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Vincristine potentiates the anti-proliferative effect of an aurora kinase inhibitor, VE-465, in myeloid leukemia cells

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ABSTRACT

Aurora kinases play an essential role in the regulation of mitosis. The kinases are overexpressed in a variety of cancer cells and are involved in tumorgenesis. Although aurora kinase inhibitors are potential agents for treatment of leukemia, the establishment of efficacious combination therapies is an attractive approach for making good use of these agents. In this study, we examined the effects of a specific aurora kinase inhibitor, VE-465, in combination with various conventional anti-leukemia agents, including doxorubicin, daunorubicin, idarubicin, mitoxantron, cytosine arabinoside, vincristine and etoposide, on acute myeloid leukemia cell lines (HL60, U937, THP-1 and KY821), chronic myeloid leukemia cell lines (KCL22, K562 and KU812) and primary leukemia cells. We found that a combination of VE-465 and vincristine had a synergistic/additive inhibitory effect on the growth of leukemia cells. VE-465 initially increased G2/M-phase cells, followed by induction of sub-G1 cells. Vincristine enhanced this effect of VE-465. The combination of VE-465 and vincristine increased the levels of cleaved caspase 3, cleaved caspase 7, cleaved caspase 9, cleaved PARP and Phospho-Chk2, suggesting that the combination caused Chk2-mediated activation of the G2/M checkpoint, resulting in sequential induction of apoptosis. Interestingly, the combination markedly decreased the level of Phospho-ERK1/2, suggesting that the combination alters a network of cellular signaling pathways. In contrast, combinations of VE-465 and other agents showed no synergistic inhibitory effect but rather had an antagonistic effect. In conclusion, our results indicate the utility of the combination of VE-465 and vincristine as a potential therapy for myeloid leukemia.

1. Introduction

Recently, many kinds of small-molecule agents targeting specific leukemogenetic molecules have been developed and studied at preclinical or clinical levels for application to treatment of leukemia [1]. The efficacy of BCR/ABL kinase inhibitors, including imatinib, nilotinib and dasatinib, against BCR/ABL-positive leukemia has indicated the potential of specific kinase inhibitors for clinical application [2]. However, many small-molecule agents have shown only limited clinical efficacy when they are used alone, and development of combination therapies may therefore be needed for making good use of these agents.

Aurora serine/threonine kinases play essential roles in regulation of cell mitosis [3–5]. Aurora-A mediates mitotic spindle formation and centosomal duplication. Aurora-B is a chromosomal passenger protein that contributes to proper chromosomal segregation and cytokinesis. Histone H3, which is involved in chromosome condensation, is phosphorylated by Aurora-B. Aurora-C is known to be predominantly expressed in germ cells, but its function remains unclear. Activity of these aurora kinases changes depending on the cell cycle phase and is mostly up-regulated at the G2/M phase [6]. It has been shown that deregulation of aurora kinases is involved in tumorgenesis and that overexpression of aurora kinases occurs in many types of human tumor cells [4,7,8]. These findings raised the possibility that inhibition of aurora kinase activity will induce blockage of the cell cycle, resulting in suppression of tumor cell proliferation. Indeed, several aurora kinase inhibitors have been developed and these agents have shown suppressive effects on the growth of

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cancer cells *in vitro* [8,9]. Certain agents, including MK-0457, have shown potent anti-leukemia activity against imatinib-resistant BCR/ABL-positive leukemia cells [10–13]. These findings suggest that aurora kinase inhibitors are potential small-molecule agents against various tumors, including leukemia. On the basis of these findings, clinical trials of several aurora kinase inhibitors against certain types of tumors are currently being carried out [14,15]. Recently, the effects of combinations of an aurora kinse inhibitor, SNS-314, and common chemotherapeutics have also been reported [16], and the results of that study indicated the possibility that combinations of an aurora kinase inhibitor and other anti-cancer agents would enhance anti-cancer activity.

In this study, we examined *in vitro* the cytotoxic effects of VE-465, a specific aurora kinase inhibitor, in combination with various conventional anti-leukemia agents. We found that vincristine, which is a vinca-alkaloid anti-cancer agent, potentiated the anti-proliferative effect of VE-465 by enhancement of apoptosis, resulting in effective inhibition of the growth of various myeloid leukemia cell lines as well as primary myeloid leukemia cells. In contrast to the combination of VE-465 and vincristine, however, combinations of VE-465 and most of the other anti-leukemia agents tested showed no synergistic inhibitory effect but rather had antagonistic effects on growth. Our findings suggest that combinations of an aurora kinase inhibitor and most of the DNA-damaging anti-leukemia agents, except vincristine, have little therapeutic efficacy, whereas the combination of an aurora kinase inhibitor and vincristine is a potential therapy for myeloid leukemia.

