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# Combinatorial Effects of PARP Inhibitor PJ34 and Histone Deacetylase Inhibitor Vorinostat on Leukemia Cell Lines

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**Abstract.** *Background: Poly (ADP-ribose) polymerase (PARP) inhibitors and histone deacetylase (HDAC) inhibitors are new promising anticancer drugs. The aim of the present study was to investigate the effect of combination treatment with PARP inhibitor PJ34 and HDAC inhibitor vorinostat on human leukemia cell lines. Materials and Methods: Proliferation, apoptosis, mitochondrial membrane potential ($\psi_m$) and cell cycle were assessed in HL60, MOLT4, U937 and K562 cells cultured with each drug alone and with both drugs. Results: PJ34 alone at 0.2-0.4 μM did not influence the examined parameters. Vorinostat alone at 1.0-2.5 μM reduced proliferation, increased apoptosis rate, lowered $\psi_m$ and increased the percentage of sub-$G_1$ cells in all cell lines. Incubation with both drugs caused further inhibition of proliferation and increase in apoptosis associated with a decrease in $\psi_m$ and sub-$G_1$ arrest in HL60, MOLT4 and K562 cells, but not in U937 cells. Conclusion: Combination of PARP and HDAC inhibitors can exert a synergistic effect on inhibition of proliferation and increase apoptosis of leukemia cells.*

Targeted therapies have become a hot topic in anticancer research. Recently, considerable interest has been given on drugs that target histone-modifying enzymes and cells with impaired DNA repair: histone deacetylase (HDAC) inhibitors and poly (ADP-ribose) polymerase (PARP) inhibitors.

PARP is a family of nuclear enzymes involved in DNA damage recognition and repair. PARP1 is the best known protein of this family which participates in single-strand breaks repair and may also be involved in the repair of double-strand breaks through the homologous recombination pathway (1, 2). Inhibition of its activity results in accumulation of single-strand breaks and ultimately leads to double-strand breaks. PARP inhibitors have been extensively studied as agents preventing DNA repair in cancer cells, especially in cells with mutated breast cancer-associated gene-1 and -2 (BRCA1/2), involved in the repair of double-strand breaks (3). However, PARP inhibitors, such as PJ34, ABT-888 and KU-0058948, have been found to exert anti-proliferative and pro-apoptotic effects when used alone or in combination with potential chemotherapeutic agents in various cancer types. PJ34 enhanced the antitumor effects of doxorubicin in HeLa cells (4). ABT-888 potentiated irinotecan treatment of colon cancer cell lines (5) and enhanced the cytotoxic effect of temozolomide in leukemia cells *in vitro* and in a melanoma model *in vivo* (6, 7). PJ34 and KU-0058948 induced cell-cycle arrest and apoptosis of primary myeloid leukemia cells and cell lines *in vitro* (8).

HDAC inhibitors are promising new anticancer agents with low toxicity toward normal non-malignant cells. They inhibit deacetylation of proteins, especially those involved in cell proliferation, differentiation and apoptosis (9). Vorinostat is a broad-spectrum HDAC inhibitor used in clinical trials (10, 11) shown to induce production of reactive oxygen species, growth arrest and apoptosis of leukemia cells (12). It has been postulated that the effect of vorinostat on cancer cells is due to induction of DNA damage, including single- and double-strand breaks (12, 13). Furthermore, impaired repair of double-strand DNA breaks is a common feature of acute myeloid leukemia and myelodysplastic syndromes (14).

To our knowledge, there are only two studies demonstrating the effects of combinatorial treatment of tumor cells with PARP inhibitors and HDAC inhibitors (8, 15) and only one of them concerns leukemia cells. Gaymes *et al*. demonstrated that the HDAC inhibitor, MS275, enhanced the cytotoxic effect of the PARP inhibitor KU-0058948 in leukemia cells *in vitro* (8). Therefore, the aim of the present study was to demonstrate the effect of PARP1 inhibitor PJ34 in combination with DNA-damaging HDAC inhibitor vorinostat on proliferation, apoptosis and cell cycle of human leukemia cell lines.

