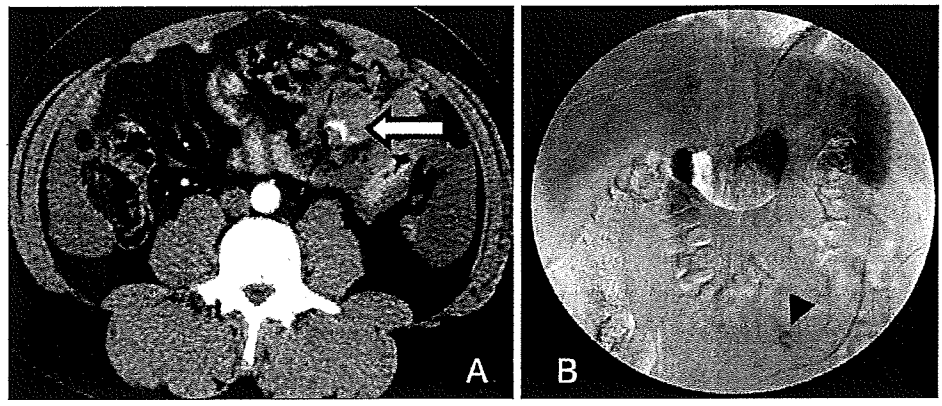

Fig. 1 **a** Computed tomography scanning with intravenous contrast detected hematoma in greater omentum and pseudovessel (*arrow*), which showed active bleeding. **b** Angiography showed extravascular leakage of the contrast medium from a branch of the left gastroepiploic artery (*arrow head*)

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Combination of tipifarnib and rapamycin synergistically inhibits the growth of leukemia cells and overcomes resistance to tipifarnib via alteration of cellular signaling pathways

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ABSTRACT

Small molecules are attractive agents for the treatment of leukemia. We found that a combination of a farnesyltransferase inhibitor, tipifarnib, and an mTOR inhibitor, rapamycin, synergistically inhibited the growth of myeloid leukemia cell lines and primary leukemia cells by inducing apoptosis and cell-cycle blockage. The combined agents reduced the level of phospho-ERK1/2, suggesting that they altered the network of signaling pathways. They also showed synergistic effects in tipifarnib-resistant K562/RR cells. The results support the utility of this combination as a potential therapy for leukemia. The combination might also be effective in overcoming resistance to tipifarnib.

1. Introduction

Recent studies have revealed that deregulation of a variety of cellular signaling pathways and of the expression of genes that control cell proliferation, survival, apoptosis, and differentiation is involved in the development of leukemia. Recently, many kinds of small molecules aimed at restoring the regulation of these pathways have been developed and studied at preclinical or clinical levels for use in the treatment of leukemia [1]. The most successful examples of this strategy are trans-retinoic acid and BCR/ABL kinase inhibitors, including imatinib, nilotinib, and dasatinib, which show substantial clinical effects against acute promyelocytic leukemia and BCR/ABL-positive leukemia, respectively [2]. Unfortunately, many other small molecules have shown only limited efficacy for the treatment of leukemia in clinical studies, possibly because the accumulation of a variety of cellular abnormalities is involved in the development and progression of leukemia [3]; thus, treatment of leukemia with a single agent of small molecule weight can hardly overcome all of these abnormalities. There is, therefore, a need to develop effective combination therapies for clinical application.

Tipifarnib (Zarnestra™, Johnson & Johnson Pharmaceutical Research & Development, Titusville, NJ), a farnesyltransferase inhibitor, has been shown to inhibit the growth of tumor cells including leukemia cells *in vitro* [4]. Some cellular proteins require farnesylation to become active, and it is thought that tipifarnib suppresses the function of some oncogenic proteins by inhibiting farnesylation and thus, inhibiting abnormally activated signaling pathways. However, the results of clinical studies have shown that tipifarnib alone had only a moderate effect against various hematologic disorders [4–11]. In addition, acquisition of drug resistance is an important consideration in patients being treated with tipifarnib. In light of this, the development of efficacious combination therapies involving tipifarnib and other agents appears to be an attractive approach for making good use of tipifarnib in the treatment of leukemia. Indeed, previous studies have shown that combination therapy with tipifarnib and certain other drugs had synergistic or additive inhibitory effects on the growth of leukemia cells [12,13].

In this study, we examined *in vitro* the cytotoxic effects of tipifarnib in combination with three agents of small molecular weight – rapamycin (an inhibitor of the mammalian target of rapamycin [mTOR]), LY294002 (an inhibitor of PI3-kinase), and U0126 (an inhibitor of MEK1/2) – in efforts to identify effective therapeutic combinations. We found that the combination of tipifarnib and rapamycin, which has also been shown to inhibit the growth of leukemia cells [14,15], had a synergistic inhibitory effect on the growth of BCR/ABL-negative leukemia cell lines as well as BCR/ABL-positive cell lines. Importantly, this combination also showed

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synergistic antiproliferative effects in tipifarnib-resistant K562/RR cells. These findings suggest that the combination of tipifarnib and rapamycin is a potential therapy for various types of leukemia and is also effective for overcoming resistance to tipifarnib.