2. Materials and methods

2.1. Cell lines

BCR/ABL-positive human leukemia cell lines (KCL22, K562 and KU812) and BCR/ABL-negative human myeloid leukemia cell lines (THP-1, HL60, U937 and KY821) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and split every 4 days. Cell numbers were counted using a Cell Counting Kit-8 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in accordance with the manufacturer's instructions. On the basis of cell numbers, a dose response curve was created and the concentration that gives rise to 50% cell numbers was designated as IC_{50}.

2.2. Reagents

VE-465 was kindly provided by Merck & Co., Inc. (West Point, PA). Cytosine arabinoside, daunorubicin, idarubicin, mitoxantron, doxorubicin, vincristine and etoposide were purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Cytotoxic effects of combinations of VE-465 and conventional anti-leukemia drugs

Cytotoxic effects of the combinations of VE-465 and various conventional anti-leukemia agents were evaluated by a Steel and Peckham isobologram as described previously [17–19]. The basis of the theory and the detailed procedure of this analysis have been described in a previous report [19]. In this analysis, when the points lie outside the left margin of the envelope, the combination treatment is considered to have a synergistic inhibitory effect on cell growth. In contrast, if the points lie outside the right margin of the envelope, the combination treatment is considered to have an antagonistic effect. When the points lie within the envelope, the combination treatment is considered to have an additive effect.

2.4. Flow cytometry

Flow cytometric analysis was performed as described previously [20]. Briefly, the cells were incubated with propidium iodide for 30 min and analyzed by flow cytometry using a FACScan/CellFIT system (Becton Dickinson, San Jose, CA).

2.5. Western blot analysis

Whole cell lysates were prepared from 1×10^7 cells according to a method described previously [21]. Then 20 μg of lysates was separated electrophoretically using 10% polyacrylamide gel. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously [21]. A mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase, which was used as an internal control, was purchased from Chemicon International (Temecula, CA). Rabbit polyclonal antibodies against anti-cleaved caspase-3, anti-cleaved caspase-7, anti-cleaved caspase-9, anti-cleaved PARP, anti-Phospho-Chk2, anti-Phospho-p53 (Ser 37), anti-ERK1/2, anti-Phospho-ERK1/2, anti-STAT5, anti-Phospho-STAT5, anti-JNK/SAPK and anti-Phospho-JNK/SAPK were purchased from Cell Signaling Technology (Beverly, MA).

3. Results

3.1. Combination of VE-465 and vincristine effectively inhibited cell growth in various human leukemia cell lines

Ki values of VE-465 against Aurora-A, Aurora-B and Aurora-C were all low (1.0 nM, 26.0 nM and 8.7 nM, respectively) [22], indicating that VE-465 effectively inhibits activity of Aurora family kinases. We first examined the cytotoxic effects of VE-465 in combination with conventional anti-leukemia agents, including cytosine arabinoside, daunorubicin, idarubicin, mitoxantron, doxorubicin, vincristine and etoposide, by Steel and Peckham isobologram analysis. As shown in Table 1, IC_{50} values of VE-465 against leukemia cells are almost the same in various human leukemia cell lines. Isobolograms were then created on the basis of the results of the dose–response curves of VE-465 and various conventional anti-leukemia agents. The results of isobologram analyses are summarized in Table 2. Representative isobolograms showing the cytotoxic effects of VE-465 in combination with vincristine or cytosine arabinoside on THP-1, HL60, KY821 and KCL22 cells are shown in Fig. 1A. Among the agents tested, only vincristine showed an additive/synergistic inhibitory effect on the growth of cells when it was combined with VE-465. Combined

Table 1
IC_{50} values of VE-465 against various human myeloid leukemia cell lines.

	Cell lines						
	HL60	KY821	THP-1	U937	K562	KCL22	KU812
IC_{50} (nM)[a]	30.3 ± 8.7	40.5 ± 28.5	35.4 ± 13.0	24.9 ± 4.1	34.8 ± 22.9	33.6 ± 5.8	67.6 ± 12.5

[a] Cells were incubated in the presence of various concentrations of VE-465 for 96 h. IC_{50} values were determined from dose–response curves. Data are shown as means ± SEM of eight independent experiments.

