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Depsipeptide Enhances Imatinib Mesylate-induced Apoptosis of Bcr-Abl-positive Cells and Ectopic Expression of Cyclin D1, c-Myc or Active MEK Abrogates this Effect

TAKESHI KAWANO[1], JUNKO HORIGUCHI-YAMADA[2], SATSUKI IWASE[1], MASAHARU AKIYAMA[1], YUSUKE FURUKAWA[3], YASUHIKO KANO[4] and HISASHI YAMADA[1]

[1]*Department of Molecular Genetics and*
[2]*Department of Oncology, Institute of DNA Medicine, Jikei University School of Medicine, Tokyo 105-8461;*
[3]*Division of Molecular Hematopoiesis and Molecular Biology, Jichi Medical School, Tochigi;*
[4]*Division of Hematology and Medical Oncology, Tochigi Cancer Center, Tochigi, Japan*

Abstract. *Background: Imatinib mesylate (STI571) is the first-line drug for chronic myeloid leukemia (CML), but development of resistance to this drug is a clinical problem. To explore the effective use of STI571, we studied the combination treatment with histone deacetylase inhibitor (depsipeptide, FK228). Materials and Methods: FK228 and trichostatin A (TSA) were studied with respect to apoptosis of two Bcr-Abl-positive cell lines, K562 and TCC-S. Genetically-modified K562 cells by any of cyclin D1, c-Myc and active MEK genes were also studied. Apoptosis was examined by nuclear-morphology under a fluorescent microscope and by the expression of annexin V. Changes of apoptosis-regulating genes and acetylated histone H4 were studied by immunoblot. Results: FK228 showed cytotoxicity at the nano-molar level. Combination treatment with STI571 and FK228 enhanced the induction of apoptosis significantly compared with each single treatment, although the histone acetylation level was not changed by the co-treatment. The combination treatment activated caspase-3 and cleaved PARP, but it did not induce any notable change in the expression of Bcl-X_L, Bcl-2 and Bax compared with each single treatment. Enhanced apoptosis by the co-treatment was abrogated by ectopic expression of cyclin D1, c-Myc or active MEK. Conclusion: The combination of FK228 with STI571 is a promising treatment for Bcr-Abl-positive CML, but the activation of the MEK/ERK pathway and its downstream target genes may bring resistance to the co-treatment in leukemic cells.*

Correspondence to: Hisashi Yamada, MD, Department of Molecular Genetics, Institute of DNA Medicine, Jikei University School of Medicine, Nishi-Shinbashi 3-25-8, Minato-ku, Tokyo 105-8461, Japan. Fax: 81-3-3435-1922, e-mail: hyamad@jikei.ac.jp

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Chronic myeloid leukemia (CML) is a disease caused by the Bcr-Abl fusion gene, which is generated by the t(9;22)(q34;q11) chromosomal translocation (1,2). Treatment of CML was advanced by the introduction of type I interferons and hydroxyurea, but the response rate to such agents is still unsatisfactory (3). Imatinib mesylate (STI571) is designed to inhibit Bcr-Abl tyrosine kinase activity (4). STI571 binds to Bcr-Abl-protein at the ATP-binding site, so that the kinase activity of Bcr-Abl is blocked specifically. Although tyrosine kinases, c-Abl, c-Kit and PDGF-receptor are inhibited by STI571, the outcome of CML treatment in the chronic phase is satisfactory without the occurrence of serious adverse effects (5). However, the development of resistance to STI571 has been demonstrated (6) and its efficacy is lower for CML in blastic crisis and Philadelphia chromosome-positive acute lymphoblastic leukemia (7).