2. Materials and methods

2.1. Cell lines

K562, KCL22, and KU812 are BCR/ABL-positive cell lines established from the peripheral blood of patients with chronic myelogenous leukemia (CML) in blast crisis [16–18]. K562/RR is a tipifarnib-resistant cell line cloned from K562 in our laboratory [19]. U937 and THP-1 are BCR/ABL-negative human myeloid leukemia cell lines [20,21]. These cells were grown in RPMI-1640 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.

2.2. Reagents

Tipifarnib was kindly provided by Johnson & Johnson Pharmaceutical Research & Development (Titusville, NJ). Rapamycin, LY294002, and U0126 were purchased from Sigma Chemical Co. (St. Louis, MO).

2.3. Cytotoxic effects of combinations of tipifarnib and other small molecules

Cells were incubated with various concentrations of agents for 4 days, and cell numbers were then counted using a Cell Counting Kit-8. The cytotoxic effects of the combinations of tipifarnib and other compounds of small molecular weight were

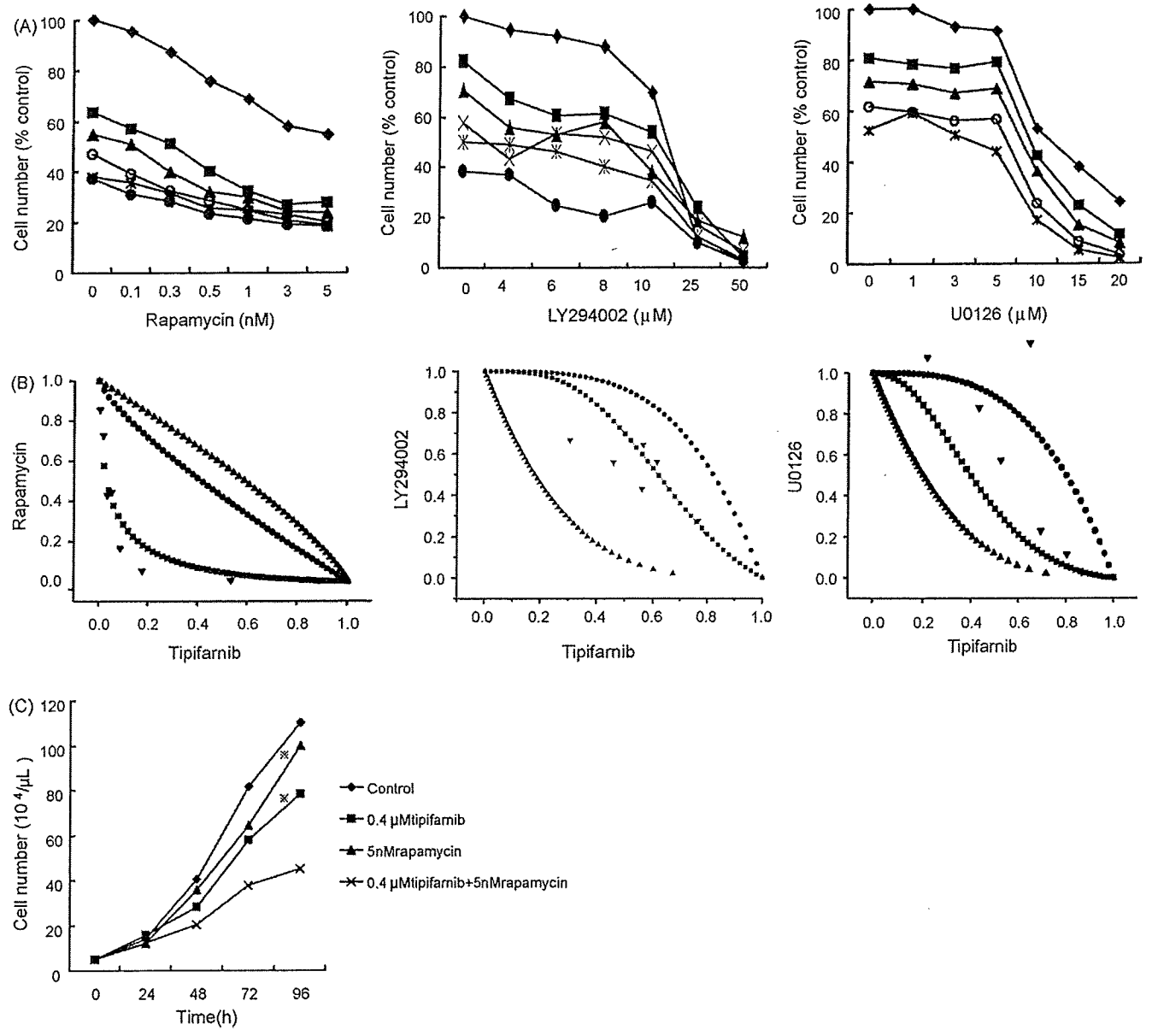

Fig. 1. Inhibitory effects of tipifarnib in combination with other agents on growth of K562 cells. (A) Dose–response curves of tipifarnib in combination with rapamycin, LY294002, and U0126. Cells were cultured with various concentrations of tipifarnib for 4 days in the presence of the indicated concentrations of each agent, and the number of viable cells was then counted with a Cell Counting Kit-8. The concentrations of tipifarnib were as follows: 0 μM (♦); 0.2 μM (■); 0.4 μM (▲); 0.6 μM (○); 0.8 μM (×); 1.0 μM (●) for rapamycin; 0 μM (♦); 0.2 μM (■); 0.4 μM (▲); 0.5 μM (○); 0.6 μM (×); 1.0 μM (●) for LY294002; and 0 μM (♦); 0.05 μM (■); 0.10 μM (▲); 0.25 μM (○); 0.50 μM (×) for U0126. (B) Steel and Peckham isobologram analyses of the combinations of tipifarnib with rapamycin, LY294002, and U0126 in K562 cells were performed as described in Section 2. The combination of tipifarnib and rapamycin showed a synergistic effect, whereas other combinations showed only additive effects. (C) K562 cells were incubated with 0.4 μM tipifarnib, 5 nM rapamycin, or with a combination of 0.4 μM tipifarnib and 5 nM rapamycin. 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 the data between cells treated with a combination of tipifarnib and rapamycin and cells treated with tipifarnib alone, and between cells treated with a combination of tipifarnib and rapamycin and cells treated with rapamycin alone (Asterisk denotes $P < 0.05$).

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evaluated by Steel and Peckham isobolograms as described previously [22]. 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. If the points lie within the envelope, the combination treatment is considered to have an additive effect.