Table 2
Results of isobologram analyses of the combinations of VE-465 and various anti-leukemia agents in human myeloid leukemia cell lines.

	THP-1	HL60	U937	KY821	KCL22	K562	KU812
Ara C	A	A	A	A	A	Add	A
DNR	A	A	A	A	A	A	Add
IDR	A	A	A	A	A	A	A
MIT	A	A	A	A	Add	A	Add
DXR	A	A	A	A	A	A	A
VCR	Add	Add	Add	Syn	Syn	Add	Add
VP16	Add	A	Add	A	A	Add	Add

Syn, synergistic effect; Add, additive effect; A, antagonistic effect; Ara-C, cytosine arabinoside; DNR, daunorubicin; IDR, idarubicin; MIT, mitoxantrone; DXR, doxorubicin; VCR, vincristine; VP-16, etoposide.

treatment with VE-465 and other conventional drugs resulted in no synergistic inhibition but rather had an antagonistic effect on cell growth.

Consistent with these results, treatment of THP-1, KY821 and KCL22 cells with the combination of VE-465 and vincristine resulted in significant inhibition of cell growth compared to the effect of VE-465 or vincristine alone (Fig. 1B). This inhibitory effect was almost the same when VE-465 or vincristine was added to the medium prior to the addition of another reagent (data not shown), suggesting that the order of addition of the reagents did not influence the combination-mediated inhibitory effect.

3.2. Induction of apoptosis in THP-1 cells by the combination of VE-465 and vincristine

To reveal the mechanisms underlying the inhibitory effect of the combination of VE-465 and vincristine on growth of leukemia cells, we performed flow cytometric analysis using THP-1 cells. When VE-465 was added to the culture medium of THP-1 cells as a single agent, the fraction of cells in G2/M-phase was significantly increased and the percentage of S-phase cells was reduced at 12 h (Fig. 2). At 48 h, however, the percentage of sub-G1 cells was increased with a decrease in the percentage of G2/M-phase cells. The same results were obtained when VE-465 was added to the culture medium of KY821 cells (data not shown). These results suggest that VE-465 initially induced blockage of the cell cycle at M-phase, which may have been caused by VE-465-mediated inhibition of aurora kinase activity, and that apoptosis of cells at G2/M arrest was subsequently induced. Although vincristine alone caused only a moderate increase in the size of the G2/M-phase fraction and had little effect on the population of sub-G1 cells, vincristine significantly enhanced the VE-465-mediated induction of the sub-G1 fraction. Such an effect of vincristine on VE465-induced apoptosis was also shown when KY821 cells were used for flow cytometric analysis (data not shown). These results thus suggest that vincristine potentiated the effect of VE-465 by enhancement of apoptosis and that this induction of apoptosis is involved in the combination-mediated growth inhibition.

3.3. Vincristine enhanced the VE-465-mediated induction of apoptosis by activation of the caspase pathway

We next examined the effects of VE-465 and vincristine on the levels of molecules related to apoptosis. When VE-465 was added as a single agent, the levels of cleaved caspase 3, cleaved caspase 7, cleaved caspase 9 and cleaved PARP were all increased in THP-1 cells (Fig. 3). In contrast, vincristine moderately increased the levels of these molecules, when compared to the effect of VE-465. Consistent with the results of flow cytometric analysis, the combination of VE-465 and vincristine significantly enhanced the increase in levels of these molecules. This combination also markedly increased the levels of cleaved caspases in KY821 cells (Fig. 3). Taken together, the results suggest that vincristine effectively enhanced the VE-465-mediated induction of apoptosis by activation of the caspase pathway.

Since Chk2 is a key molecule for regulation of the G2/M checkpoint, we examined the effect of the combination on the level of Phospho-Chk2 in THP-1 cells. As shown in Fig. 4, while the level of Phospho-Chk2 was increased by either treatment with VE-465 or vincristine, it was significantly increased by the combination at 12 h. Furthermore, the phosphorylation level of p53, which is one of the downstream molecules of Chk2, had started to increase at 12 h and was markedly increased 48 h after the start of combination treatment. When KY821 cells were used instead of THP-1 cells, the levels of Phospho-Chk2 and Phospho-p53 were also increased by the combination (Fig. 4), suggesting that the combination-induced phosphorylation of Chk2 activates the downstream signaling. These results thus suggest that Chk2-mediated activation of the G2/M checkpoint is involved in initial blockage of the cell cycle at G2/M phase, followed by the induction of apoptosis.

