Pan-RAF inhibitor LY3009120 is highly synergistic with low-dose cytarabine, but not azacitidine, in acute myeloid leukemia with *RAS* mutations

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Abstract. Alterations in *RAS* oncogenes have been implicated in various types of cancer, including acute myeloid leukemia (AML). Considering that currently, there are no targeted therapies for patients with *RAS*-mutated AML despite the poor outcomes, RAF may be a potential target for AML. In this study, we first analyzed the efficacy of different MAPK inhibitors in AML cell lines. We found that LY3009120, a pan-RAF inhibitor, significantly decreased cell survival in *RAS*-mutated AML cell lines. We then investigated the synergistic effects of LY3009120 with either cytarabine or azacitidine. We found that the combination of low-dose cytarabine and LY3009120 showed a synergistic effect in *NRAS*-mutated HL-60 cells and *KRAS*-mutated NB4 cells. This effect was caused by a decrease in proliferation, induction of apoptosis, and cell growth arrest through a decrease in phosphorylated MEK and ERK along with a cytotoxic response occurring specifically for the *RAS* mutation of the pan-RAF inhibitor LY3009120. In addition, we confirmed that combination treatment with low-dose cytarabine and LY3009120 led to an increase in apoptosis in primary AML cells. Our findings indicate that combination therapy with pan-RAF inhibitor LY3009120 and low-dose cytarabine may be a promising treatment strategy for *RAS*-mutated AML.

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Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous and highly aggressive hematological malignancy with an average onset age of 67 years (1). Patients with AML harbor distinct genetic and molecular abnormalities caused by various chromosomal aberrations in about 50-60% of *de novo* AML cases and 80-95% of secondary cases (2). Classically, mutated genes in AML are classified according to their characteristics, and the gene group that affects cell proliferation includes *KIT*, *FLT3*, and *RAS* (3,4). MAPK, induced Ras signaling, is a major oncogenic pathway in AML.

Alterations in *RAS* are involved in the progression of various cancers. Missense *RAS* mutations causing a gain-of-function phenotype occur in 25% of human cancers (5) and 20-25% of AMLs. *KRAS* and *NRAS* mutations are present in 5 and 11% of AMLs, respectively (6). Patients with *RAS*-mutated AML have shorter overall survival and AML-free survival, higher median age, and lower complete remission rates (7). In addition, abnormalities in Ras proteins that regulate various functions, including growth, migration, adhesion, survival, and differentiation in normal cells, affect leukemia primarily and result in secondary effects such as chemotherapy resistance and cancer recurrence (8-11). Therefore, the *RAS* status of a patient has prognostic value and is a potent cancer biomarker (12-15). However, it has been challenging to target Ras directly. While drugs targeting the downstream signaling pathway, including RAF, mitogen-activated protein kinase/ERK (extracellular signal-regulated kinase) kinase (MEK), are being developed mainly for solid tumors (12,16-19), none of them are proven successful (20-22). Developed as a pan-RAF inhibitor of RAF, downstream of Ras, LY3009120 inhibits the activity of all RAF isoforms (A-RAF, B-RAF, and c-RAF) and RAF dimers, and it is currently in the first phase of clinical trials for solid tumors (23).

Therefore, in this study, using *RAS* mutant AML cells, we investigated the synergistic effects of MAPK inhibitors in combination with low-dose cytarabine or azacytidine, currently used as first-line treatments for AML in elderly patients.

Materials and methods

Cell culture and treatment. The three AML cell lines (HL-60, NB4, and KG-1) used in the study were obtained from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). KG-1 and HL-60 cell lines were authenticated using the AmpFISTR® Identifiler® PCR Amplification Kit and the NB4 cell line using the PowerPlex®21 System Kit. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) and incubated overnight at 37˚C in 5% CO_2. Cells were seeded for 24 h and treated with cytarabine, azacytidine, LY3009120, LXH254, dabrafenib, and trametinib. For single treatment, each inhibitor was treated at indicated concentrations ranging from 0.001 to 10 μM for 72 h. After that, the combined treatment was treated with cytarabine 50 nM or/and LY3009120 6/50 nM concentration for 72 h. Each experiment was repeated five times.

Patient samples. Bone marrow mononuclear cells (MNCs) from patients with AML were isolated using the Ficoll gradient method and cryopreserved in Cell banker 2, a serum-free medium. The MNCs of the patients were thawed at 37˚C in Iscove's modified Dulbecco's media containing 10% fetal bovine serum and incubated for 24 h.

Agents and antibodies. Cytarabine (Cytosar-U®, Ara-C, Arabinosylcytosine); the pan-RAF inhibitors, LY3009120 and LXH254; the B-RAF inhibitor, dabrafenib; and the MEK inhibitor, trametinib were purchased from Selleck Chemicals, and azacitidine was procured from Sigma-Aldrich. The following antibodies were purchased from Cell Signaling Technology: phospho-c-RAF (Ser338; cat. no. 9427), c-RAF (cat. no. 9422), B-RAF (cat. no. 9434), phospho-MEK1/2 (Ser217/221; cat. no. 9121), MEK1/2 (cat. no. 9122), phospho-p44/42 mitogen-activated protein kinase (MAPK; Erk1/2; Thr202/Tyr204; cat. no. 9101), p44/42 MAPK (Erk1/2; cat. no. 4696), caspase-3 (cat. no. 9662), caspase-9 (cat. no. 9502), and poly(ADP-ribose) polymerase (PARP; cat. no. 9542). GAPDH (SC-25743) antibodies were purchased from Santa Cruz Biotechnology. The secondary antibodies, goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L), were purchased from Jackson ImmunoResearch Laboratories.

Proliferation assay. Cells were cultured in 96-well plates for 1 day and treated with each drug six times for 72 h. Cell proliferation was assessed using the Quanti-Max WST-8 Cell Viability Assay kit (Biomax). DMSO was used as a control for all experiments. The optical density of each well was measured at 450 nm using a microplate reader (Molecular Devices) (24). Each experiment was repeated five times.

Western blotting. Protein concentrations were measured using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein (15 μg) were loaded on 10 and 12% polyacrylamide gels containing SDS, transferred to PVDF membranes, and blocked for 1 h in 5% skim milk. The membranes were incubated overnight at 4˚C with the appropriate primary antibody and then with the HRP-conjugated secondary antibody for 1 h at 37˚C. GAPDH was used as a control marker in all the blots.