Modulators of the chromatin structure have been attracting attention as potent anticancer agents (8-10). Transcription and replication are largely regulated by the acetylation and deacetylation of histones (11). Histone deacetylase inhibitors (HDACIs) block deacetylation of the lysine residues of histones and have been shown to inhibit cell proliferation, as well as inducing the differentiation and apoptosis of malignant cells (8-10). Therefore, HDACIs seem to be a promising treatment for solid tumors and hematological malignancies. Tricostatin A (TSA), derived from *Streptomyces* sp., was shown to induce apoptosis and differentiation by promoting cell cycle arrest at both G1- and G2/M-phases (12,13). One of the HDACIs, depsipeptide (FK228), structurally unrelated to TSA, also induces apoptosis in several tumor cell lines (14). Interestingly, the response regarding cell cycle and apoptosis of cells treated by FK228 seems to differ according to the raf/MEK/ERK activation status of the treated cells (15).

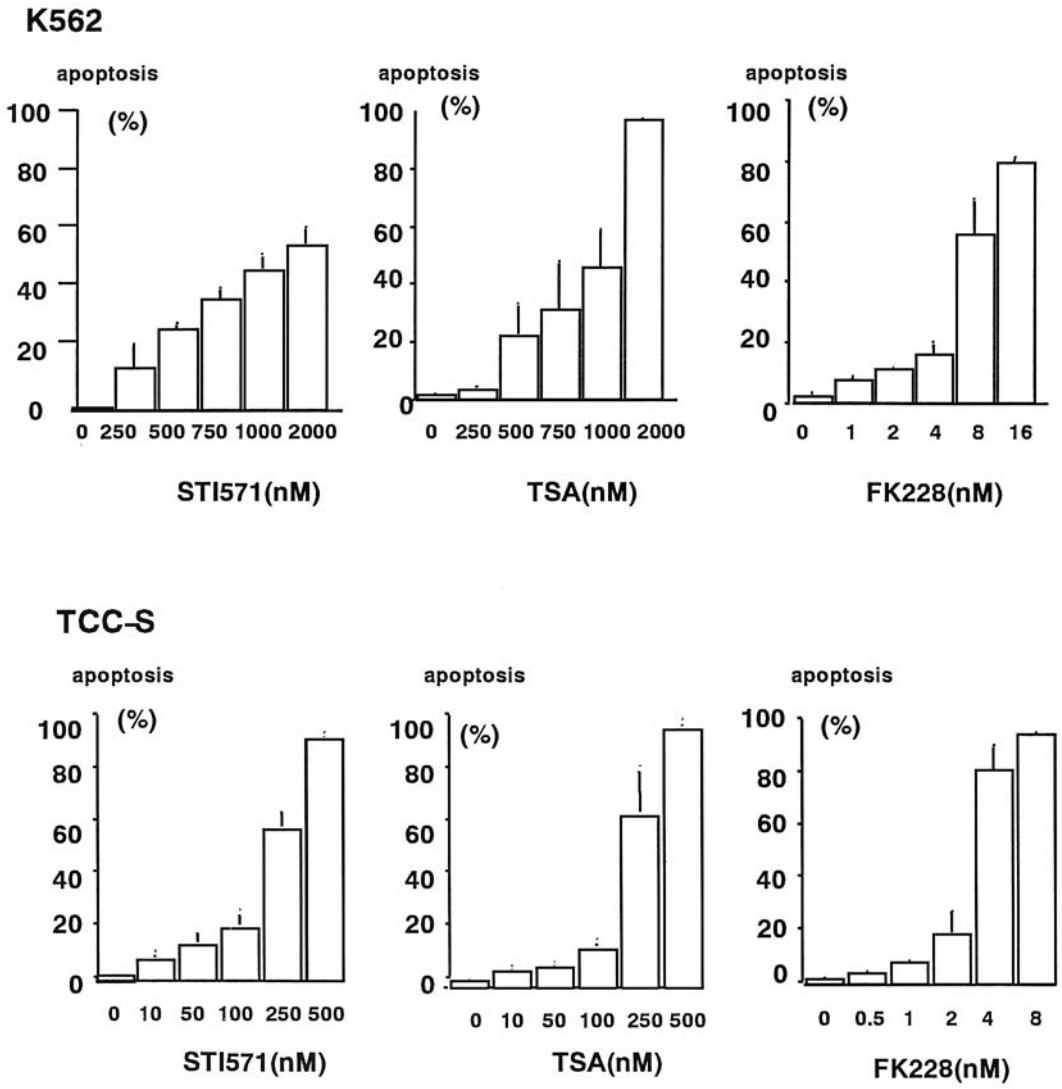

Figure 1. *Induction of apoptosis by STI571 or each HDACI. K562 cells and TCC-S cells were treated with each drug at the designated concentration for 48 h. Cells were double-stained as demonstrated in the text and apoptotic cells were counted under the fluorescent microscope. Experiments were done three times independently and the mean and SD were demonstrated.*