3.4. VE-465 induced ERK1/2 phosphorylation

Since the induction of apoptosis is also under the control of cellular signaling pathways, we also examined the effects of the combination on levels of phosphorylation forms of ERK1/2, JNK/SAPK and STAT5 using THP-1 cells. Interestingly, VE-465 alone and

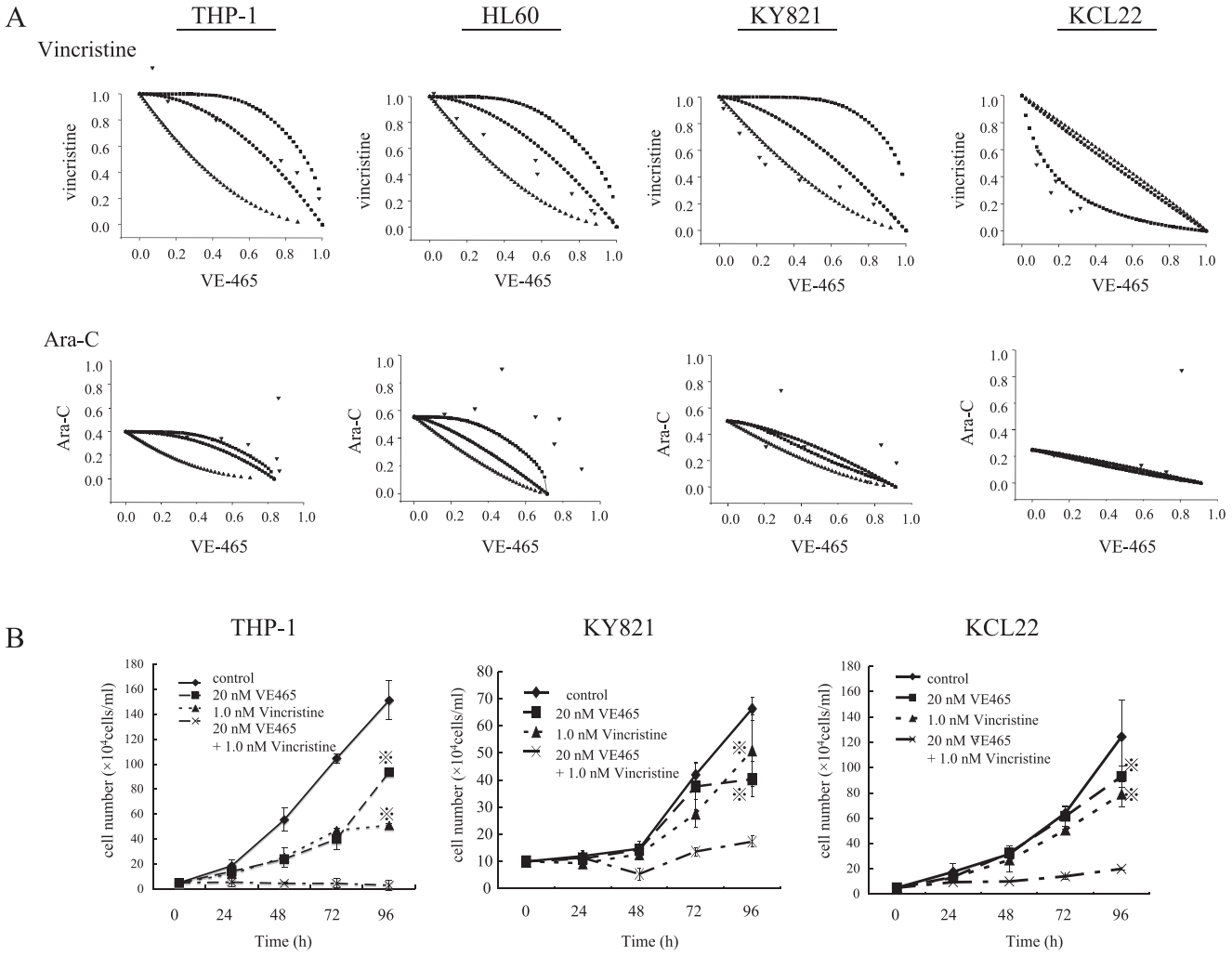

Fig. 1. Effects of VE-465 in combination with vincristine and cytosine arabinoside on the growth of THP-1, HL60, KY821 and KCL22 cells. (A) Cells were cultured with various concentrations of VE-465 for 4 days in the presence of various concentrations of vincristine or cytosine arabinoside, and the number of viable cells was then counted with a Cell Counting Kit-8. Isobolograms were created on the basis of the dose–response curves. The concentrations that produced 50% cell growth inhibition were expressed as 1.0 on the ordinate and the abscissa of the isobolograms. The combination of VE-465 and vincristine had a synergistic or additive effect, whereas the combination of VE-465 and cytosine arabinoside showed an antagonistic effect. (B) THP-1, KY821 and KCL22 cells were incubated with the indicated concentrations of VE-465, vincristine or a combination of these agents. The number of viable cells was counted at each time point using trypan blue staining. Each point represents the mean value of three independent experiments. Statistical analysis was carried out using Scheffe's test for comparison of data for cells treated with a combination of VE-465 and vincristine and cells treated with VE-465 alone and for comparison of data for cells treated with a combination of VE-465 and vincristine and cells treated with vincristine alone (asterisk denotes $P < 0.05$).

