Combination of SK-7041, one of novel histone deacetylase inhibitors, and STI571-induced synergistic apoptosis in chronic myeloid leukemia

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Although STI571 still plays a key role in the treatment of chronic myeloid leukemia, emergence of resistance to STI571 is a major obstacle to successful outcome. Therefore, new agents that increase the sensitivity of chronic myeloid leukemia cells to STI571 are urgently required. SK-7041 is a novel hybrid synthetic histone deacetylase inhibitor derived from the hydroxamic acid of trichostatin A and pyridyl ring of MS-275. Its cytotoxic effects were examined both as a single agent and in combination with STI571 in acute and chronic myeloid leukemia. SK-7041 exhibited growth inhibition of leukemia cells by downregulation of CDK4, cyclin E and cyclin B1 expression, and by upregulation of $p21^{CIP1=WAF1}$ expression with subsequent activation of the mitochondria-mediated caspase pathway. SK-7041 showed synergism on growth inhibition, cell cycle arrest and induction of apoptosis in chronic myeloid leukemia (K562) when combined with STI571. The synergistic effect was mediated through the same mechanism as in SK-7041 alone, involving reduction of cyclin D1 and induction of $p21^{CIP1=WAF1}$. Taken together, our findings suggest that SK-7041 is active against leukemia and offers new prospects for overcoming STI571 resistance in chronic myeloid leukemia. *Anti-Cancer Drugs* **18:641–647 © 2007 Lippincott Williams & Wilkins.**

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Introduction

STI571 (glivec, gleevec, imatinib) is a well-known inhibitor of Bcr/Abl, the oncogenic tyrosine kinase that causes chronic myeloid leukemia [1,2]. STI571 achieved an unprecedented 96% of hematologic responses and 76% of complete and major cytogenetic remissions after a median follow-up of 18 months in chronic-phase chronic myeloid leukemia (CML) patients. A small percentage of these patients and most patients in advanced phase (accelerated phase and blast crisis), however, relapsed on STI571 therapy [3,4]. Therefore, novel therapies are required to overcome STI571 resistance. One approach is the combination of STI571 with additional agents such as conventional anticancer agents, histone deacetylase (HDAC) inhibitors and proteasome inhibitors [5–8].

Histone acetylation status is reciprocally regulated by the activities of HDACs and histone acetylases. HDAC inhibitors induce acetylation of several histones (e.g. H3 and H4), events that favor uncoiling and relaxation of the chromatin structure [9]. Chromatin relaxation, in turn, permits the expression of diverse genes, including those involved in the differentiation process (e.g. $p21^{CIP1=WAF1}$) [10]. Even though many HDAC inhibitors have been developed including trichostatin A, MS-275, valproic acid and suberoylanilide hydroxamic acid, most HDAC inhibitors have the drawbacks of instability and low bioavailability. A great demand to develop more selective and less-toxic HDAC inhibitor derivatives exists. SK-7041 is a novel hydroxamic acid-based HDAC inhibitor developed for the purpose of enhancing the pharmaceutical properties and the enzyme selectivity [11].

HDAC inhibitors have been shown to induce terminal differentiation of Bcr/Abl-positive leukemic cell lines by a mechanism that involves the downmodulation of mitogen-activated protein kinase [12]. This suggests that both Bcr/Abl and HDAC inhibition may show synergism in CML cells (K562). In this study, we investigated the

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cellular and molecular effects of SK-7041 alone in acute and chronic leukemia as well as in combination with STI571 on K562 human chronic myeloid leukemia cells *in vitro*.

Materials and methods

Cell line and reagent

Human leukemia cell lines, K562 (CML), HEL (acute erythroleukemia) and HL-60 (acute promyelocytic leukemia) were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Roswell Park Memorial Institute 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin/streptomycin. Cell lines were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. All experiments were performed using cells in logarithmic growth phase. SK-7041 was dissolved in sterile dimethyl sulfoxide at a final concentration of 10 μmol/l and STI571 was dissolved in sterile phosphate-buffered saline (PBS) at a final concentration of 3.2 mmol/l.