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*Key Words:* PARP inhibitors, HDAC inhibitors, PJ34, vorinostat, apoptosis, cell cycle, leukemia cell lines.

## Materials and Methods

*Drugs*. PJ34 was purchased from Merck Chemicals (Darmstadt, Germany) and vorinostat from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of drugs were aliquoted and kept frozen at −20ºC.

*Cell culture*. Cultures of HL60, MOLT4, U937 and K562 cells (all obtained from ECACC, Salisbury, UK) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum ( FBS), 2 mM L-glutamine and 50 μg/ml gentamicin (all from Life Technologies, Paisley, UK). Cells were cultured at 37˚C in a humidified 5% $CO_2$ atmosphere and used for experiments in the exponential growth phase.

*PARP activity assay*. Cellular PARP activity was measured using the Universal Colorimetric PARP Assay kit (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The assay measures incorporation of biotinylated poly(ADP-ribose) into histone proteins in 96-well plates. Briefly, cell lysates were prepared from $5\times10^6$ cells and 30 μg of protein was added in triplicates to the wells containing PARP buffer and PARP cocktail. Following incubation at room temperature for 1 h and a short wash with 0.1% Triton X-100, streptavidin horseradish peroxidase was added to the wells, and the mixture was incubated at room temperature for 20 min. After washing the plate with 0.1% Triton X-100, TACS-Sapphire colorimetric substrate was added and plate was incubated in the dark for 30 min. Absorbance of samples was measured at 450 nm using microplate fluorescence reader FL600 (Bio-Tek Instruments, Inc., Winooski, VT , USA).

PARP activity was calculated using the standard curve obtained from readings of the standards and expressed as arbitrary absorbance units per mg of protein.

*Cell proliferation and viability assays*. Cell growth was assessed by the alamar blue assay (Biosource, Camarillo, CA, USA), as described previously (16). Briefly, the cells were seeded into 96-well plate at a density of $2\times10^4$ cells per well (100 μl) in culture medium alone or supplemented with the studied drugs and incubated for a specified amount of time (48 h with PJ34 and 24 h with vorinostat). After the incubation, alamar blue was added to the wells to a final concentration of 10% and the plates were incubated for 4 h at 37˚C in a humidified atmosphere with 5% $CO_2$. The intensity of fluorescence emitted due to reduction of alamar blue was measured using FL600 microplate reader (excitation: 530 nm, emission: 590 nm).

Cell viability was determined using the trypan blue exclusion method. Cell proliferation and viability were expressed as the percentage of that of the control (100%).

*Apoptosis assay*. Apoptotic cells were analyzed using Muse Annexin V and Dead Cell Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The assay utilizes annexin V to detect phosphatidylserine on the cell membrane surface of apoptotic cells and a dead cell marker, 7-aminoactinomycin D (7-AAD), as an indicator of cell membrane integrity. Briefly, $5\times10^5$ cells were resuspended in 100 μl of culture medium containing 1% FBS and incubated with 100 μl of Muse Annexin V and Dead Cell Reagent for 20 min at room temperature in the dark. Annexin V–positive/7-AAD–negative cells represented early apoptotic cells, while annexin V–positive/7-AAD–positive cells demonstrated late apoptotic cells. The cells were quantified using the Muse Cell Analyzer and Muse analysis software (Millipore).

*Change of mitochondrial membrane potential ($\Delta\psi_m$)*. Mitochondrial membrane potential was determined using Mitochondrial Permeability Transition Detection kit MitoPT™ JC-1 (AbD Serotec, Oxford, UK) according to the manufacturer's instructions. JC-1 dye exhibits potential-dependent accumulation in mitochondria indicated by a fluorescence emission shift from green to red (590 nm). Consequently, mitochondrial de-polarization is indicated by a decrease in the red/green fluorescence intensity ratio. Briefly, cells were resuspended at a density $1\times10^6$ cells in 1 ml of MitoPT™ JC-1 solution and incubated for 15 minutes at 37˚C in an atmosphere containing 5% $CO_2$ in the dark. After incubation, cells were resuspended in the assay buffer and seeded in a 96-well clear bottom black plate at a density $1\times10^5$ cells/100 μl per well. Fluorescence was assessed in the microplate reader by measuring both the monomer (527 nm emission; green) and J-aggregate (590 nm emission; red) forms of JC-1 following 488 nm excitation. $\Delta\psi_m$ was calculated as J-aggregate to monomer fluorescence intensity ratio. The membrane potential of mitochondria in treated cells was expressed as a percentage of that in control cells (100%).