VE-465 in combination with vincristine decreased the level of Phospho-ERK1/2 at 12 h after the start of treatment (Fig. 5A). Furthermore, the combination of VE-465 and U0126, a potent MEK1/2 inhibitor, had an additive effect (Fig. 5B), indicating the possibility that down-regulation of MAPK signaling is important for VE-465 functions. In addition, the level of Phospho-JNK/SAPK was decreased by the combination as well as by either treatment alone. In contrast, single agent treatment or the combination had little effect on the levels of Phopho-STAT5. These results suggest that both VE-465 and vincristine alter a network of signaling pathways, and the possibility that these alterations are involved in either activation of the G2/M checkpoint or induction of apoptosis could not be ruled out.

3.5. VE-465 and vincristine synergistically inhibited the growth of leukemia cells from patients with acute myeloid leukemia

To clarify whether the combination effectively inhibits growth of primary leukemia cells, we next examined the effect of the combination of VE-465 and vincristine on the growth of primary leukemia cells from two patients with acute myeloid leukemia. Written informed consent for the examination was obtained from the patients. Percentages of blood blast cells at the time of collection were 80.5% and 90%, respectively. Cell culture was started immediately after collection. Five days after the start of treatment, the number of viable cells was significantly decreased when the cells were treated with the combination (Fig. 6A). Furthermore, Steel and Peckham isobologram analysis demonstrated that combined treatment of the cells with VE-465 and vincristine had a synergistic ~ additive anti-proliferative effect (Fig. 6B). Although statistical analysis could not be carried out because of the small number of repetitions of the experiments, these results suggest that the combination is also effective against primary leukemia cells.

4. Discussion

The aim of this study was to reveal the effects of an aurora kinase inhibitor in combination with various anti-leukemia agents on leukemia cells. Since VE-465 mainly targets aurora kinase, we