2.4. Western blot analysis

Nuclear extracts were prepared from 1×10^7 cells according to a method described previously [23]. Then, 10 μg of nuclear extract was separated electrophoretically using a 10% polyacrylamide gel. Immunoblotting and detection by enhanced chemiluminescence were performed as described previously [23]. 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 caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, PARP, cleaved PARP, cytochrome c, phospho-cdc2, Chk2, phospho-Chk2, Mcl-1, BCR, phospho-BCR, phospho-CrkL, p44/42 (ERK1/2) MAP kinase, phospho-p44/42 (ERK1/2) MAP kinase, JNK, phospho-JNK, STAT5, and phospho-STAT5 as well as mouse monoclonal antibodies against CDK4, cyclin D1, and cyclin D3 were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-$p27^{KIP1}$ monoclonal antibody was purchased from BD Biosciences (San Jose, CA).

2.5. Flow cytometry

Flow cytometric analysis was performed as described previously [19]. Briefly, the cells were incubated with propidium iodide for 30 min and analyzed by flow cytometry with a FACScan/CellFIT system (Becton Dickinson, San Jose, CA).

3. Results

3.1. Combined treatment of K562 cells with tipifarnib and rapamycin resulted in synergistic inhibition of cell growth

To examine the cytotoxic effects of tipifarnib in combination with other agents of small molecular weight, including rapamycin, LY294002, and U0126, Steel and Peckham isobologram analysis was performed using K562 cells. The IC_{50} values of tipifarnib, rapamycin, LY294002, and U0126 against K562 cells are 0.4 μM, 5 nM, 12 μM, and 13 μM, respectively. The dose–response curves for tipifarnib in combination with those small molecules are shown in Fig. 1A. Isobolograms were then created on the basis of the results of the dose–response curves. As shown in Fig. 1B, all points for a combination of tipifarnib and rapamycin lay outside the left margin of the envelope, indicating a clear synergistic inhibitory effect on the growth of the cells. Consistent with these results, combined treatment of K562 cells with the IC_{50} concentrations of tipifarnib (0.4 μM) and rapamycin (5 nM) resulted in more significant inhibition of growth than did either tipifarnib or rapamycin alone (Fig. 1C). In contrast, combinations of tipifarnib and LY294002 or tipifarnib and U0126 showed no synergistic inhibition but had an additive effect on the growth of the cells (Fig. 1B). These results indicate that although combinations of tipifarnib and all three other small molecules enhanced the cytotoxic effect, rapamycin was the most effective partner of tipifarnib for combination therapy.