Cell cycle analysis. To investigate the cell cycle profiles of the treated and untreated cells after the treatment, the cells were collected, washed with DPBS, and fixed with 70% ethanol. The fixed cells were treated with 0.4 mg/ml RNase (Promega) and 5 μl of 50 μg/ml propidium iodide (Invitrogen) (25). The stained cells were analyzed using FACSCanto II (BD Biosciences) and the apoptotic cells were detected using flow cytometry. For cell cycle analysis, flow cytometry data was analyzed using 'ModFit' software. Each experiment was repeated three times.

Apoptosis assay. Apoptosis induction after single or combined treatment with cytarabine and LY3009120 was measured using the Dead Cell Apoptosis Kit with Annexin V/FITC and PI (Thermo Fisher Scientific), and DMSO was used as a control. Cells were collected 24 or 72 h after their respective treatment and stained with Annexin V/PI dye (26). Each experiment was repeated three times.

Statistical analysis. Statistical significance among the groups was determined using GraphPad Prism version 5.0 (GraphPad Software Inc.). Statistical analyses for the efficacy of the drugs were performed using the two-tailed unpaired Student's t-test. One-way ANOVA followed by Dunnett's post hoc test were used for multiple comparisons. Combination index (CI) values were calculated using the Chou-Talalay equation (27). CI values were used to validate combinatorial effects between the two drugs; CI>1.00 indicates antagonism, CI=1.00 indicates additivity, and CI<1.00 indicates synergism (28). Western blot bands were quantified using ImageJ software (1.53a), and comparisons between the two groups were analyzed using Student's t-test using a GraphPad. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. P<0.05 was considered to indicate a statistically significant difference.

Results

Pan-Raf inhibitor specifically inhibits proliferation of RAS-mutant AML cells. We evaluated the effects of cytarabine, azacytidine, and the MAPK inhibitors, LY3009120, LXH254, dabrafenib, and trametinib on the proliferation of three AML cell lines. To determine whether the inhibitors had differential effects on the AML cells with *RAS* mutations and those with wild-type (WT) *RAS*, we treated the HL-60, NB4, and KG-1 cells with varying concentrations of the inhibitors. We found a slight increase in proliferation after treatment with low concentrations of all the inhibitors except trametinib, but a dose-dependent decrease in all cell lines (Fig. 1A-F) (****P<0.0001). In addition, after treatment with 0.1 μM of the pan-RAF inhibitors, LY3009120 and LXH254, the proliferation rate in the *RAS*-mutated cells was significantly lower than that in the WT *RAS* KG-1 cells (Fig. 1C and D). In contrast, dabrafenib and trametinib treatments did not show a difference in the proliferation rate of the *RAS*-mutated and WT *RAS*-containing cells (Fig. 1E and F). The IC50 value for each inhibitor can be found in Table SI.

Treatment with a combination of low-dose cytarabine and LY3009120 decreases cell proliferation in RAS-mutated