*Cell-cycle analysis*. Cell cycle distribution was determined using the Muse Cell Cycle Kit (Millipore) according to the manufacturer's instructions. The assay is based on the measurement of DNA content in nuclei labeled with propidium iodide (PI). Briefly, $1\times10^6$ cells were harvested and fixed with 70% ice cold ethanol at −20˚C for 3-12 h. After washing with phosphate buffered saline (PBS), cell pellets were resuspended in 200 μl of Cell Cycle Reagent and incubated for 30 min at room temperature in the dark. Cells were analyzed by Muse Cell Analyzer and the cell cycle phase distribution was quantified using Muse analysis software. Graphs were prepared with freeware Flowing Software 2.5 (http://www.flowingsoftware.com).

*Statistical analysis*. The results are expressed as means±SD of three independent experiments. Statistical analysis was performed by using two-tailed Student's *t*-test. *p*-Values of less than 0.05 were considered statistically significant. Data were analyzed using the Prism 5.0 software (GraphPad, La Jolla, CA, USA).

## Results

*Effect of PJ34 on PARP activity, cell proliferation and viability*. HL60, MOLT4, U937 and K562 cell lines were exposed to increasing concentrations of the PARP1 inhibitor PJ34 (0.05 to 1.6 μM) for 48 h. PARP activity was significantly reduced in cells in a dose-dependent manner (Figure 1A). In untreated control cells, the highest PARP activity was observed in the MOLT4 line (~80 U/mg of protein) and the lowest in the U937 line (~50 U/mg of protein) (Figure 1A). Cell proliferation and viability were also inhibited in a dose-dependent manner (Figure 1B and C). Concentrations of 0.2 μM PJ34 for HL60 and MOLT4 cells and 0.4 μM for U937 and K562 cells were chosen for further studies, being the highest concentrations having a relatively low effect on proliferation and viability (~20% decrease in