Growth inhibition assay

Growth inhibition of leukemia cells by SK-7041 was examined using cell counting kit-8 (DOJINDO Laboratories, Kumamoto, Japan). Briefly, 1×10^4 cells of K562, HL-60 and HEL cells, respectively, were seeded in each well of 96-well microtiter plates. After 24 h, the medium was replaced with medium containing various concentrations of SK-7041. After 24, 48 and 72 h of incubation, respectively, 10 μl of cell counting kit solution was added and incubated for 4 h in a CO_2 incubator. The absorbance of each well was measured in a microplate reader (Becton Dickinson Labware, Le Pont de Claix, France) at 450 nm. Means and standard deviations were generated from three independent experiments. Absorbance values were normalized to the values obtained from the control group to determine the value for percentage of survival. Each assay was performed in triplicate.

Cell cycle analysis

K562, HEL and HL-60 cells were treated with 1 μmol/l of SK-7041 for 6, 18 and 24 h, respectively. Then, the cells were washed and fixed in 4% paraformaldehyde until analysis. After overnight incubation at 20°C, cells were washed with PBS (without Ca^{2+} or Mg^{2+}) before staining with propidium iodide (PI), and then suspended in staining buffer (10 μg/ml PI; 0.5% Tween 20; 0.1% RNase in PBS). The stained cells were analyzed in the Flow Cytometry Core at the Cancer Research Institute, Seoul National University College of Medicine.

Immunoblotting

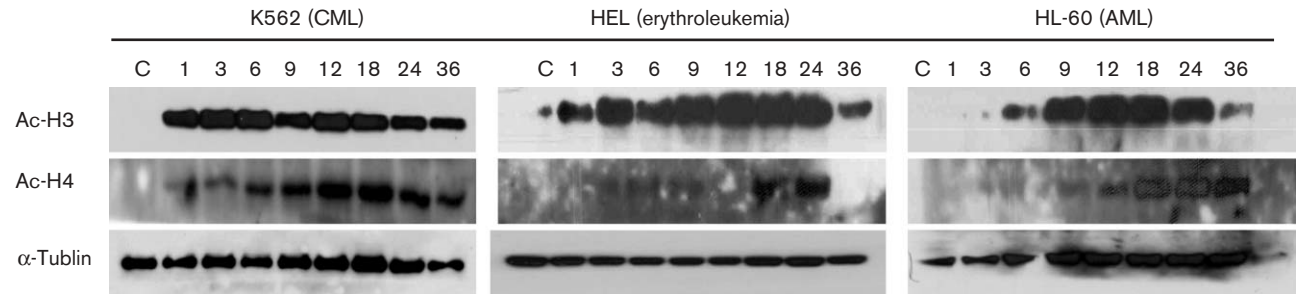
Immunoblot analysis was performed in cultured cells treated with SK-7041, STI571 or a combination of both. Cultured cells were washed twice in ice-cold PBS and pelleted by centrifugation at 12 500 r.p.m. for 10 min. Cell pellets were incubated in lysis buffer (20 mmol/l Tris–HCl, pH 8.0, 137 mmol/l NaCl, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 10 mmol/l ethylenediaminetetraacetic acid, 10 μg/ml leupeptin, 100 mmol/l NaF, 2 mmol/l Na_3VO_4 and 1% NP-40) at 4°C for 20 min. Insoluble fractions were removed by centrifugation at 12 500 r.p.m. for 20 min and the supernatants were frozen at −70°C until used. Protein concentration of the lysate was measured by bicinchoninic acid protein assay reagent (Pierce, Rockford, Illinois, USA). Equal amounts of protein were resolved in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). Membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk for 1 h at room temperature and incubated with appropriate primary antibody for 2 h. Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Following antibodies were used in this study; Ac-H3, Ac-H4 (Upstate, Lake Placid, New York, USA), proliferation cell nuclear antigen (PCNA), cytochrome *c*, caspase-3, poly(ADP ribose) polymerase (PARP), cyclin D1, cyclin B1, CDK4, cyclin E, CDK2, cdc2 (Santa Cruz, Santa Cruz, California, USA) and α-tubulin (Sigma, St Louis, Missouri, USA).