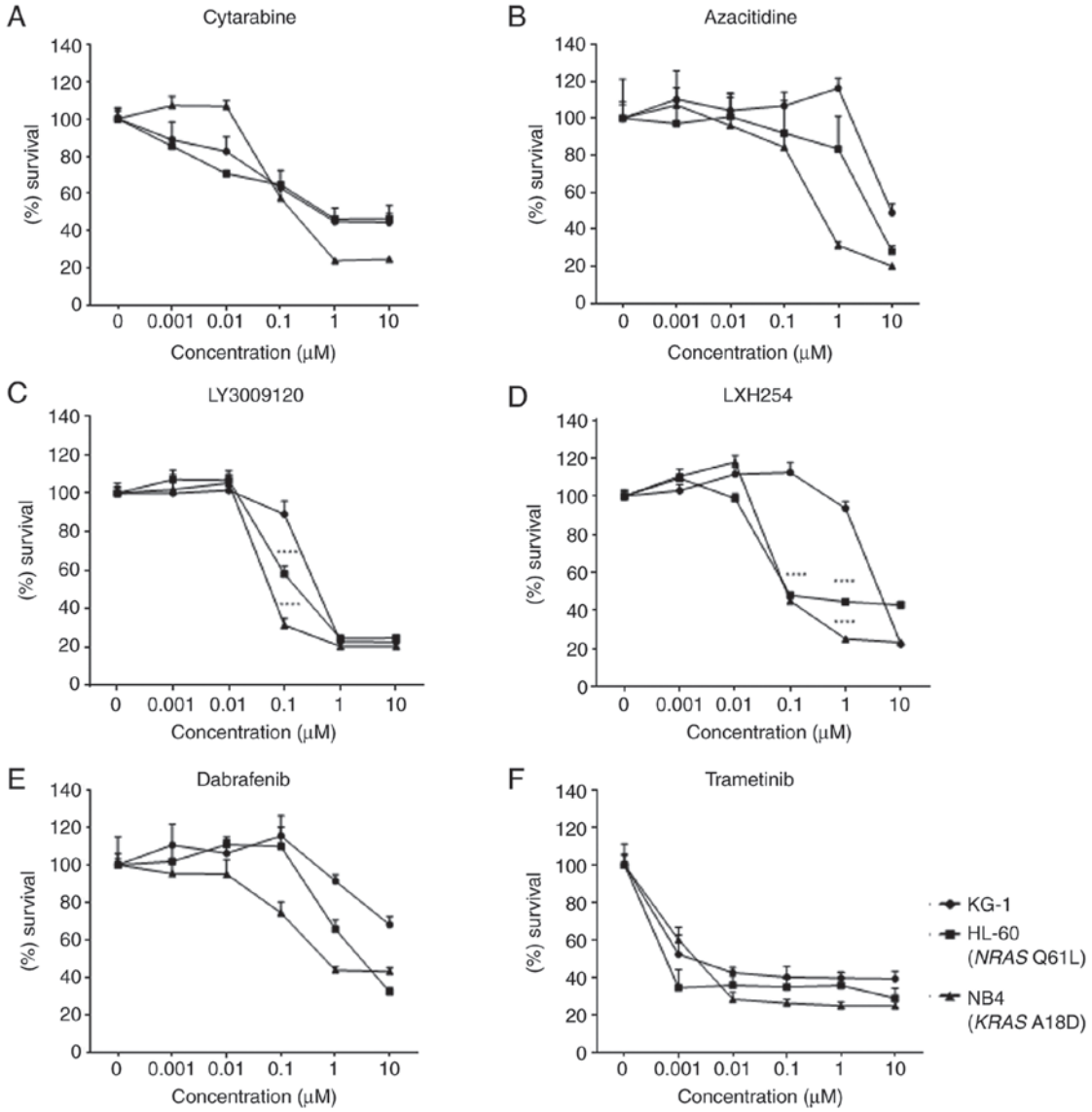

Figure 1. Effect of cytarabine and MAPK inhibitors on the proliferation of AML cells with wild-type (KG-1) or mutated *RAS* (HL-60 and NB4). To confirm the individual effect of (A) cytarabine, (B) azacitidine, (C) LY3009120, (D) LXH254, (E) dabrafenib and (F) trametinib on AML cell lines, the cells were treated with each inhibitor at the indicated concentrations ranging from 0.001 to 10 μM for 72 h, and cell proliferation assays were performed. Following treatment with 0.1 μM LY3009120 and LXH254, HL-60 and NB4 cells showed significantly lower cell proliferation compared to that in KG-1 cells. ****P<0.0001. AML, acute myeloid leukemia.

AML cells. After testing the individual effects of cytarabine, azacitidine, and the MAPK inhibitors, we tested whether the combination of either low-dose cytarabine or azacitidine with pan-RAF inhibitors has a synergistic effect in *RAS*-mutated AML cells. The dose of cytarabine was chosen based on that used in clinical practice and previous studies (29-32). We tested the combinations of low-dose cytarabine (0-70 nM) or azacitidine (0-200 nM) with LY3009120 or LXH254 (0-100 nM) in HL-60, NB4, and KG-1 cells (Figs. S1A and B and S2A and B).

Analysis of the CI value for the combined treatment of cytarabine and LY3009120 showed synergism in HL-60 and NB4 cells (Table I). In the HL-60 cells, a stronger synergistic effect was seen after combination treatment with low-dose cytarabine and LY3009120 compared to that seen after individual treatments. In the NB4 cells treated with the combination, the proliferation rate was slightly reduced with mild synergism. Furthermore, the inhibitory effect of the combination of azacytidine with a pan-RAF inhibitor was not as prominent as that of the cytarabine combination (Fig. S1A and B). In comparison with each single treatment group, antagonism was exhibited in the IC values under the combined treatment conditions with no change in cell proliferation.

These results reveal that the two pan-RAF inhibitors, LY3009120 and LXH254, specifically affect *RAS*-mutated AML cells. However, they do not exhibit the same synergism with cytarabine, and only LY3009120 shows a synergistic effect with cytarabine in the *RAS*-mutated AML cell lines.

Low-dose cytarabine in combination with LY3002190 inhibits oncogenic MAPK signaling in RAS-mutated AML cell lines. Next, we investigated whether the observed synergism affected the Ras/RAF/MEK/ERK signaling pathways.

Table I. Evaluation of the synergistic effect of low-dose cytarabine and LY3009120.

A, HL-60 (*NRAS* Q61L) cells

Cytarabine, nM	LY3009120, nM	Fa	CI
50	6.25	0.47	0.49
50	12.50	0.44	0.53
50	25.00	0.36	0.71
50	50.00	0.35	0.96
50	100.00	0.39	1.01

B, NB4 (*KRAS* A18D) cells

Cytarabine, nM	LY3009120, nM	Fa	CI
50	6.25	N/A	N/A
50	12.50	N/A	N/A
50	25.00	0.50	1.00
50	50.00	0.70	0.75
50	100.00	0.71	1.18

After the AML cell line was treated with the indicated concentrations of the drug, the proliferation rate was measured via a proliferation assay, and then CI and Fa values were calculated using CalcuSyn software. AML, acute myeloid leukemia; Fa, fraction affected; CI, combination index; N/A, measurement not possible due the value being too low.

Here, synergistic doses were selected based on proliferation inhibition data (Table I) and treated the HL-60 and NB4 cells with cytarabine and/or LY3002190 for 24 h before assessing the signaling pathway components by western blotting. The combination treatments led to a significant decrease in the levels of phosphorylated c-RAF in HL-60 cells (Fig. 2A), whereas the levels of p-c-RAF increased in NB4 cells after single treatments by limited paradoxical activation. Finally, the level of phosphorylated MEK and ERK decreased with combined treatment in both the *RAS*-mutated cells (Fig. 2A). While the differences in the NB4 cells were not as statistically significant as that in the HL-60 cells, a significantly reduced value was confirmed (Fig. 2B)(*P<0.05).

These results indicate that compared to the individual drug treatments, the combined treatment with cytarabine and LY3009120 showed a marked effect on the phosphorylation of proteins required for active MAPK signaling.

Combined treatment with low-dose cytarabine and LY3009120 results in cell cycle inhibition. To investigate whether the combination treatment alters cell cycle progression in *RAS*-mutated AML cells, we performed cell cycle analysis under the same conditions. Cells were treated cytarabine, LY3009120, or a combination of both (as described earlier) for 24 and 72 h, stained with PI, and analyzed using flow cytometry.

Notably, in the HL-60 cells treated for 24 h, the percentage of cells in the G0G1 phase increased while that of cells in the S phases decreased after combined treatment. NB4 cells showed a slight increase in the percentage G0G1 phase cells and decrease in the S phase cells after combined treatment for 24 h (Fig. 3A) (*P<0.05; ***P<0.001, ****P<0.0001).

In the HL-60 cells treated for 72 h, the percentage of cells in the G0G1 phase increased remarkably and that of the cells in the S phases decreased after the combined treatment (Fig. 4A). In the NB4 cells treated for 72 h, there was an increase in the G0G1 cells in the combination treatment group as opposed to the individual treatment groups, and there was a slight increase in the percentage of S phase cells in the combination treatment group (Fig. 4A). Figs. 3B and 4B show that the cell cycle arrest was statistically significant) (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

In HL-60 cells, cell cycle inhibition was observed after treatment for 24 h. Compared to the HL-60 cells, the NB4 cells showed weaker cell cycle arrest at 24/72 h. These results are consistent with the results of the combined treatment observed in the proliferation assays.