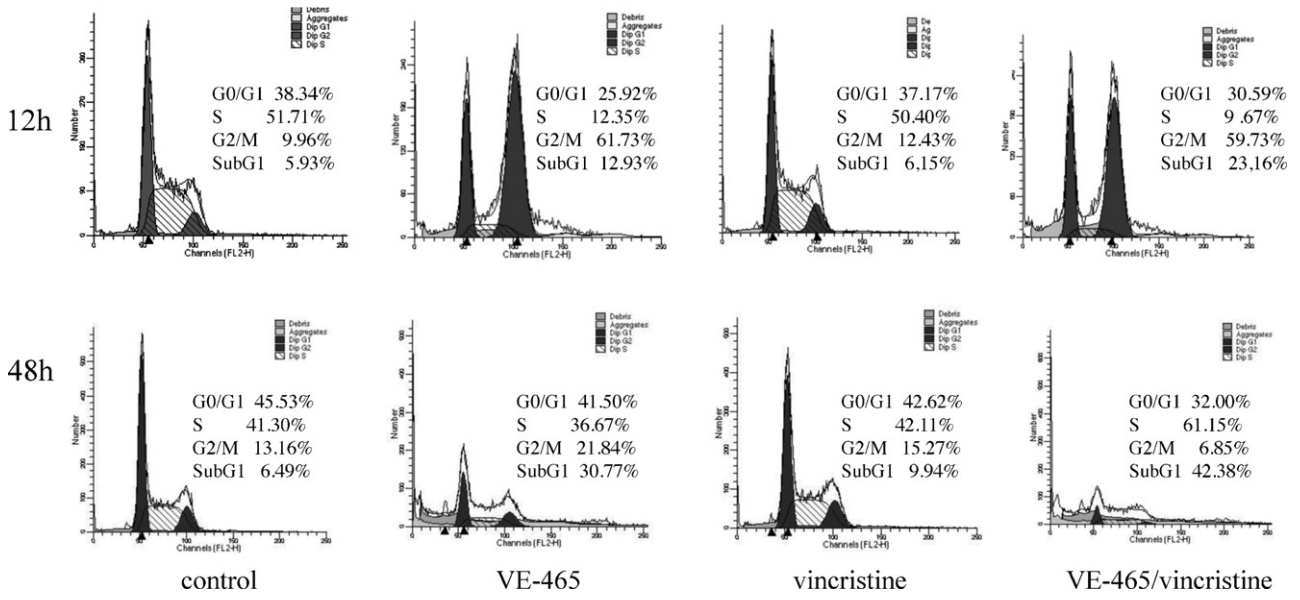

Fig. 2. Effect of the combination of VE-465 and vincristine on induction of apoptosis and blockage of the cell cycle. After THP-1 cells had been incubated for 12 and 48 h with VE-465, vincristine, or a combination of VE-465 and vincristine, the cells were harvested and incubated with propidium iodide for 30 min and analyzed by flow cytometry with a FACScan/CellFIT system (Becton Dickinson, San Jose, CA). The results shown are representative of three independent experiments.

thought that it would be a good reagent for understanding the pharmaceutical effect of aurora kinase inhibition. VE-465 alone had an inhibitory effect on growth of leukemia cell lines, consistent with the results of previous studies showing that VE-465 has anti-myeloma activity [23] and that MK-0457, another aurora kinase inhibitor, inhibits the growth of hematological malignant cells [10–13].

Against our expectation, however, the results of Steel and Peckham isobologram analysis, which provides very strict and reliable results for cytotoxic effects of combination treatments [19], showed that the combinations of VE-465 and most of the conventional anti-leukemia agents, except vincristine, had antagonistic effects on growth. Most of the DNA-damaging conventional anti-leukemia agents, including cytosine arabinoside and anthracyclins, have less effect on quiescent cells than on dividing cells. Therefore, it is likely that VE-465-mediated inhibition of cell mytosis at M-phase reduced sensitivity to these drugs. Since the two reagents are required to be added simultaneously to the medium in isobologram analysis, it would be interesting to clarify whether an alternative order of addition of the reagents influences the effect on growth.

Among conventional anti-leukemia agents, however, vincristine is an exception. The combination of VE-465 and vincristine had an additive/synergistic inhibitory effect on the growth of a variety of cell lines as well as primary leukemia cells from two patients with acute myeloid leukemia. Since vincristine is not a DNA-damaging anti-leukemia agent but inhibits mitotic division through polymerization of microtubles, it is likely that vincristine still has an effect on cells treated with VE-465. A previous study also showed that combinations of the aurora kinase inhibitor SNS-314 and mitotic spindle-targeted anti-cancer agents such as vincristine and docetaxel had synergistic effects and suggested that vincristine-mediated activation and aurora kinase inhibitor-mediated bypass of the spindle assembly checkpoint may induce apoptosis [16]. Consistent with these

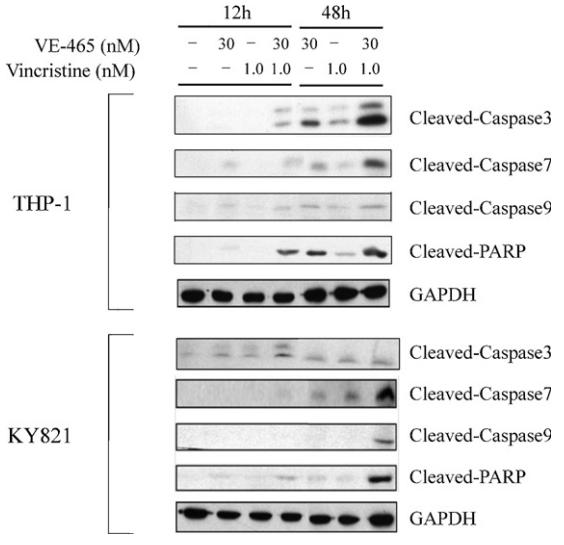

Fig. 3. Effect of the combination of VE-465 and vincristine on levels of molecules related to apoptosis. THP-1 and KY821 cells were treated with VE-465, vincristine, or a combination of VE-465 and vincristine for 12 and 48 h. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used as a control for loading.