Result

SK-7041 as a single agent in acute and chronic leukemia

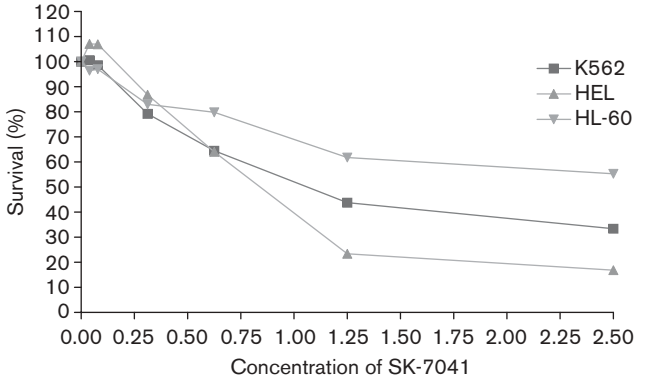
To examine the ability of SK-7041 to inhibit HDAC in K562 (chronic myeloid leukemia blast crisis), HEL and HL-60 (acute promyelocytic leukemia) cells, we performed immunoblotting to determine intracellular levels of acetylated H3 and H4. SK-7041-induced hyperacetylation of H3 and H4 in a time-dependent manner until 18 h, and then the expression level slowly decreased over 24–36 h (Fig. 1). Growth inhibition assay was performed in three different leukemia cell lines. HEL cells were most significantly inhibited when treated with 1.25 μmol/l of SK-7041 at 72 h (75% growth inhibition), and percentages of growth inhibition in K562 and HL-60 cells were 50 and 35%, respectively (Fig. 2). Even though the effect of SK-7041 was different on three different leukemia cell lines, growth inhibition by SK-7041 was dose-dependent in all the three cell lines. We quantified apoptosis by evaluating the sub-G_1 fraction of the K562, HEL, and HL60 cell populations with PI staining of permeabilized cells. Proportion of the cells at the sub-G_1 phase from K562, HEL, and HL-60 were 48.66, 38.95 and 19% at 24 h, respectively (Fig. 3). Like in the growth inhibition assay, SK-7041 caused a gradually increasing sub-G_1 fraction regardless of leukemia cell lines. Subsequently we investigated the apoptosis-related gene

Fig. 1



Effect of SK-7041 on the expression patterns of acetylation of histones H3 and H4 in leukemia cell lines. Thirty micrograms of whole-cell lysate were obtained from three different leukemia cells (K562, HEL and HL-60 cells) treated with 1 μmol/l of SK-7041 for various times for determining acetyl H3 and H4 using Western blot. α-Tubulin was used as a loading control. The results are representative of duplicate experiments.

Fig. 2



Effect of SK-7041 on proliferation of human leukemia cell lines *in vitro*. The effect of SK-7041 on the proliferation of leukemia cell lines was determined by cell counting kit-8. Logarithmically growing K562, HEL and HL-60 cells were exposed to the indicated concentrations of SK-7041 for 72 h. Cell viability was determined by measuring the absorbance. The results shown are representative of at least three independent experiments.

expression after treating cells with 1 μmol/l of SK-7041. Induction of cytochrome *c*, indicating the release of cytochrome *c* from the mitochondria, was detected in K562, HEL and HL-60. Intracellular levels of pro-PARP and procaspase-3 gradually decreased time dependently. PCNA expression level remained suppressed with the treatment of SK-7041 (Fig. 4). As the presence of functional cyclin/CDK complex is required for the cell cycle progression and dysfunction of cyclins is strongly associated with the growth of leukemia cells, we examined cell cycle-related proteins. The expression of CDK4 was dramatically decreased, whereas $p21^{CIP1=WAF1}$ expression was increased; however, SK-7041 did not affect the expression of cyclin E, CDK2, cyclin B1, and cdc2. (Fig. 5).