Combined treatment with low-dose cytarabine and LY3009120 causes cell death in RAS-mutated AML cells. We used Annexin V and PI staining and analyzed the apoptotic HL-60 and NB4 cells after individual and combined treatments with cytarabine and LY3002190 for 24 and 72 h. Of the HL-60 cells analyzed after 24 h treatment, there was a slight increase in the early apoptotic cells in the combined treatment group and not in the control and individual treatment groups (Fig. 5A). At 72 h after treatment of the HL-60 cells, there was an increase in the early/late apoptotic cells in the combined treatment group (Fig. 5B). In contrast, NB4 cells showed only slight changes in the apoptotic cells at both 24 and 72 h (Fig. 5) (****P<0.0001).

Next, to identify the precise mechanism underlying cell death, we examined the expression of molecules related to cell death signaling. Specifically, we analyzed the cleavage of PARP, caspase-3, and caspase-9. After co-treatment of cytarabine and LY3009120 in HL-60 cells, the levels of caspase-3 and caspase-9 decreased and the level of cleaved PARP increased. In NB4 cells, no change in caspase was seen, but PARP levels decreased and the cleaved form increased in the combination treatment group (Fig. 6). We additionally confirmed changes in autophagy-related molecules, but did not confirm the significant difference of the combination treatment (Fig. S3).

These results and the immunoblotting data confirm the underlying mechanisms of synergism in HL-60 cells. Furthermore, immunoblotting analysis for NB4 cells showed a slight molecular change consistent with the slight increase in apoptotic cells.

The combination treatment of low-dose cytarabine and LY3009120 induced apoptosis in primary AML cells bearing the RAS mutation. We tried to confirm the apoptosis effect of the combination treatment of low-dose cytarabine and LY3009120 in primary AML cells and HL-60 and NB4 cell lines. We analyzed apoptosis in the primary cells of three patients with AML with *NRAS* mutants and one patient with *KRAS* mutants (Table SII). Even if it was analyzed using a small number of samples, in the primary cells of patients with AML with *NRAS* or *KRAS* mutations, the ratio of apoptotic cells increased after the combination treatment with the two drugs (Figs. 7 and S4 and S5) (*P<0.05, **P<0.01).

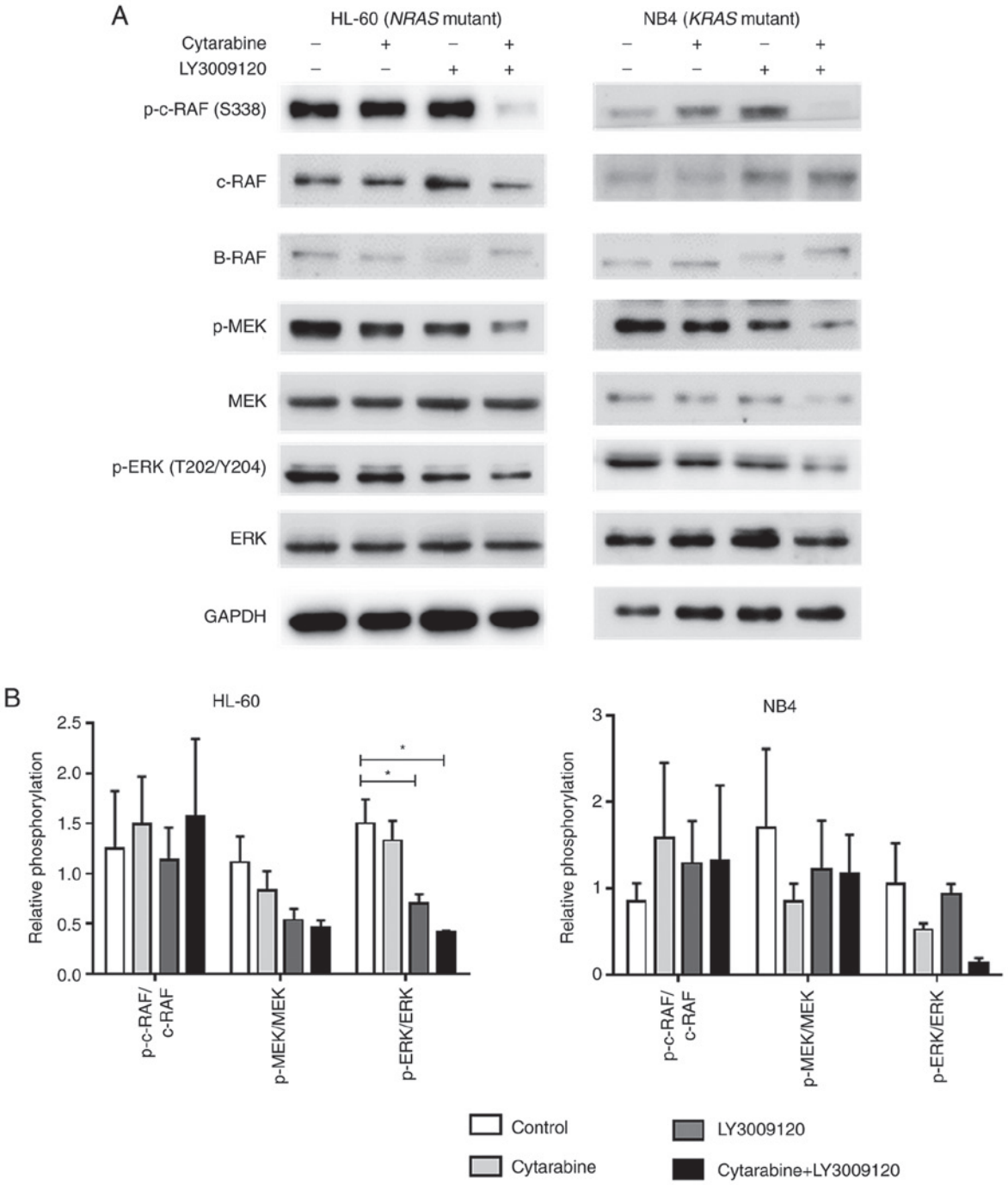

Figure 2. Inhibition of the MAPK pathway protein in *RAS* mutant cells following combined treatment with LY3009120 and low-dose cytarabine. (A) *NRAS*-mutated HL-60 and *KRAS*-mutated NB4 cells were treated with cytarabine and/or LY3009120 for 24 h, and the levels of the MAPK pathway molecules, including p-c-RAF, c-RAF, B-RAF, p-MEK1/2, MEK1/2, p-ERK1/2 and ERK1/2, were analyzed via western blotting. The HL-60 cell line was treated with 50 nM cytarabine, 6 nM LY3009120 or 50 nM cytarabine + 6 nM LY3009120. The NB4 cell line was treated with 50 nM cytarabine, 50 nM LY3009120 or 50 nM cytarabine + 50 nM LY3009120. (B) Semi-quantification of protein phosphorylation in HL-60 and NB4 cells. *P<0.05. p-, phosphorylated.