3.2. Induction of apoptosis and cell-cycle blockage in K562 cells by the combination of tipifarnib and rapamycin

We next performed flow cytometry analysis to clarify whether induction of apoptosis and blockage of the cell cycle are involved in the synergistic inhibition of cell growth (Fig. 2). When tipifarnib was added to K562 cells as a single agent, the percentage of sub-G1 cells was increased, whereas that of G0/G1 cells was unchanged. In contrast, rapamycin alone increased the percentage of G0/G1 cells, which is consistent with the results of a previous study showing that rapamycin induces G0/G1 arrest [14], with a decrease in the percentage of sub-G1 cells. Combined treatment of K562 cells with tipifarnib and rapamycin also resulted in an increase in the percentage of G0/G1 cells at 24 h ($P = 0.028$). Furthermore, the combination showed a tendency toward induction of sub-G1 cells, although this effect was not significant ($P = 0.197$ and 0.226 at 24 h and 48 h, respectively). Therefore, it is likely that the synergistic inhibition

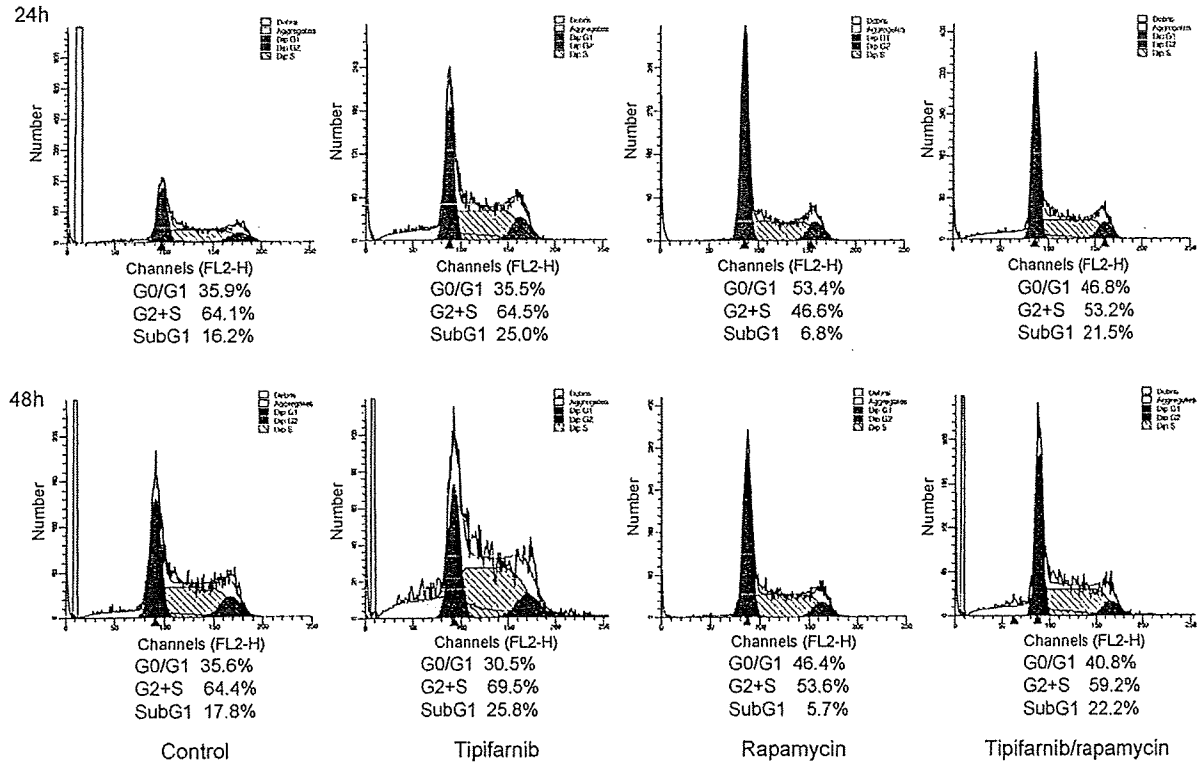

Fig. 2. Effect of the combination of tipifarnib and rapamycin on the induction of apoptosis and cell-cycle blockage. After cells were incubated for 24 and 48 h with tipifarnib, rapamycin, or a combination of tipifarnib and rapamycin, the cells were harvested and incubated with propidium iodide for 30 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.

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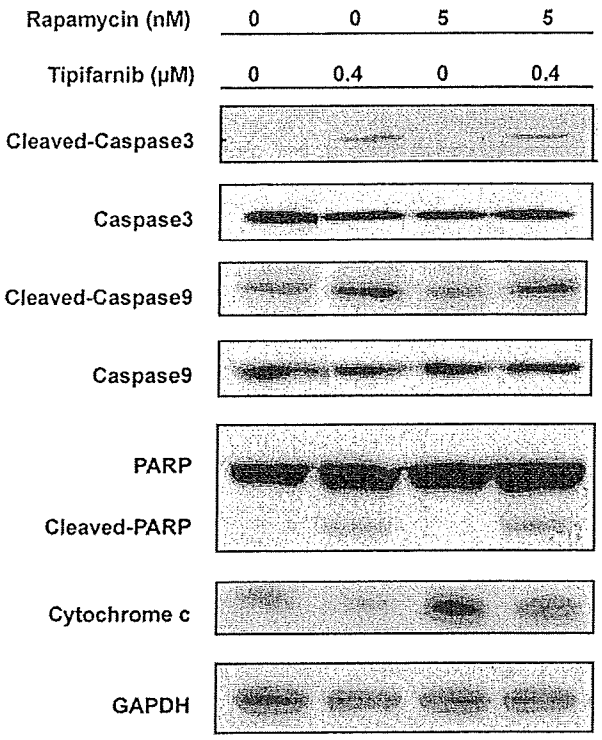

Fig. 3. Effect of the combination of tipifarnib and rapamycin on the levels of molecules related to apoptosis. Cells were treated with tipifarnib, rapamycin, or a combination of tipifarnib and rapamycin for 24 h. Total cell lysates were prepared and subjected to Western blot analysis using antibodies against cleaved caspase-3, caspase-3, cleaved caspase-9, caspase-9, cleaved PARP, and PARP. Mitochondrial lysates were prepared and subjected to Western blot analysis using anti-cytochrome c antibody. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as an internal control.