SK-7041 combined with STI571 in chronic leukemia

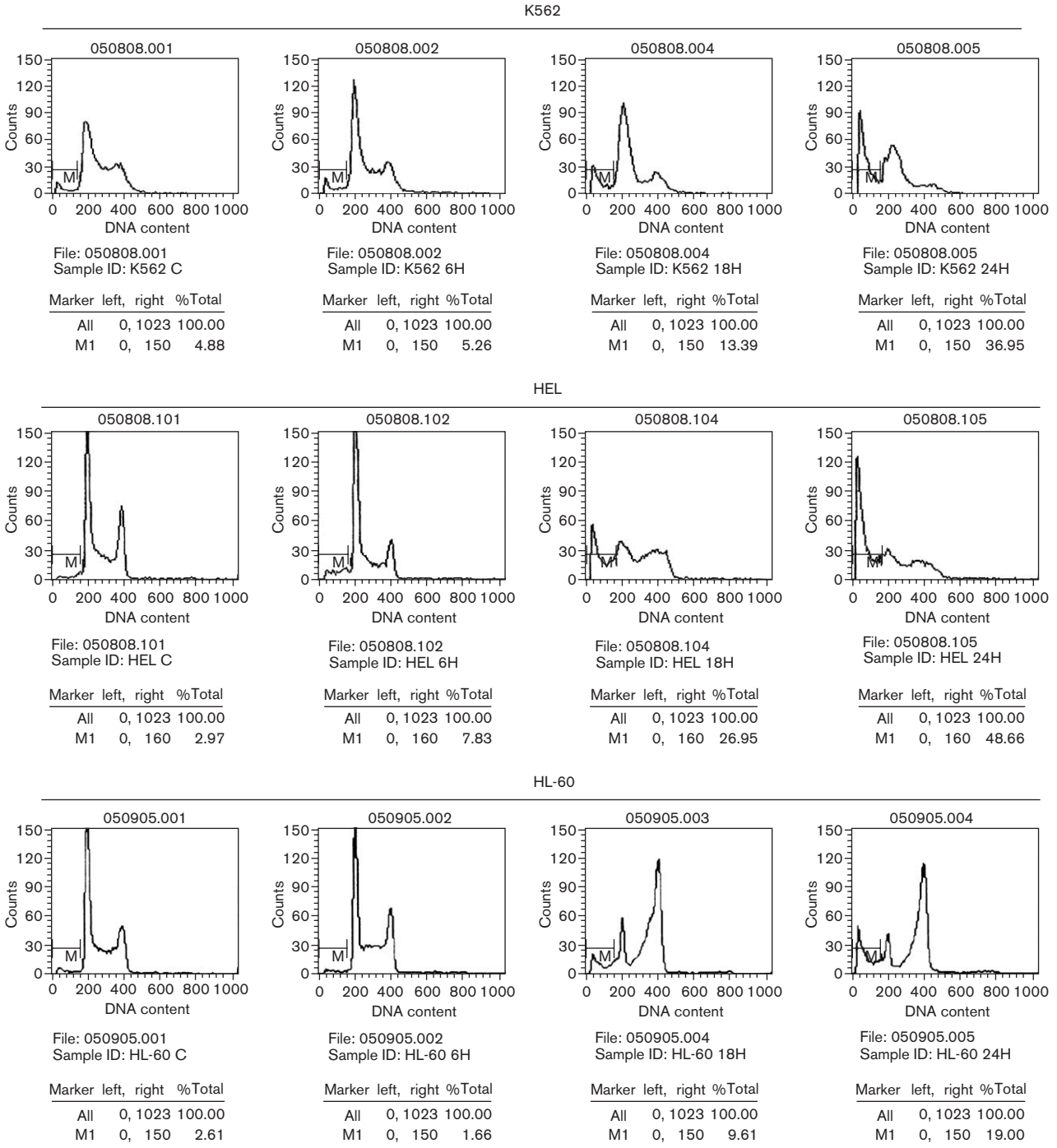
Exposure of cells to STI571 at 160 nmol/l for 24 h negligibly induced apoptosis, and 0.2 μmol/l SK-7041 alone was also minimally toxic. When the cells were exposed to 0.2 μmol/l SK-7041 in combination with 160 nmol/l STI571, a significant growth inhibition was, however, observed at 72 h incubation time (Fig. 6). Apoptosis was increased when the cells were exposed to combined treatment as indicated by the increased sub-G_1 DNA content compared with control (Table 1). Then, we focused on the changes of molecular effectors of apoptosis and cell cycle arrest with combined treatment. Combined treatment activated caspase-3 and PARP, as noted by the presence of proteolytic fragments, whereas their activation was negligible with either STI571 or SK-7041 alone. Expression of $p21^{CIP1=WAF1}$ dramatically increased when the cells were treated with the combination of STI571 and SK-7041. The expression of cyclin D1 and CDK4 was decreased with combined treatment but not with either treatment alone (Fig. 7).