These results confirm that the combination treatment of low-dose cytarabine and LY3009120 could be a potential new treatment for patients with AML with *RAS* mutations by confirming its effect on primary cells as well as AML cell lines.

Discussion

AML predominantly affects elderly people, and the *RAS* mutation often results in a poor prognosis of AML. Since cytarabine and azacitidine are commonly used to treat AML in the elderly, we investigated the combined effect of these two drugs with the pan-RAF inhibitor LY3009120 in AML cells carrying the *RAS* mutations. The AML cells we used had several genetic modifications, including *RAS* mutations, and the WT *RAS* KG-1 cells are less sensitive to anticancer drugs. Thus, in this study, we intended to confirm the combination effect of the anticancer agents and the Raf inhibitor used for AML treatment and confirmed the reduction of down-signaling by treatment with the Raf inhibitor in all three cell lines. However, this drug

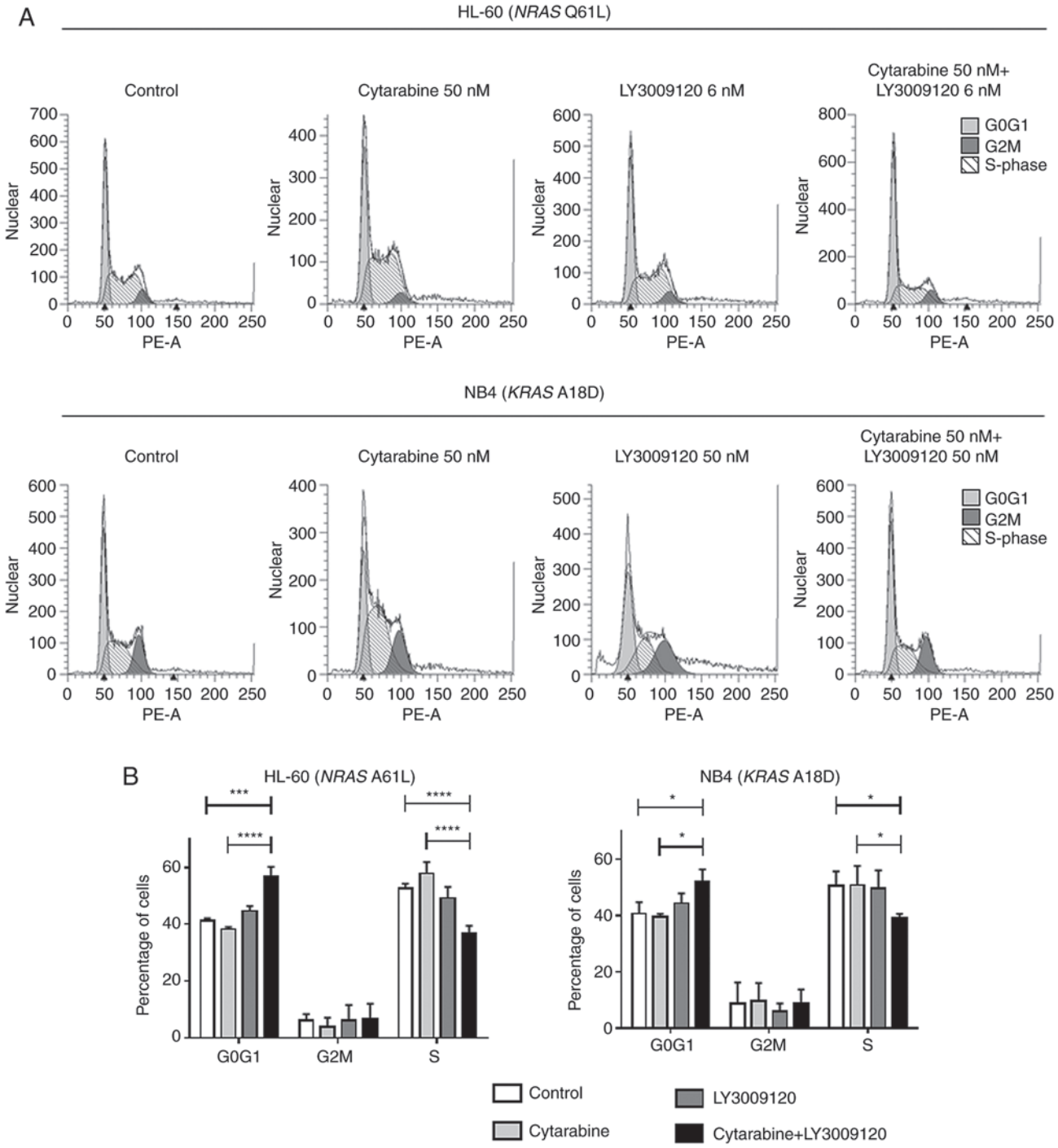

Figure 3. Cell cycle arrest of *RAS* mutant acute myeloid leukemia cells following combined treatment for 24 h. (A) Cells were treated with drugs alone or in combination for 24 h. The cell cycle profiles of HL-60 and NB4 cells were analyzed using PI staining and flow cytometry. The HL-60 cell line was treated with 50 nM cytarabine, 6 nM LY3009120 or 50 nM cytarabine + 6 nM LY3009120. The NB4 cell line was treated with 50 nM cytarabine, 50 nM LY3009120 or 50 nM cytarabine + 50 nM LY3009120. (B) The changes in the number of cells in different phases of the cell cycle were statistically analyzed for both HL-60 and NB4 cells. *P<0.05, ***P<0.001, ****P<0.0001.

combination synergistically affects only the *RAS*-mutated AML cells, and the synergism with cell cycle inhibition and increased apoptosis was confirmed in the HL-60 cells with *NRAS* mutation. In the NB4 cells with *KRAS* mutation, a slight cell cycle inhibition was observed in terms of increased G0G1 phase cells, decreased S phase cells, and increased apoptotic cells. Minimal effect was observed in NB4 cells as opposed to the HL-40 cells; however, cell cycle inhibition was observed after the 24 h treatment, which is a relatively short time, and apoptosis induction was induced after the 72 h treatment in NB4 cells. This indicates that the combined treatment of drugs causes cell death after cell cycle arrest.