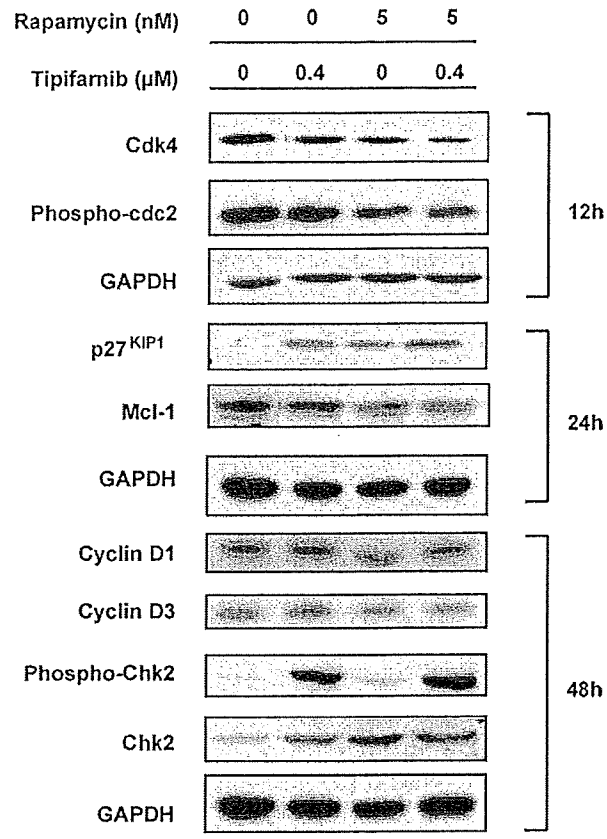

Fig. 4. Effect of the combination of tipifarnib and rapamycin on the levels of molecules related to the cell cycle. Cells were cultured with tipifarnib, rapamycin, or a combination of tipifarnib and rapamycin for the indicated times. Total cell lysates were prepared and subjected to Western blot analysis using indicated antibodies. The expression of GAPDH is shown as an internal control.

of growth mediated by the combination involved both apoptosis and blockage of the cell cycle.

3.3. Effect of the combination of tipifarnib and rapamycin on the levels of molecules related to apoptosis

We then examined the effect of the combination of tipifarnib and rapamycin on the levels of molecules related to apoptosis. The combination induced cleaved caspase-3, cleaved caspase-9, and cleaved PARP to levels similar to those induced when tipifarnib was added as a single agent (Fig. 3). In contrast, rapamycin alone had no effect on the levels of these molecules. The level of cytochrome c in a mitochondrial fraction was somewhat increased by rapamycin alone but was decreased when tipifarnib was added as a single agent or in combination with rapamycin. These results suggest that either tipifarnib alone or the combination of tipifarnib and rapamycin induced apoptosis by the release of cytochrome c from mitochondria with subsequent activation of the caspase pathway.

3.4. Effect of the combination of tipifarnib and rapamycin on the levels of molecules related to the cell cycle

The combination of tipifarnib and rapamycin also influenced the levels of molecules involved in the regulation of the cell cycle. When rapamycin was added to K562 cells as a single agent, a notable increase in the level of p27 and slight reductions in the levels of cdk4, phospho-cdc2, Mcl-1, and cyclin D3 were observed (Fig. 4). Tipifarnib alone also increased the level of p27, but it reduced the level of cdk4 to a lesser extent and had no effect on the levels of phospho-cdc2, Mcl-1, and cyclin D3. Importantly, the combination of tipifarnib and rapamycin increased the level of p27 and decreased the level of cdk4 to greater extents than did tipifarnib or rapamycin alone (Fig. 4). Taken together, these results suggest that tipifarnib contributed primarily to the induction of apoptosis and influenced cell-cycle regulation to a lesser extent, whereas rapamycin was mainly involved in blockage of the cell cycle in the cells treated with the combination. In this case, neither reagent reduced the effect of the other. Interestingly, the level of phospho-Chk2 was significantly increased by tipifarnib alone or in the combination, whereas it was not changed with rapamycin alone. These results suggest that the blockage of the cell cycle induced by rapamycin was not mediated by activation of Chk2 (Fig. 4).