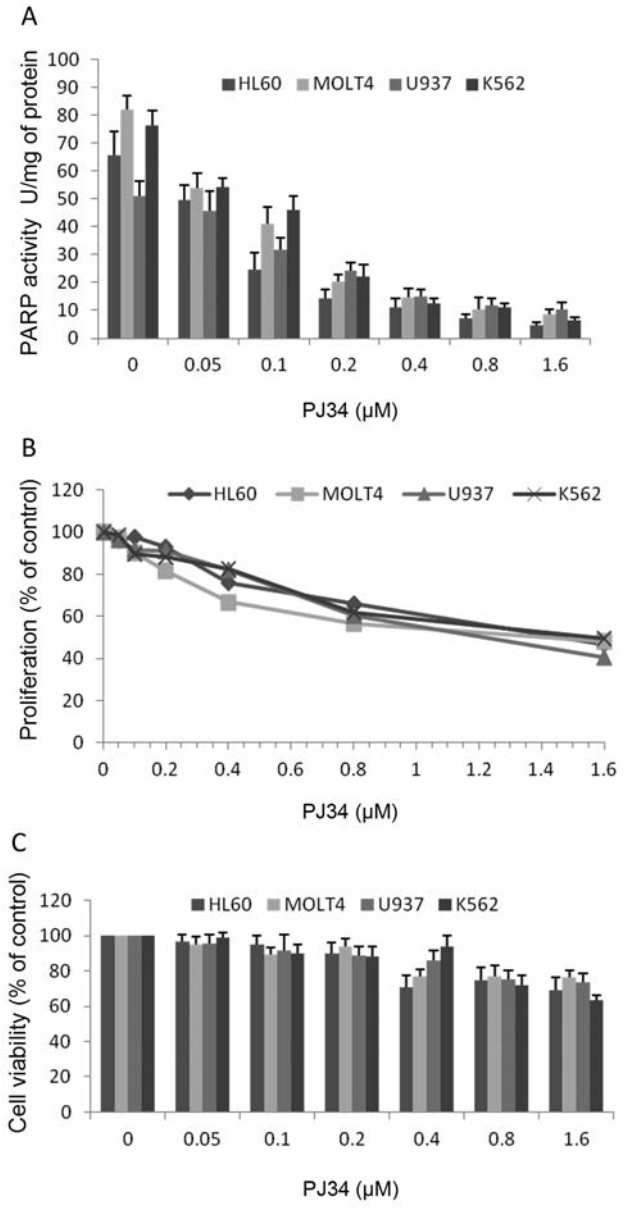


Figure 1. *Effect of PJ34 on poly (ADP-ribose) (PARP) activity (A), proliferation (B) and viability (C) of HL60, MOLT4, U937 and K562 cell lines. PJ34 lowered PARP activity, and reduced cell proliferation and viability in a dose-dependent manner.*

proliferation and ~90% viability) (Figure 1B and C). At these doses of PJ34, PARP activity was reduced to approximately 15 U/mg of protein in all cell lines (Figure 1A).

*Effects of combined PJ34 and vorinostat treatment on cell proliferation and apoptosis*. Cells were treated with appropriate doses of PJ34 and different concentrations of vorinostat, causing approximately 20% of total apoptosis (1 μM for HL60

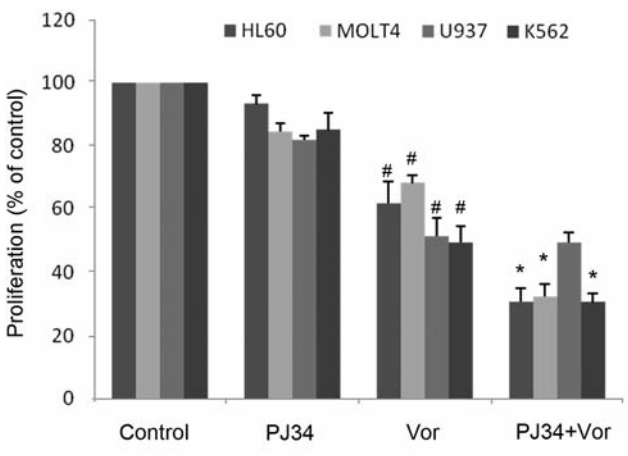


Figure 2. *Effect of PJ34 and vorinostat (Vor) on the proliferation of HL60, MOLT4, U937 and K562 cells. Each value is the mean±SD of three experiments. #Significantly different from the corresponding group of untreated (control) cells ($p<0.04$); *significantly different from the corresponding group of vorinostat-treated cells ($p<0.05$).*

and MOLT4 cells; 2 μM U937 cells and 2.5 μM for K562 cells, data not shown) whether separately or in combination. In the latter case, PJ34 was added 24 h before vorinostat and then the cells were exposed to both drugs for the next 24 h.

At the concentrations used, PJ34 alone did not significantly influence proliferation in the studied cell lines, whereas treatment with vorinostat alone caused its significant reduction in all lines. Treatment with both drugs further significantly lowered proliferation of HL60, MOLT4 and K562 cells compared to treatment with vorinostat-alone. In U937 cells, this effect was not observed (Figure 2).

Treatment with PJ34-alone had no significant effect on apoptosis in either cell line: the total apoptotic rate ranged from 4.3% in HL60 cells to 5.5% in MOLT4 cells. Vorinostat-induced apoptosis was markedly potentiated after a combined treatment with PJ34 and the percentage of apoptotic cells nearly doubled, ranging from 37.6% in K562 cells to 48.5% in MOLT4 cells. This effect was, however, not observed in U937 cells (Figure 3). Similar results were obtained when early and late apoptotic cells were assessed separately (data not shown).