Many anticancer agents have been combined with STI571, some of them enhancing its activity, but some decreased its anti-proliferative effect (16). Another study showed that HDACIs increased apoptosis in acute promyelocytic leukemia and neuroblastoma by co-operating with all-trans retinoic acid (ATRA) (17, 18). Recently, one of the HDACIs, known as SAHA, has been shown to enhance apoptosis of Bcr-Abl-positive cells when used in combination with STI571 (19, 20), but the effect of FK228 still remains unclear. Moreover, the enhancing mechanism underlying this co-treatment is unknown.

In this investigation, we studied FK228 in combination with STI571. We found that a low concentration of FK228 enhanced the apoptosis of two Bcr-Abl-positive cells in combination with STI571. Furthermore, the potentiation of apoptosis by the co-treatment was abrogated by ectopic expression of cyclin D1, c-Myc or active MEK genes.

Materials and Methods

Cells and culture. K562 cells were obtained from the Riken cell bank (Tsukuba, Japan) and TCC-S cells were described elsewhere (16). K562 cells, which were ectopically expressing cyclin D1, c-Myc or constitutively active MEK, were described elsewhere (21, 22). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) containing 100U/ml of penicillin and 100 μg/ml of streptomycin (Gibco BRL, Gaithersburg, MD, USA). The cells were maintained at 37˚C under 5% CO_2 in a humidified incubator and were used for