The pan-RAF inhibitor, LY3009120, inhibits all RAF isoforms and binds to both protomers of the RAF dimer to prevent phosphorylation of the downstream signaling molecules MEK and ERK, with limited paradoxical activation in *RAS*-mutated cells (23). Consistent with this, we found a reduction in the levels of phosphorylated MEK and ERK in cells treated with LY3009120,

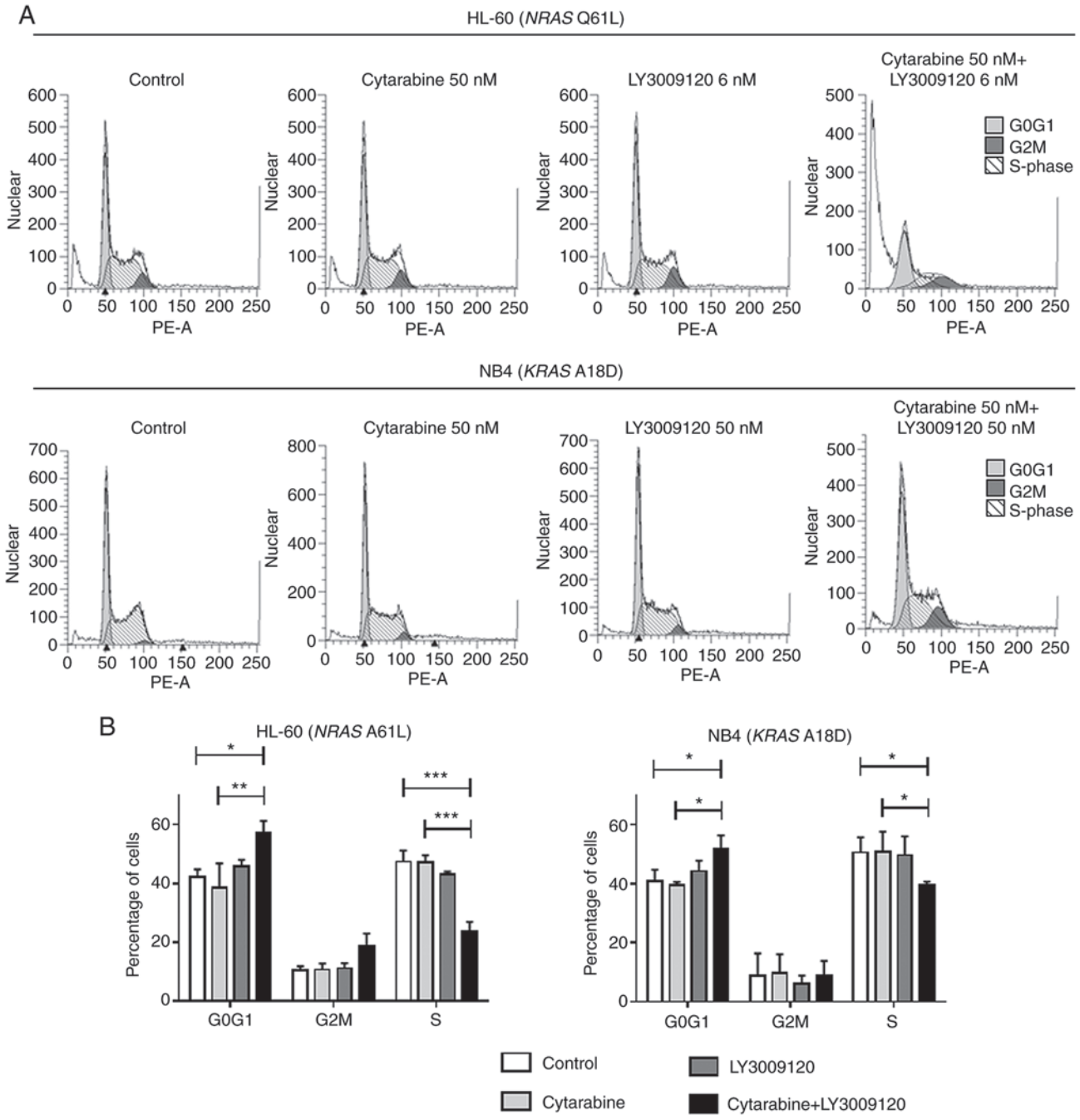

Figure 4. Cell cycle arrest of *RAS* mutant acute myeloid leukemia cells following combined treatment for 72 h. (A and B) Cells were treated with the indicated doses for 72 h. Cell cycle profiles of HL-60 and NB4 were analyzed by flow cytometry and changes in the number of cells in different phases of the cell cycle were statistically analyzed for both HL-60 and NB4 cells. The HL-60 cell line was treated with 50 nM cytarabine, 6 nM LY3009120 or 50 nM cytarabine + 6 nM LY3009120. The NB4 cell line was treated with 50 nM cytarabine, 50 nM LY3009120 or 50 nM cytarabine + 50 nM LY3009120. *P<0.05, **P<0.01, ***P<0.001.

indicating that the RAF kinase activity was impaired by the inhibitor, despite increased levels of phosphorylated c-RAF at specific inhibitor concentrations in NB4 cells, a phenomenon that has been observed in other cells (33). High concentrations of inhibitors induce nonspecific effects; however, compared with other MAPK inhibitors, the two pan-RAF inhibitors showed a specific inhibitory effect on *RAS*-mutated AML cells.