Discussion

The emergence of drug resistance continues to be a major obstacle to the successful treatment of cancer patients. Novel agents or drug combinations that overcome this problem are eagerly sought. A growing body of evidence now exists that in-vitro treatment of human chronic myeloid leukemia cells with STI571 either alone or with HDAC inhibitors is effective in inhibiting proliferation and inducing apoptosis. HDACs are recognized as a promising target for new potent and nontoxic selective HDAC inhibitors. Many researchers are focusing on derivatives from several HDAC inhibitors. SK-7041 synthesized from hydroxamic acid of trichostatin A and pyridyl ring of MS-275 was used in this study [11].

Our data suggested that SK-7041 effectively inhibited the growth of leukemia cells via deregulation of histone

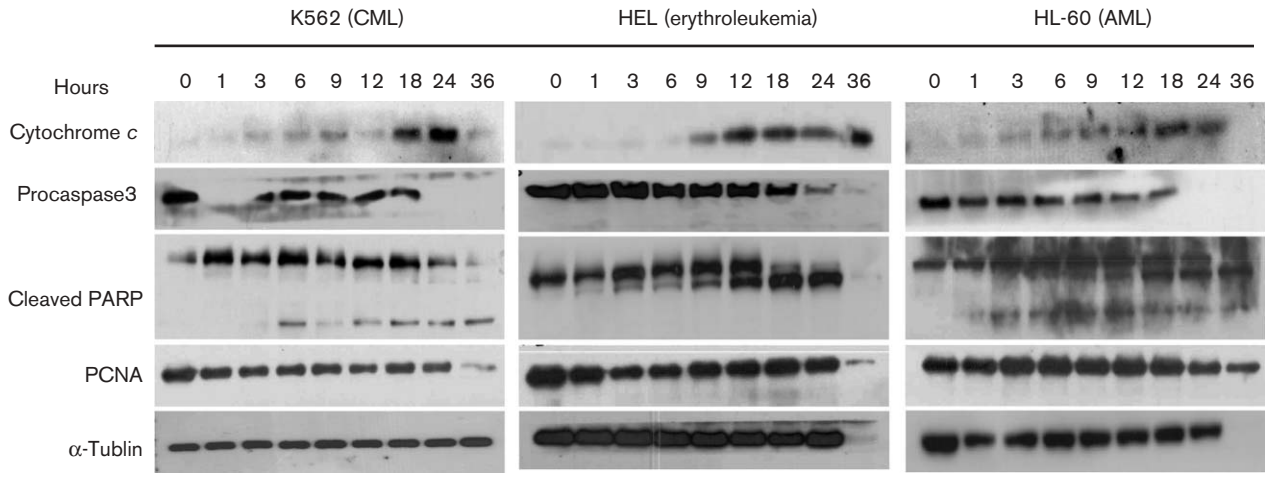
Fig. 3



Effect of SK-7041 on cell cycle. After K562, HEL and HL-60 cells were treated with 1 μmol/l SK-7041, the cells were harvested and fixed with 4% paraformaldehyde. Fixed cells were stained with propidium iodine and were analyzed for the sub-G_1 fraction using flow cytometric analysis. The results shown are representative of at least three independent experiments.