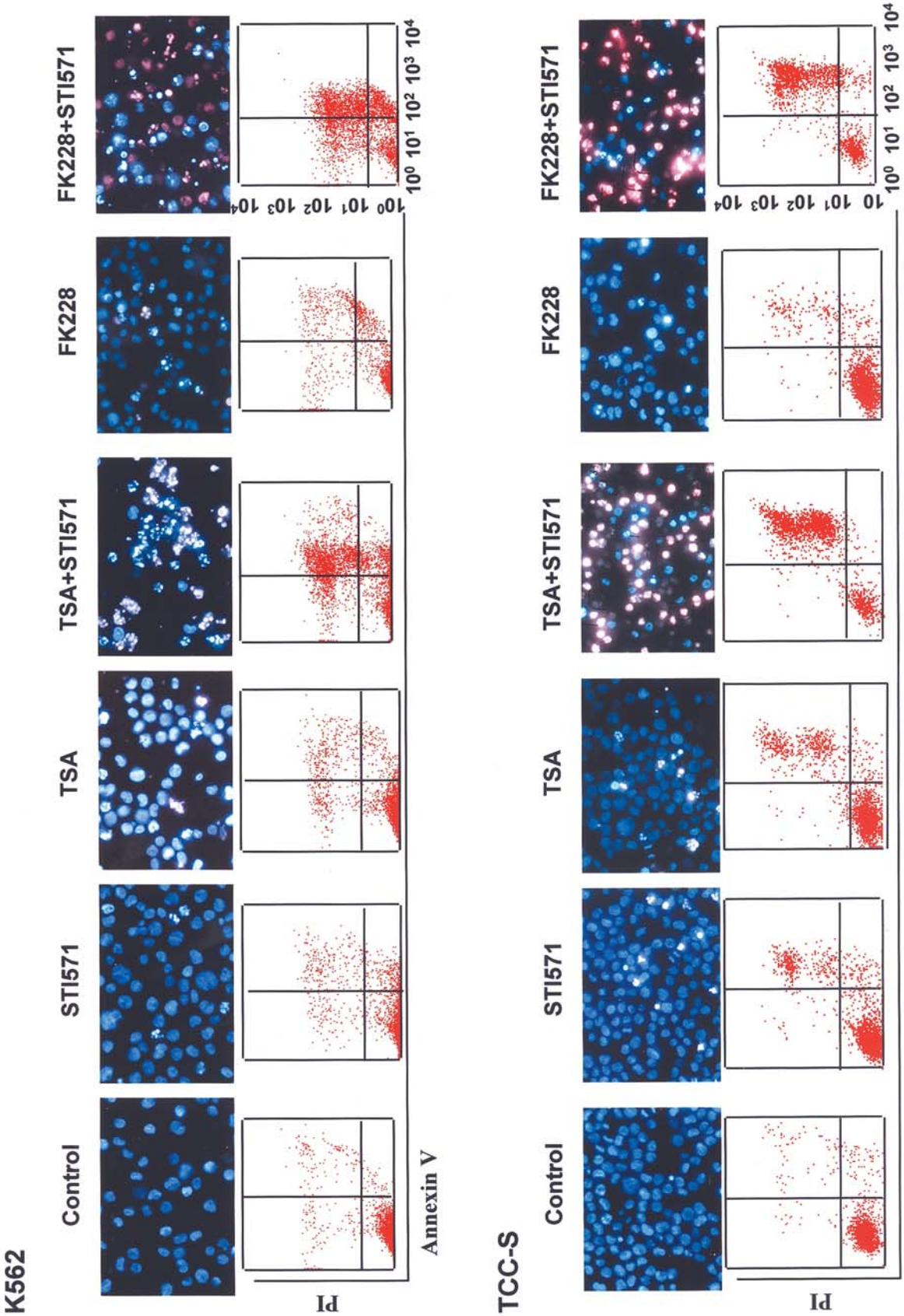

Figure 2. *The effect of combination between STI571 and each HDACI. K562 cells and TCC-S cells were treated with each drug for 48 h and apoptosis was examined by nuclear-morphology (upper panel) and by flow cytometry (lower panel) as mentioned in the text. Fragmented cells were increased markedly by each combination treatment. Similarly, the annexin V-positive population was increased by the combination treatment. The following drugs-concentrations were used for treatment: 250 nM STI571, 500 nM TSA and 2 nM FK228 for K562 cells and 100 nM STI571, 100 nM TSA and 1 nM FK228 for TCC-S cells.*