*Effects of combined PJ34 and vorinostat treatment on mitochondrial membrane potential*. Treatment with PJ34 alone did not cause significant changes in $\psi_m$, an early indicator of mitochondrial involvement in apoptosis, while vorinostat-alone significantly reduced $\psi_m$ in all cell lines compared to untreated cells. The combined treatment of cells with both drugs resulted in a significant decrease in $\psi_m$ compared to treatment with vorinostat alone in all cell lines except U937 cells (Figure 4).

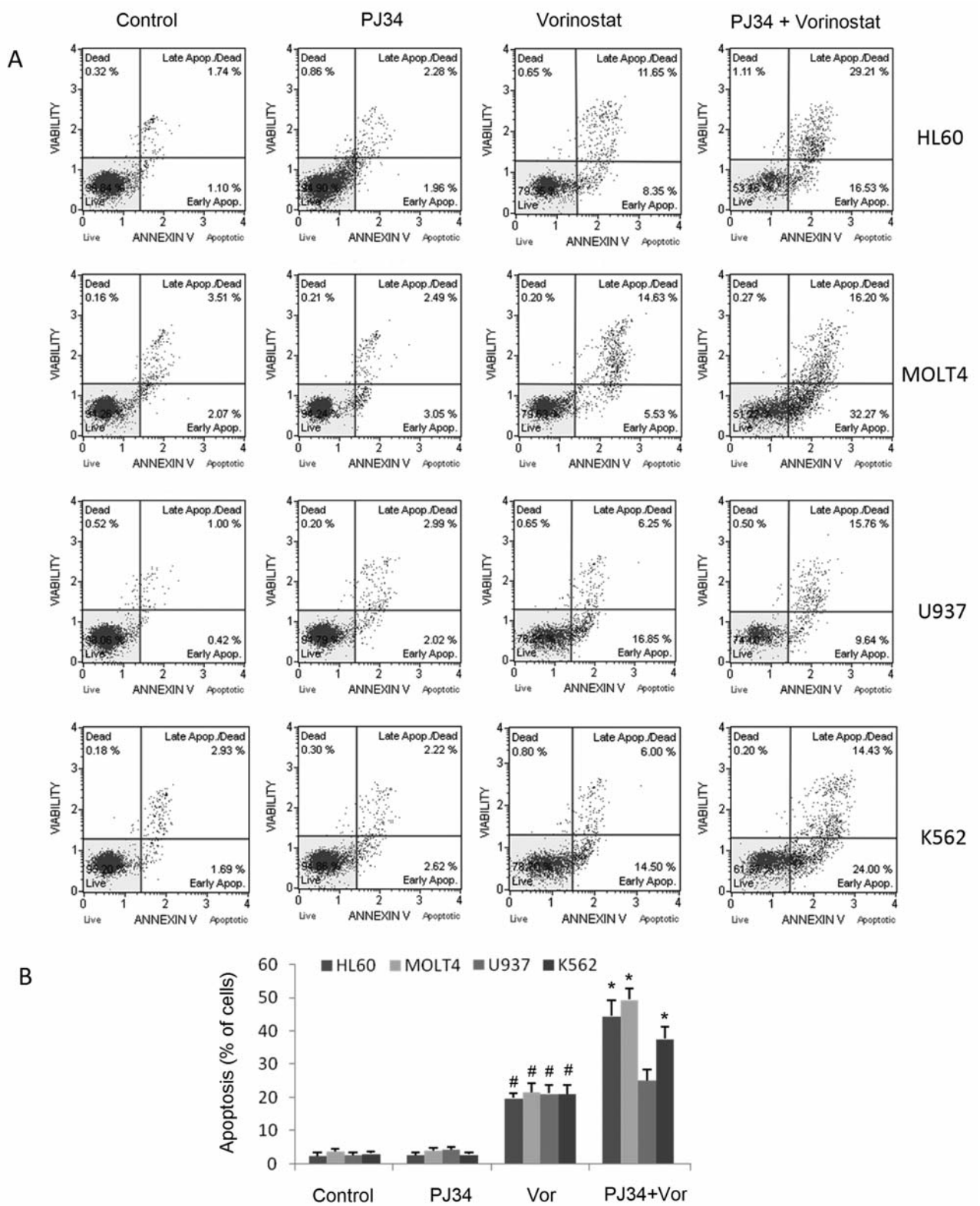


Figure 3. *Effect of PJ34 and vorinostat (Vor) on apoptosis of HL60, MOLT4, U937 and K562 cells. Representative dot plots of annexin V/7-aminoactinomycin D (7-AAD) apoptotic assay (A) and graph showing the percentage of apoptotic cells (B). Each value is the mean±SD of three experiments. #Significantly different from the corresponding group of untreated (control) cells (p<0.03); *significantly different from the corresponding group of vorinostat-treated cells (p<0.03).*

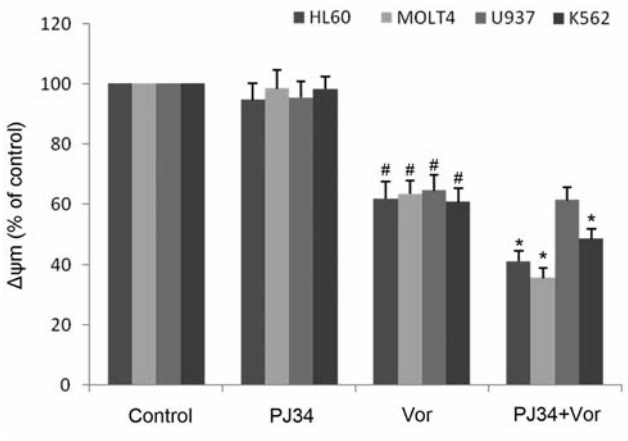


Figure 4. *Effect of PJ34 and vorinostat (Vor) on the mitochondrial membrane potential ($\psi_m$) of HL60, MOLT4, U937 and K562 cells. Each value is the mean±SD of three experiments. #Significantly different from the corresponding group of untreated (control) cells (p<0.03); *significantly different from the corresponding group of vorinostat-treated cells (p<0.04).*