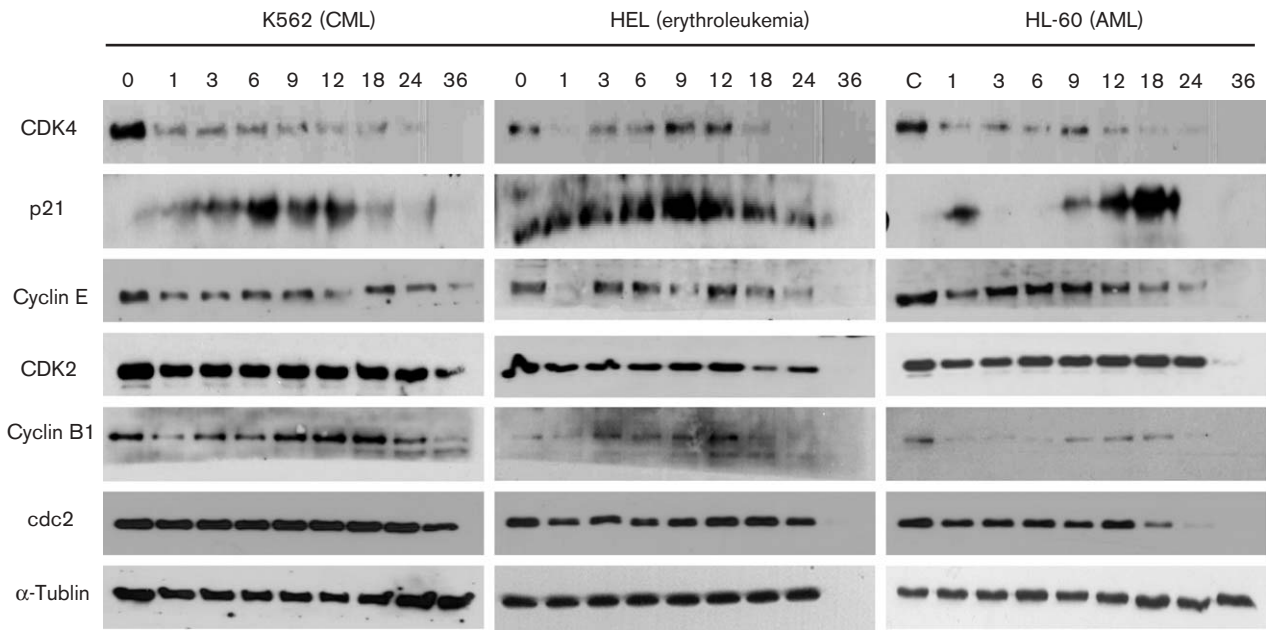
deacetylation. Our results showed that SK-7041-induced release of cytochrome *c* from mitochondria, activation of PARP, and activation of caspase-3. Previous studies suggested that HDAC inhibitors in STI571-resistant cells synergistically induced apoptosis [5,6]. Our data also showed that combined treatment of SK-7041 and STI571 exerted synergistic effects on apoptosis. On the basis of our data, the enhanced ability of the combined

Fig. 4



Effect of SK-7041 on expression levels of apoptosis-related proteins. Thirty micrograms of whole-cell lysate were obtained from three different leukemia cells (K562, HEL and HL-60) treated with 1 μmol/l SK-7041 for various times for determining cytochrome *c*, caspase-3, PARP and PCNA using Western blot. α-Tubulin was used as a loading control. The results are representative of duplicate experiments.

Fig. 5

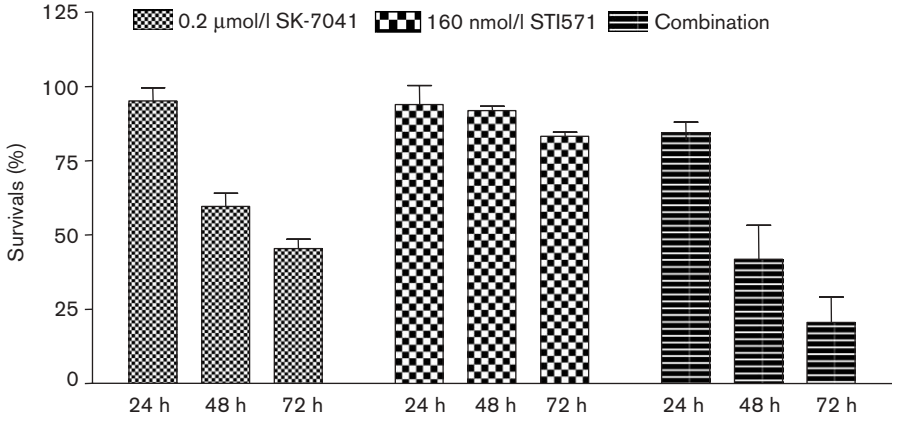


Effect of SK-7041 on expression levels of cell cycle-related proteins. Thirty micrograms of whole-cell lysate were obtained from three different leukemia cells (K562, HEL and HL-60 cells) treated with 1 μmol/l of SK-7041 for various times, and used for determining CDK4, cyclin E, CDK2, cyclin B1, cdc2, and $p21^{CIP1=WAF1}$ using Western blot. α-Tubulin was used as a loading control. The results are representative of duplicate experiments.

treatment to trigger apoptosis in K562 cells may result from interference with the actions of Bcr/Abl downstream antiapoptotic genes or, alternatively, from deregulation of signaling/cell cycle-regulatory pathways by induction of $p21^{CIP1=WAF1}$.