3.5. Combination of tipifarnib and rapamycin inhibited STAT5, JNK, and ERK1/2 activities

We then examined the effect of the combination of tipifarnib and rapamycin on cellular signaling pathways. As shown in Fig. 5, tipifarnib alone and tipifarnib in combination with rapamycin markedly reduced the level of phospho-STAT5. The level of phospho-STAT5 was also decreased by treatment with rapamycin at 6 h, but this effect was modest and transient. In contrast, rapamycin induced a higher level of phospho-JNK/SAPK at 12 h. Therefore, suppression of STAT5 activity might be involved in the tipifarnib-mediated induction of apoptosis, whereas activation of the JNK-SAPK pathway might play a role in the blockage of the cell cycle induced by rapamycin.

Interestingly, treatment of the cells with a combination of tipifarnib and rapamycin resulted in pronounced reduction of the

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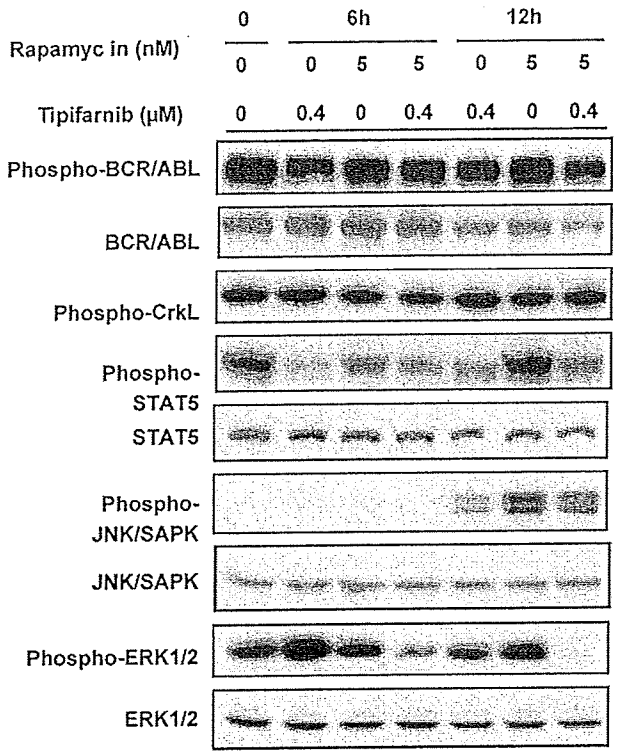

Fig. 5. Effect of the combination of tipifarnib and rapamycin on the levels of molecules related to signaling pathways. Cells were treated with tipifarnib, rapamycin, or a combination of tipifarnib and rapamycin and harvested at each time point. Total cell lysates were prepared and subjected to Western blot analysis using the indicated antibodies.

phospho-ERK1/2 level from 6 h to 12 h after the start of treatment, whereas the level of phospho-ERK1/2 was increased transiently and slightly by tipifarnib alone and was not changed when rapamycin was added as a single agent. Therefore, it is possible that a potent suppression of ERK1/2 activity is involved in the synergy displayed by the combination. In contrast, the combination had no effect on the levels of phospho-BCR/ABL and phospho-CrkL, which is a substrate of BCR/ABL, suggesting that the combination had no effect on BCR/ABL activity.

3.6. Combination of tipifarnib and rapamycin synergistically inhibited growth of various types of leukemia cells

Drug resistance is a serious problem for patients being treated with agents of small molecular weight such as tipifarnib. It was, therefore, important to reveal whether the combination treatment also effectively inhibited the growth of tipifarnib-resistant cells. To address this question, we examined the effect of the combination on growth of the tipifarnib-resistant cell line K562/RR, which was recently cloned from K562 in our laboratory [19], by isobologram analysis. Combined treatment of K562/RR cells with tipifarnib and rapamycin resulted in synergistic inhibition of growth with the accumulation of both G0/G1 phase and sub-G1 phase cells (Fig. 6A, data not shown). In contrast, tipifarnib in combination with LY294002 or U0126 merely additively inhibited growth of K562/RR cells (Fig. 6A). We further examined the cytotoxic effect of the combination in the other human BCR/ABL-positive leukemia cell lines KCL22 and KU812 as well as in the human BCR/ABL-negative leukemia cell lines U937 and THP-1. Fig. 6B shows that the combination of tipifarnib and rapamycin also synergistically inhibited the growth of all cell lines examined. These results suggest that the effect of the combination of tipifarnib and rapamycin is not restricted to K562 cells but that this combination is effective for various types of leukemia cells, including tipifarnib-resistant cells, regardless of the presence or absence of BCR/ABL protein.