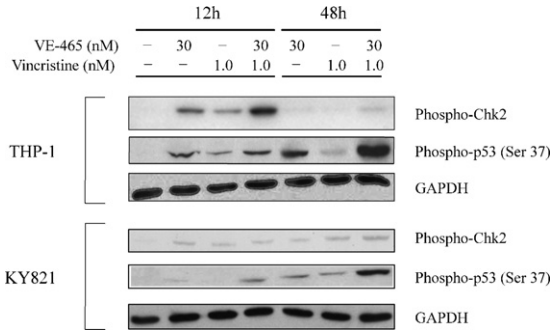

Fig. 4. Effect of the combination of VE-465 and vincristine on levels of Chk2. THP-1 and KY821 cells were cultured with VE-465, vincristine, or a combination of VE-465 and vincristine for 12 and 48 h. Total cell lysates were prepared and subjected to Western blot analysis using antibodies against Phospho-Chk2 and Phospho-p53 (Ser 37). The expression of GAPDH is shown as an internal control.

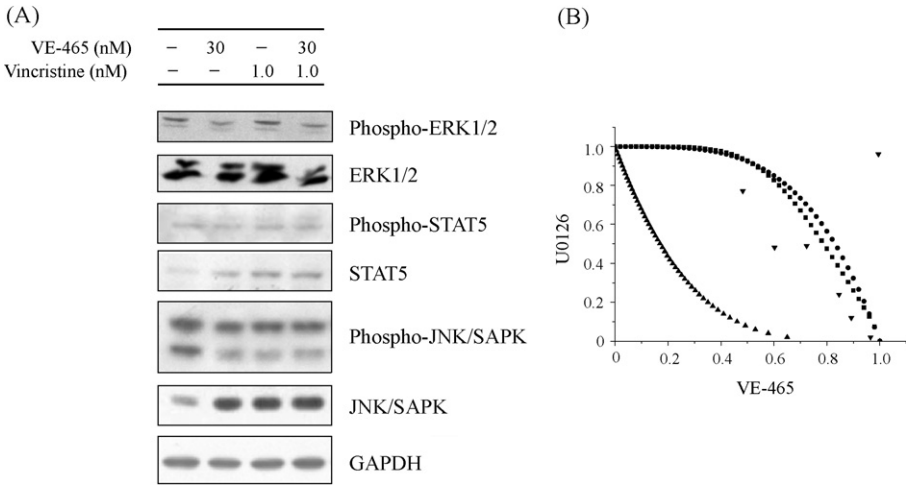

Fig. 5. Effect of the combination of VE-465 and vincristine on levels of molecules related to signaling pathways. (A) THP-1 cells were treated with VE-465, vincristine, or a combination of VE-465 and vincristine for 12 h. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. The expression of GAPDH is shown as an internal control. (B) Steel and Peckham isobologram analysis of the combination of VE-465 and U0126 in THP-1 cells was performed as described in Section 2.

findings, our results showed that vincristine markedly enhanced the effect of VE-465 on accumulation of sub-G1-phase cells. Furthermore, co-administration of these agents increased the levels of molecules related to apoptosis. These results thus suggest that VE-465-mediated inhibition of aurora kinase activity induced apoptosis after blockage of the cell cycle at M-phase and that vincristine effectively potentiated the process leading to apoptosis.