*Effects of combined PJ34 and vorinostat treatment on the cell cycle*. PJ34 alone did not significantly influence the cell cycle in the studied lines (Figure 5). After treatment with vorinostat alone, the percentage of S-phase cells was significantly reduced in HL60 and U937 lines (Figure 5A-C) and all cell lines showed an increase in the percentage of cells in the sub-$G_1$ phase (Figure 5). Compared to the effect of vorinostat-alone, the combined treatment of cells with vorinostat and PJ34 resulted in further decrease in the percentage of S-phase cells in all cell lines and of cells in the $G_2$/M phase for U937 and K562 lines, while the percentage of cells in $G_0/G_1$ phase was significantly increased for U937 and K562 cells and of those in sub-$G_1$ phase cells for all except U937 cells (Figure 5).

## Discussion

Combining drugs which target DNA repair and epigenetic control of gene transcription seems to be an encouraging approach to anticancer treatment. The present study has demonstrated that combined exposure of human leukemia cell lines to the PARP inhibitor PJ34 and the HDAC inhibitor vorinostat results in a significant decrease in cell proliferation, accompanied by an increase in both early and late apoptosis, manifested by the loss of mitochondrial membrane potential, an early event preceding phosphatidylserine externalization (17, 18), annexin V binding and 7-AAD fluorescence.