3.7. Tipifarnib and rapamycin effectively inhibited the growth of leukemia cells from a patient with acute myeloid leukemia

To clarify whether the combination effectively inhibits growth of primary leukemia cells, we next examined the cytotoxic effect of combined treatment using primary leukemia cells from the peripheral blood of a patient with acute myeloid leukemia. Written informed consent for the examination was obtained from the patient. As shown in Fig. 7, the combination of tipifarnib and rapamycin enhanced the inhibition of growth in the primary

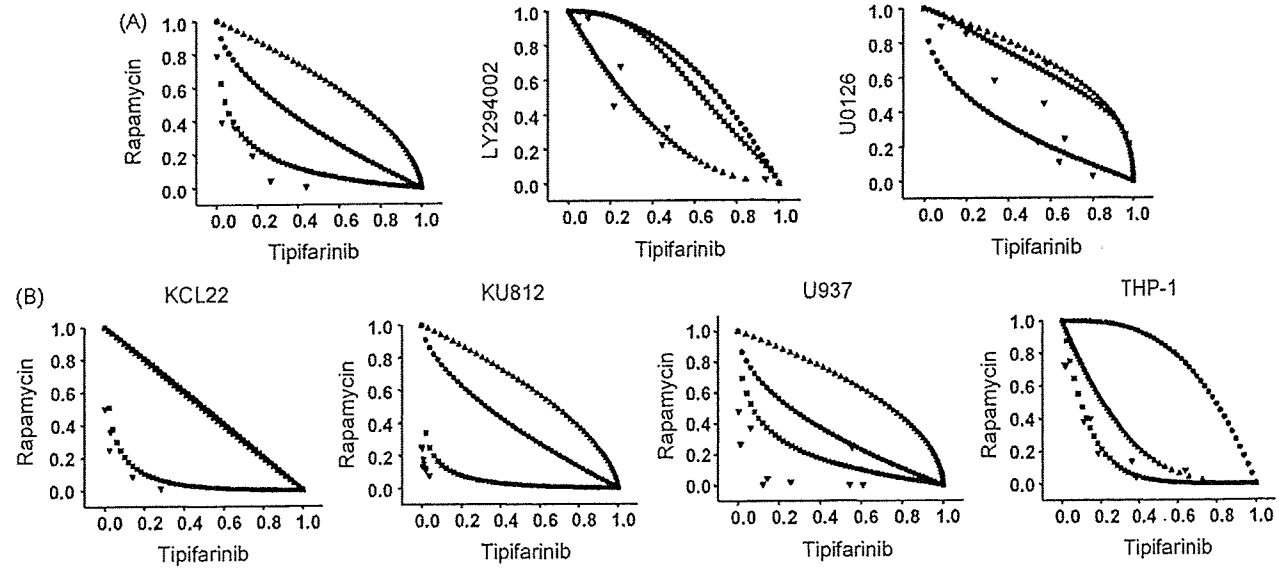

Fig. 6. Cytotoxic effect of tipifarnib in combination with rapamycin in various leukemia cell lines. (A) Steel and Peckham isobologram analyses of the combinations of tipifarnib with rapamycin, LY294002, and U0126 in tipifarnib-resistant K562/RR cells were performed as described in Section 2. The combination of tipifarnib and rapamycin showed a synergistic effect, whereas other combinations showed only an additive effect. (B) Steel and Peckham isobologram analyses of the effects of the combination of tipifarnib and rapamycin in BCR/ABL-positive KCL22 and KU812 cells and BCR/ABL-negative U937 and THP-1 cells were performed as described in Section 2. Most points lie within the area representing synergistic effects.

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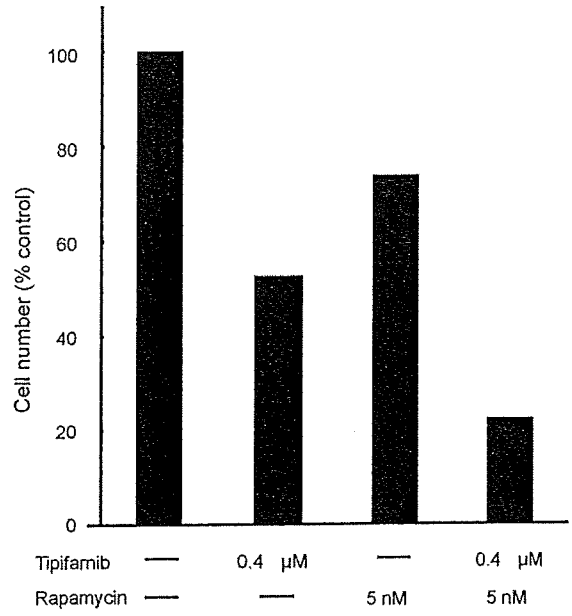

Fig. 7. Effect of the combination of tipifarnib and rapamycin on the inhibition of growth of primary leukemia cells. Primary leukemia cells from the peripheral blood of a patient with acute myeloid leukemia were incubated with 0.4 μM tipifarnib, 5 nM rapamycin, or with a combination of 0.4 μM tipifarnib and 5 nM rapamycin for 5 days. The number of viable cells was counted by trypan blue staining. Percentage of cell number is shown.

leukemia cells, suggesting that the combination is also effective against primary leukemia cells.