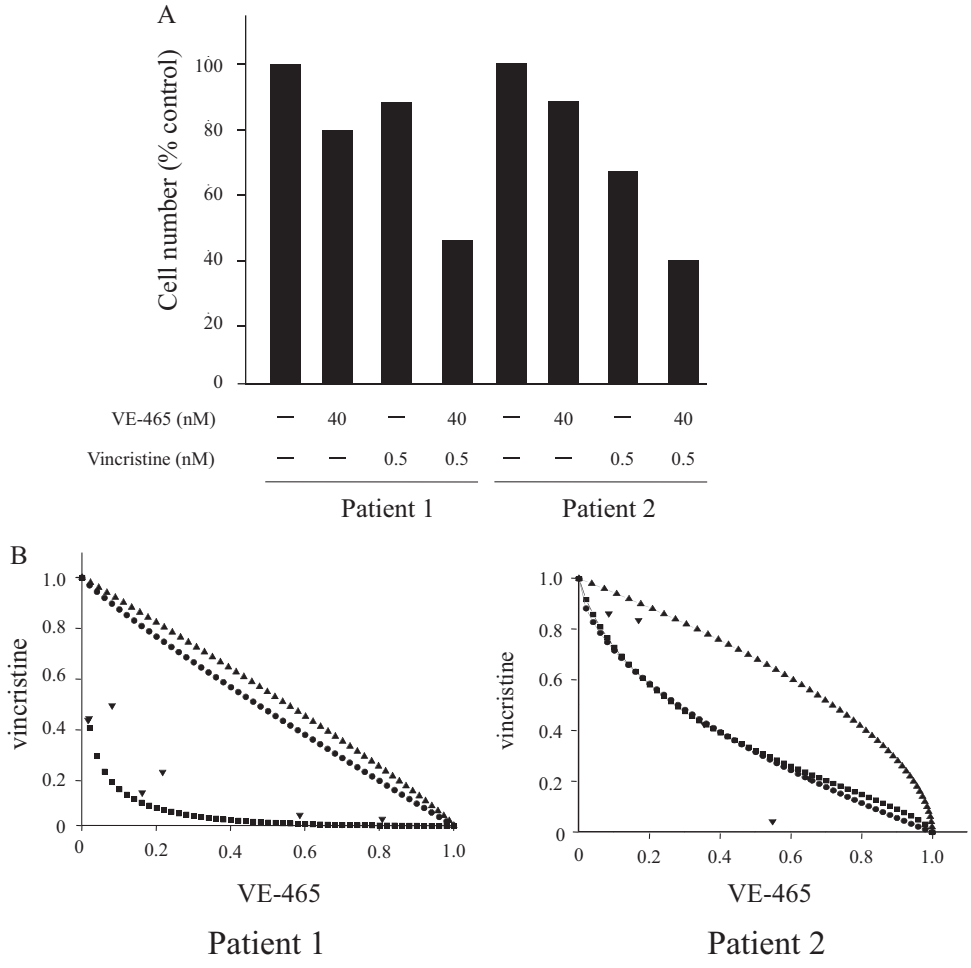

Fig. 6. Effect of the combination of VE-465 and vincristine on inhibition of growth of primary leukemia cells. (A) Primary leukemia cells from peripheral blood of two patients with acute myeloid leukemia were incubated with 40 nM VE-465, 0.5 nM vincristine, or a combination of 40 nM VE-465 and 0.5 nM vincristine for 5 days. The number of viable cells was counted using trypan blue staining. The results are expressed as percentage of viable treated cells to viable non-treated cells. (B) Steel and Peckham isobologram analyses of the combination of VE-465 and vincristine in primary leukemia cells were performed as described in Section 2.

Our results showed that both VE-465 and vincristine also influenced activities of signaling pathways. Treatment of cells with VE-465 alone and VE-465 in combination with vincristine resulted in a decrease in the level of Phospho-ERK1/2. Furthermore, Steel and Peckham isobologram analysis demonstrated that the combination of VE-465 and U0126, a potent MEK1/2 inhibitor, had an additive effect (Fig. 5B). It is thus possible that down-regulation of MAPK signaling is involved in induction of blockage of the cell cycle and apoptosis in cells treated with VE-465. In addition, the level of Phospho-JNK/SAPK was decreased by treatment with either VE-465 or vincristine alone. Several previous studies suggested that induction of G2 arrest was associated with JNK activation [24–26]. However, Liu et al. showed that inhibition of p38 MAPK resulted in attenuation of lidamycin-induced G2 arrest with increase in the level of JNK phosphorylation [27]. It is thus possible that the effect of JNK on activity of the cell cycle checkpoint is altered due to the difference in cell types or difference in causes of the cell cycle blockage. It would be interesting to clarify whether VE-465 or vincristine-mediated suppression of JNK activity is involved in activation of the G2/M checkpoint in myeloid leukemia cells.

In conclusion, our findings suggest that co-administration of VE-465 and most of the conventional anti-leukemia agents has little clinical value for the treatment of leukemia. However, vincristine effectively enhanced the anti-leukemia effect of VE-465, indicating the utility of the combination of VE-465 and vincristine as a potential therapy for myeloid leukemia. We did not use lymphoid leukemia cells in this study. Since vincristine is frequently used for treatment of lymphoid malignancies, it would be interesting to clarify whether this combination also shows a synergistic ~ additive inhibitory effect on the growth of acute lymphoblastic leukemia cells. Such efforts are now being made in our laboratory.

Acknowledgments

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