Such effects were similar in three out of the four studied cell lines: HL60, MOLT4 and K562, but was not observed in U937 line: as compared to cells treated by vorinostat as a single agent, combined treatment with vorinostat and PJ34 did not induce significant changes in cell growth, apoptosis rate and mitochondrial membrane potential. All leukemia cell lines studied revealed sensitivity to PJ34, manifested by a dose-dependent decrease in PARP activity. However, in untreated control cells, PARP activity was the lowest in U937 cells, and this could contribute to unresponsiveness of this cells to the combined treatment, although the relevant data seem to be controversial. As demonstrated in other studies on treatment of cell lines with PARP inhibitors combined with other anticancer drugs, PARP inhibitor ABT-888 did not enhance temozolomide activity in U937 cells and in some primary leukemia cells expressing low PARP activity. On the other hand, KG1 leukemia cells with low PARP activity showed a marked potentiation of temozolomide cytotoxicity in combination with ABT-888 (6). Another PARP inhibitor, GPI-15427 increased temozolomide and irinotecan activity in colon cancer xenografts and this effect was not dependent on PARP activity (19). In our previous study, etoposide-induced apoptosis of U937 cells was not potentiated after pre-treatment with HDAC inhibitors, although such potentiation was observed in HL60 cells (16).

The different effectiveness of PARP inhibitors may reflect the variability of cell lines. Leukemia cells display different phenotypes and diverse chromosomal abnormalities. Some aberrant genes, especially in cells in acute myeloid leukemia and myelodysplastic syndromes, are components of the homologous recombination pathway of double-strand DNA break repair. Moreover, leukemia cells display error-prone non-homologous end-joining of double-strand DNA break repair and mutations in other double-strand DNA break repair components (20-22). This makes them good candidates for treatment with PARP inhibitors, however, the response of different cell lines to PARP inhibitors combined with other chemotherapeutic agents can also depend upon a variety of factors, such as drug concentration, exposure conditions and the sequence of drug administration. In MCF7 cells, the PARP inhibitor NU1025 increased the antiproliferative effect of topoisomerase II inhibitor C-1305 when added to the culture as the second drug, but reversal of the schedule had the opposite result: the antiproliferative effect of C-1305 was reduced (23). The combined treatment of leukemia cells with PARP, HDAC and DNA methyltransferase inhibitors can also be schedule-specific (8).

PARP inhibitors have been postulated to affect the cell cycle. As recently demonstrated, PJ34 causes cell-cycle arrest in various cancer cell lines (24-26). The arrest of cells at the cell-cycle checkpoints is an event preceding apoptosis (27). HDAC inhibitors can also influence cell cycle. Vorinostat has been shown to induce apoptosis of leukemia cells in the $G_0/G_1$ phase of the cell cycle by inducing the cyclin-dependent kinase inhibitor p21 and to arrest cells in the $G_1$ phase (28, 29). In our study, PJ34 and vorinostat were