Moreover, CI analysis and analysis of levels of various proteins in the signaling pathway revealed the synergistic effect of cytarabine and LY3009120 in *RAS*-mutated AML cells. The latter analysis showed that levels of phosphorylated MEK and ERK were reduced in both *RAS* mutant cells, with changes in ERK being the most prominent. In addition to the differences between cell lines, we also found differences in the effects of the pan-RAF inhibitors, LY3009120 and LXH254. However, both inhibitors belong to the same family of type II RAF inhibitors. Only LY3009120 displayed a synergistic effect with low-dose cytarabine. We suggest that this response is due to differences in the mechanism and/or specificity of inhibition of the two agents. Although both the pan-RAF inhibitors act on both protomers of the RAF dimer, LXH254 acts on both B- and c-RAF (from NCI) and LY3009120 inhibits the kinase activity of all

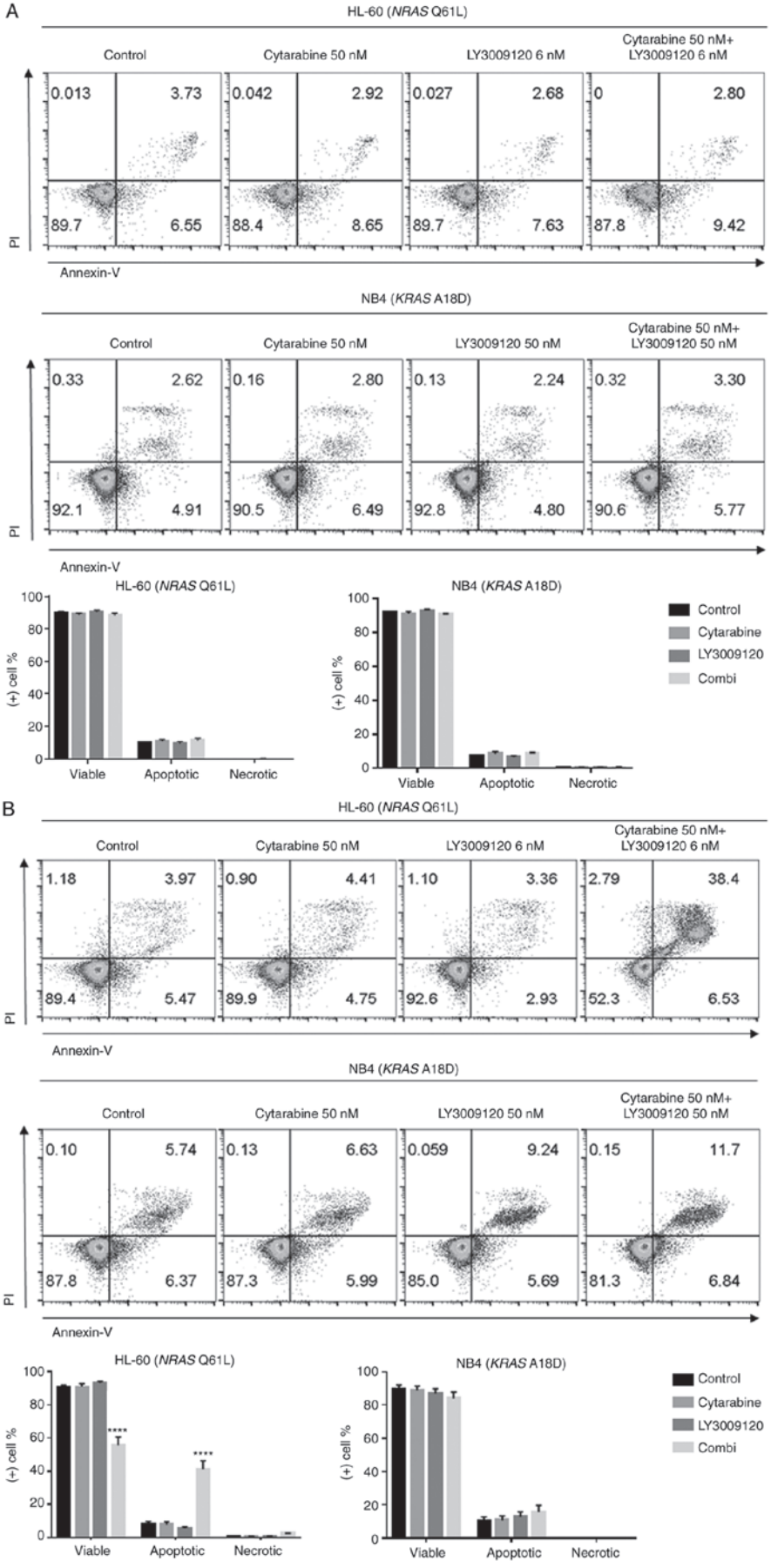

Figure 5. Induction of apoptosis in *RAS* mutant acute myeloid leukemia cells by low dose cytarabine combined with LY3009120. Cells were treated with the inhibitors at the indicated concentrations, and the apoptotic cells were analyzed by Annexin V and PI staining. (A) HL-60 and NB4 cells treated for 24 h. (B) HL-60 and NB4 cells treated for 72 h. The HL-60 cell line was treated with 50 nM cytarabine, 6 nM LY3009120 or 50 nM cytarabine + 6 nM LY3009120. The NB4 cell line was treated with 50 nM cytarabine, 50 nM LY3009120 or 50 nM cytarabine + 50 nM LY3009120. ****P<0.0001.