Among the CDKs that regulate the cell cycle, CDK2, CDK4 and CDK6 are activated in association with the D-type cyclins or cyclin E during the G_1 progression and the G_1–S transition. The reduced kinase activities of CDK4 could be accompanied by the underphosphorylation of

Fig. 6



SK-7041 combined with STI571 inhibited the growth of K562 cells. K562 cells were treated with the indicated concentrations of SK-7041, STI571 or the combination for 24 h. Cell death was determined by cell counting kit-8. The results shown are representative of at least three independent experiments.

Table 1 Cell cycle analysis of SK-7041, STI571 or a combination of SK-7041 and STI571 in K562 cell

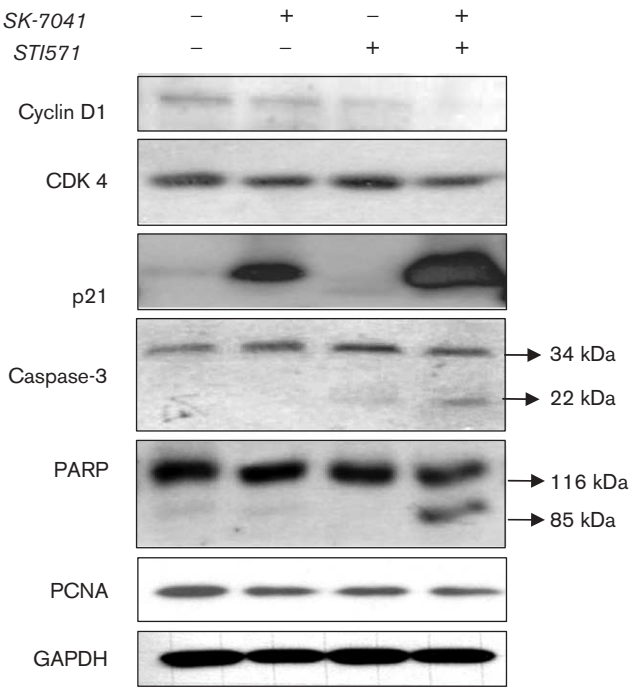
	Control (%)	SK-7041 (0.2 μmol/l) (%)	STI571 (160 nmol/l) (%)	Combination (%)
12 h	1.43	20.90	23.15	21.40
24 h	1.80	39.27	22.54	51.90
48 h	1.18	38.98	25.71	45.84

K562 cells were exposed to 160 nmol/l STI571, 0.2 μmol/l SK-7041, or the combination for 12, 24 and 48 h. After the indicated treatment times, cells were fixed with 4% paraformaldehyde, stained with propidium iodide and subjected to flow cytometric analysis. The percentage of sub-G_1 phase was calculated from the DNA content histograms.

the Rb protein, which is known to sequester the transcription factor, E2F, thereby preventing the cells from further entering the cell cycle progression [12]. Increasing $p21^{CIP1=WAF1}$ expression could affect the phosphorylation of CDK4. The blocking at G_1 from entry into the S-phase appears to be mediated by the down-regulation of the CDK4-associated kinase activity in association with the induction of $p21^{CIP1=WAF1}$ [13,14]. With combined treatment in our experiment, the expression levels of cyclin D1 and CDK4 were lower when compared with either treatment alone.

Deregulation of the $p21^{CIP1=WAF1}$ plays a critical role in synergistic interactions between STI571 and SK-7041 in the K562 cell line. The observations that acetylation of histones by SK-7041 activates the $p21^{CIP1=WAF1}$ promoter suggested that this $p21^{CIP1=WAF1}$ plays a critical role in SK-7041-mediated apoptosis. Taken together, treatment of STI571 combined with SK-7041 resulted in a dramatic increase in mitochondrial damage and apoptosis in K562 cells, accompanied by upregulation of p21 and downregulation of cell cycle-related proteins (cyclin D1 and CDK4). For further study, we are investigating mitogen-activated protein kinase and peroxisome proliferator activated receptor-γ which is involved in the control of cellular proliferation and differentiation.

Fig. 7



Modulation of the expression of cell cycle regulators by SK-7041, STI571, or the combination for 24 h. K562 cells were treated with 0.2 μmol/l SK-7041 and 160 nmol/l STI571, or the combination for 24 h. Modulation of protein expression patterns was analyzed by Western blotting. The results shown are representative of two independent experiments.

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