Representative confocal images of conjugates between NKL cells and CellTracker orange CMTMR-labeled 221 cells (blue). Conjugates were fixed, permeabilized, and then stained with phalloidin for actin (green) and anti-perforin antibody (red). Conjugates were analyzed by confocal microscopy to determine the polarization of perforin-containing granules toward target cells. Conjugates were classified into different stages according to the progression of granule polarization toward target cells. Shown are conjugates representative of each stage. (**B**) NKL cells pretreated for 30 min with 10 μM 8-AG were incubated with CMTMR-loaded 221 cells for 30 min. Cells were then stained as described in (**A**), and the percentages of NKL cells at each stage of granule polarization were measured with at least 100 NKL-target cell conjugates. Representative data of two independent experiments are shown.

lymphocytic leukemia cells *in vitro* [32], and is administered as combination therapy for patients with B cell malignancies [33]. Lenalidomide and IPH2101, a novel anti-inhibitory KIR antibody, mediate rejection of a lenalidomide-resistant tumor in mice [34]. The mechanism of action of the combination therapies is thought to be that lenalidomide augments actin remodeling and lowers the threshold of NK cell activation through CD16, an Fc receptor [35]. In the present study, we analyzed natural cytotoxicity against different blood cancer cells (*e.g.*, K562 and 221), rather than ADCC mediated by CD16. NK cell cytotoxicity is triggered by the balanced engagement of distinct and multiple receptors, including NKG2D, 2B4, NKp46, LFA-1, and others. In this respect, the efficacy of NK cell-mediated immunotherapy might be improved by 8-AG through increased adhesion to target cells and natural cytotoxicity.

In conclusion, the effect of 8-AG on NK cells shows promising results as an IMiD, increasing NK functionality through increased conjugate formation and cytolytic granule polarization. These results support further study to re-evaluate 8-AG as an anticancer drug, particularly for patients whose cancer cells show NK cell susceptibility and/or apoptotic sensitivity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.020.

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## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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