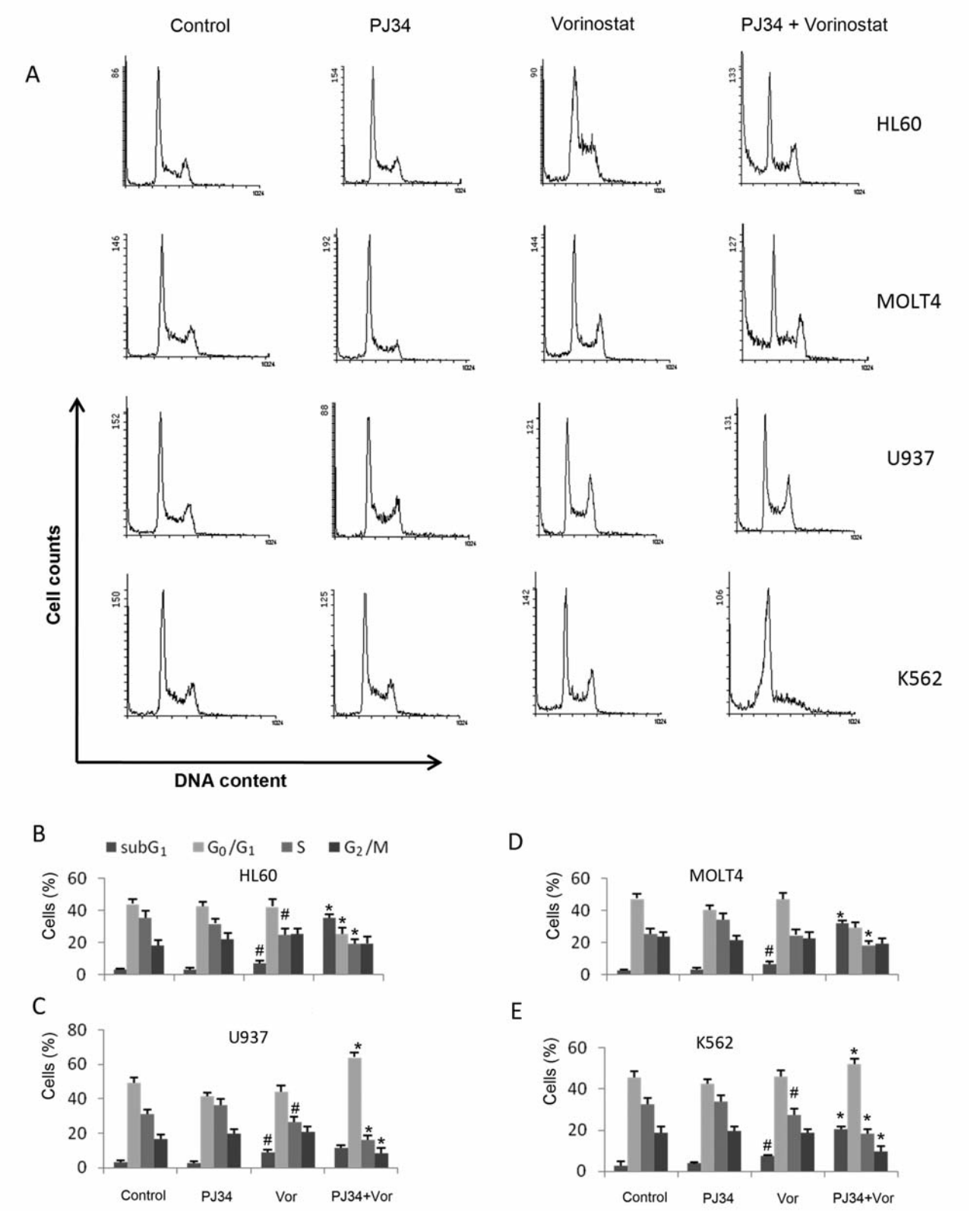


Figure 5. *Effect of PJ34 and vorinostat (Vor) on cell-cycle distribution of HL60, MOLT4, U937 and K562 cells. Representative DNA histograms (A) and graphs showing the distributions of cells in specific cell cycle phases (B, C, D, E). Each value is the mean±SD of three experiments. #Significantly different from the corresponding group of untreated (control) cells ($p<0.05$); *significantly different from the corresponding group of vorinostat-treated cells ($p<0.05$).*

used at concentrations showing low toxicity. At 0.2 and 0.4 μM, PJ34-alone did not induce significant changes in the cell cycle. The main effect of vorinostat alone and in combination with PJ34 at the studied concentrations was a significantly increased percentage of cells in the sub-$G_1$ phase, indicative of apoptosis. This result fully corresponded to the effect of the drugs on apoptosis, with all cell lines except U937 showing further significant increase in the number of sub-$G_1$ cells after combined treatment. We also observed an increased percentage of K562 and U937 cells in the $G_0/G_1$ phase after treatment with PJ34 and vorinostat. A similar effect was reported after prolonged 24-h treatment of K562 cells with vorinostat alone (30).

In conclusion, combination of PARP inhibitors with HDAC inhibitors can effectively reduce survival of leukemia cells by inhibiting proliferation and inducing apoptosis, but this effect may depend on cell type, concentration of drugs within the range showing less additive toxicity for normal cells, and administration schedule. Nevertheless, it seems to be a promising therapeutic strategy for hematological malignancies.

## Conflicts of Interest

None declared.

## Acknowledgements

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