4. Discussion

Development of therapeutic strategies using small molecular weight agents is very attractive for the treatment of hematologic malignancies. However, mono-therapy with these agents occasionally shows limited clinical efficacy. One possible approach for making good use of these agents is the establishment of effective combination therapies. For this purpose, we examined the effect of the farnesyltransferase inhibitor tipifarnib in combination with other therapeutic agents of small molecular weight on the growth of leukemia cells.

It has been shown that Steel and Peckham isobologram analysis provides stringent and reliable results for the cytotoxic effects of combination treatments [22]. Using this analysis, we found that the combination of tipifarnib and rapamycin synergistically inhibited the growth of leukemia cells. Notably, this synergistic effect was also observed in tipifarnib-resistant K562/RR cells, which are likely to acquire resistance by farnesyltransferase activity-independent mechanisms, such as continuous activation of signaling pathways [19]. Because acquisition of drug resistance is frequently found in patients treated with small molecules, it was important to show the potential to overcome resistance to tipifarnib with this combination. Acquisition of resistance to tipifarnib might also be caused by other mechanisms, such as inability to block farnesylation. Indeed, it has been shown that insertion of mutations in the farnesyltransferase β gene resulted in the acquisition of resistance to tipifarnib [24], although patients whose disease is resistant to tipifarinib because of mutations in the farnesyltransferase gene have not yet been described. Recently, Raponi et al. [25] found by analysis of gene expression profiles from patients treated with tipifarnib that a low ratio of the expression level of RASGRP1/APTX predicts a poor response to tipifarnib. It is therefore of interest to clarify whether the combination of tipifarnib and rapamycin is also effective in AML cells for which such a poor response is predicted. Unlike the combination with rapamycin, combinations of tipifarnib with other agents, including LY294002 and U0126, had additive inhibitory effects on growth, suggesting that rapamycin is a particularly good combination partner for tipifarnib.

It is likely that tipifarnib and rapamycin contributed to the inhibition of leukemia cell growth in different manners. Our results demonstrate that tipifarnib was mainly involved in the induction of apoptosis, which is consistent with the results of previous studies showing that tipifarnib induces apoptosis in hematologic malignant cells [26,27]. In contrast, rapamycin was involved in blockage of the cell cycle and slightly blocked spontaneous apoptosis. These results are consistent with the results of a previous study showing that rapamycin inhibits the growth of acute myeloid leukemia cells by blocking the cell cycle in G0/G1 phase [14].

Clarification of the molecular mechanisms of the effects of each agent is of great interest. Administration of tipifarnib alone, rapamycin alone, and co-administration of these agents all increased the level of phospho-JNK (Fig. 5). It is, therefore, possible that up-regulation of JNK activity is involved in the induction of cell-cycle blockage in K562 cells treated with rapamycin or tipifarnib. Because rapamycin has been shown to reduce JNK activity in several cell types [28–30], it is likely that rapamycin has distinct effects on JNK signaling, depending on the type of cell. In support of this hypothesis, there has been a study demonstrating that rapamycin potentiates the cytotoxicity of UCN-01, which is a Chk1 and protein kinase C inhibitor, accompanied by phosphorylation of JNK [31]. In contrast, the level of phospho-STAT5 was significantly reduced by tipifarnib alone or by tipifarnib in combination with rapamycin (Fig. 5). Although the direct target proteins through which tipifarnib mediates tumor suppression remain to be identified, our results suggest that tipifarnib affects the activities of molecules related to cellular signaling, including STAT5 and JNK.

We hypothesize that co-administration of tipifarnib and rapamycin alters a network of cellular signaling pathways. In support of this hypothesis, the combination markedly reduced the level of phospho-ERK1/2 (Fig. 5). The fact that enforced expression of MEK1, an upstream serine-threonine kinase of ERK1/2, in KCL22 cells resulted in no abrogation of the combination-mediated synergistic inhibitory effect on growth (data not shown) and the fact that the combination of tipifarnib and the MEK1/2 inhibitor U0126 showed no synergistic effect (Fig. 1) suggest that ERK1/2 activity was not the only target involved in the synergy induced by the combination. However, it is possible that alteration of the signaling pathway network mediated by the combination is required for developing the synergy. On the other hand, tipifarnib did not enhance the rapamycin-mediated inhibition of phosphorylation of 4EBP1 and p70 S6, which are downstream molecules of the mTOR pathway (data not shown). Although it has been reported that the farnesyltransferase inhibitor lonafarnib inhibits mTOR signaling [32], our results suggest that tipifarnib had no effect on the inhibition of mTOR function mediated by rapamycin and that mTOR activity was not related to the synergistic effect of tipifarnib and rapamycin.

In conclusion, the findings in this study demonstrated that the combinations of tipifarnib and rapamycin, LY294002, or U0126 have advantages for the treatment of leukemia. In particular, the combination of tipifarnib and rapamycin synergistically inhibited the growth of leukemia cells, including tipifarnib-resistant cells, by induction of apoptosis as well as blockage of the cell cycle. These findings indicate the potential clinical application of combination therapies with tipifarnib.

Conflict of interest

None

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