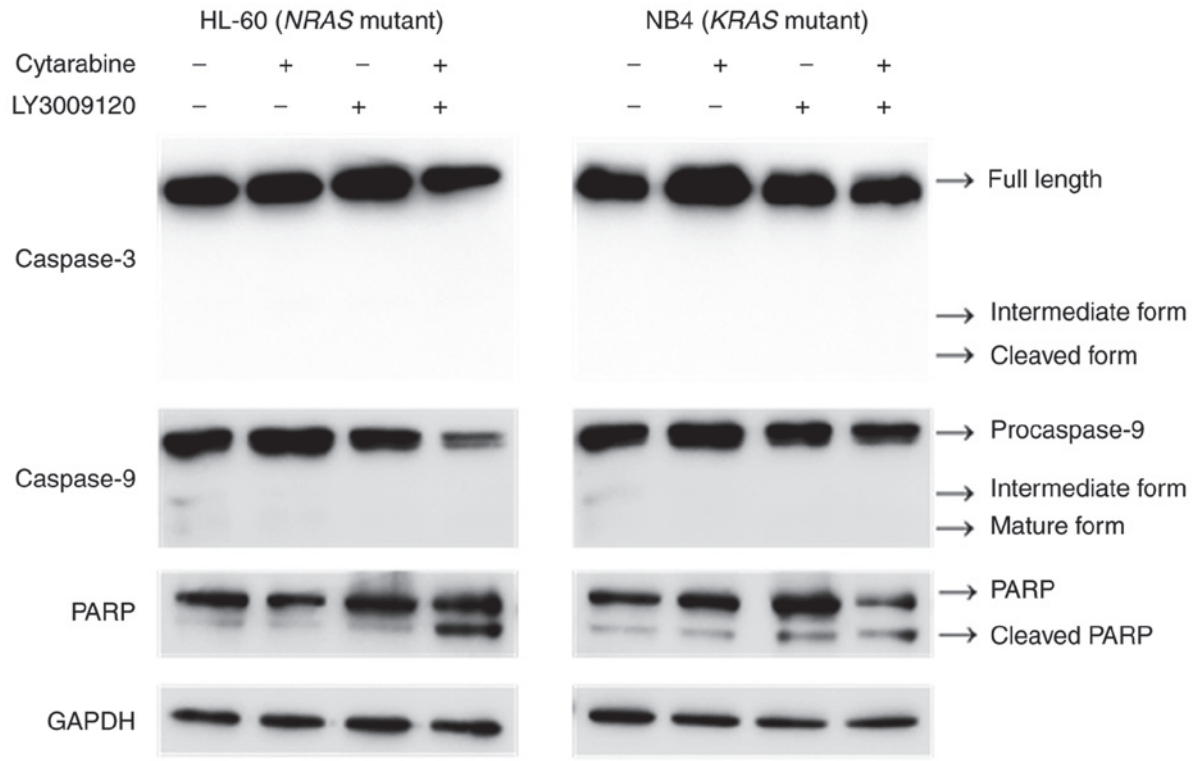

Figure 6. Identification of apoptosis pathway after treatment of *RAS* mutant acute myeloid leukemia cells with low-dose cytarabine combined with LY3009120. Cells were treated with the inhibitors at the indicated concentrations for 72 h to ensure cell death. Western blotting was performed using antibodies against caspase-3, caspase-9 and PARP. The HL-60 cell line was treated with 50 nM cytarabine, 6 nM LY3009120 or 50 nM cytarabine + 6 nM LY3009120. The NB4 cell line was treated with 50 nM cytarabine, 50 nM LY3009120 or 50 nM cytarabine + 50 nM LY3009120.

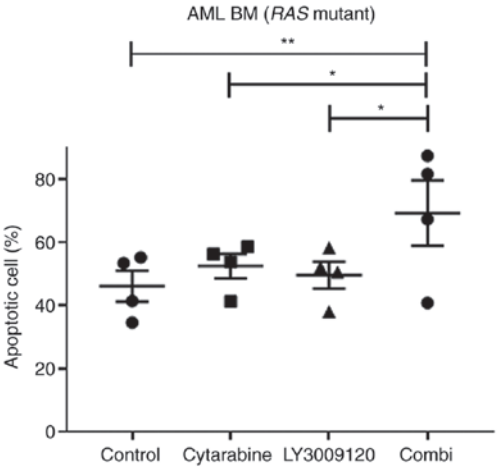

Figure 7. Induction of apoptosis in primary cells of *RAS* mutant AML by low-dose cytarabine combined with LY3009120. Cells were treated for 72 h to ensure apoptosis. The apoptotic cells were analyzed by Annexin V and PI staining. The HL-60 cell line was treated with 50 nM cytarabine, 6 nM LY3009120 or 50 nM cytarabine + 6 nM LY3009120. The NB4 cell line was treated with 50 nM cytarabine, 50 nM LY3009120 or 50 nM cytarabine + 50 nM LY3009120. *P<0.05, **P<0.01. AML, acute myeloid leukemia; BM, bone marrow.

RAF isoforms (23). It is known that the cytotoxic response of LY3009120 is specific for the *RAS* mutation, and this response is less toxic to healthy bone marrow cells. Furthermore, this drug effect persists without producing resistant cells after drug treatment (34). Therefore, the differences in the effects of these inhibitors observed in combination with low-dose cytarabine could be attributed to the mechanism of action of these inhibitors.

Although synergistic effects were observed in both the AML cell lines studied, the effects were prominent in the *NRAS*-mutated HL-60 cells, compared to *KRAS*-mutated NB4 cells. Therefore, we further investigated the response of the two cell lines to the RAF inhibitor LY3009120. In HL-60 cells, the expression of phosphorylated ERK decreased in the LY3009120-treated cells compared to the control cells. Conversely, in NB4 cells, the expression of phosphorylated ERK and phosphorylated c-RAF increased after LY3009120 treatment. Such paradoxical activity of ERK owing to a RAF inhibitor is well known (35-37). Therefore, we suggest that these paradoxical activities of LY3009120 are reflected in the differences observed in the response of HL-60 and NB4 cells to the combination therapy.

Despite this complex physiological phenomenon, our immunoblotting data show that the combination of cytarabine and LY3009120 has shown synergy in *RAS* mutant AML, and thus can be considered a novel therapeutic strategy. In order to clearly confirm the therapeutic effect, humanization experiments can show clear results for going to a clinical trial, but further studies on animal and patient-based studies are needed. Induction of specific mutations and obtaining patient samples with *RAS* mutations are not easy, so our in vitro study cannot determine the role of the tumor microenvironment in actual patients, differences between drugs, and the underlying mechanisms for cell-to-cell reactivity.

Therefore, further studies are required on this issue, and it would be interesting to test the synergy between pan-RAF

inhibitors and venetoclax, either with or without low-dose cytarabine (38) in *RAS*-mutated AML.

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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

JP acquired, analyzed and interpreted the data, and drafted the manuscript. YK, HP, JMB, JH, DYS and SSY devised the study concept and design, and provided technical guidance for all aspects of the project. All authors discussed the results and contributed to the final manuscript. SSY and YK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (approval no. IRB-1201-099-396; Seoul). Primary samples from patients with acute myeloid leukemia were obtained with written informed consent from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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