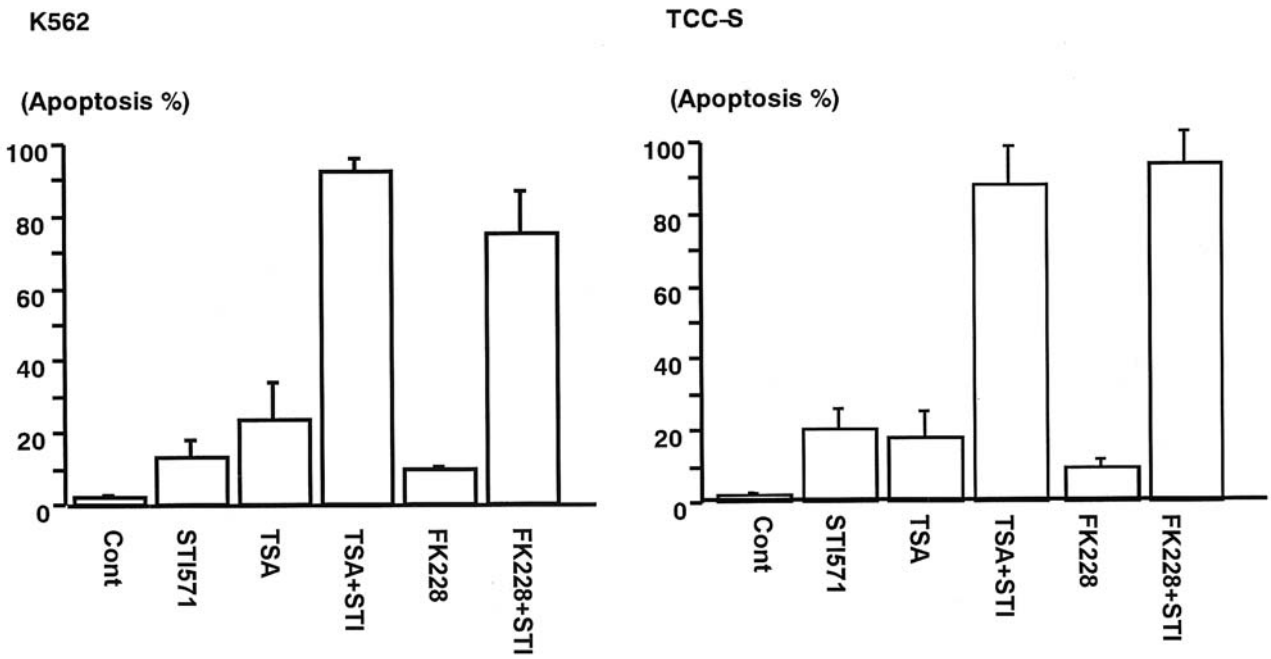

Figure 3. *Statistical analysis of apoptosis induced by the co-treatment. The percentage of apoptosis was calculated by the double-staining method. Experiments were done three times independently and the mean and SD were demonstrated. The occurrence of apoptosis increased significantly ($p<0.01$) in each co-treatment group.*

experiments in the exponential growth phase ($2x10^5$ /ml). Viable cells were counted by the erythrosine B exclusion assay (Nacalai Tesque Inc., Kyoto, Japan).

Reagents. STI571 was kindly provided by Novartis (Basel, Switzerland), and was prepared as a 1 mM stock solution dissolved in DMSO (Nacalai Tesque). FK228 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), and was prepared as a 1 mg/ml stock solution in ethanol. Tricostatin A (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was stored in a light-protected freezer at -20˚C as a 1 M stock solution in ethanol. An aliquot of the stock solution was diluted with distilled water or medium just before use.

Detection of apoptosis. Cells undergoing apoptosis were detected by double-staining with propidium iodide (PI) and bisbenzimide H33342 (Sigma, St. Louis, MO, USA). After staining, apoptotic cells were identified under a fluorescent microscope based on the nuclear morphology and cell membrane permeability. In this study, cells with fragmented nuclei were defined as apoptotic and a total of 500 cells were counted, as reported elsewhere (19). Apoptosis was also studied by staining with FITC-conjugated anti-annexin V antibody and PI according to the manufacturer's instructions (MBL, Nagoya, Japan), using FacsCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

Immunoblot analysis. Cells were washed with ice-cold TBS buffer (25 mM Tris-HCl (pH 8.0) and 150 mM NaCl) and lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and PMSF). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio Rad, Hercules, CA, USA) using a wet electroblot chamber. The membranes were blocked in TBS buffer containing 0.1% Tween-20 and 5% skim milk for at least 4 hours at 4˚C. Incubation with the primary antibodies was performed overnight at 4˚C in TBS buffer with 0.1% Tween-20 using the dilutions recommended by the manufacturer. Then 1 hour of incubation at room temperature was done with the secondary antibody conjugated to horseradish peroxidase, and proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA). The primary antibodies for caspase-3, Bcl-2, Bcl-X_L, Bax and histone H4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-PARP and anti-hyperacetylated histone H4 were obtained from Upstate USA, Inc. (Charlottesville, VA, USA).

Results

Induction of apoptosis by STI571, TSA and FK228. First, we studied the occurrence of apoptosis by STI571, TSA and FK228. Apoptosis was detected by the double-staining method after 48 hours of continuous exposure to each drug (Figure 1). STI571 induced apoptosis in a concentration-dependent manner, while apoptosis by TSA and FK228 was markedly increased at high concentrations. TCC-S cells were more sensitive to apoptosis than K562 cells.

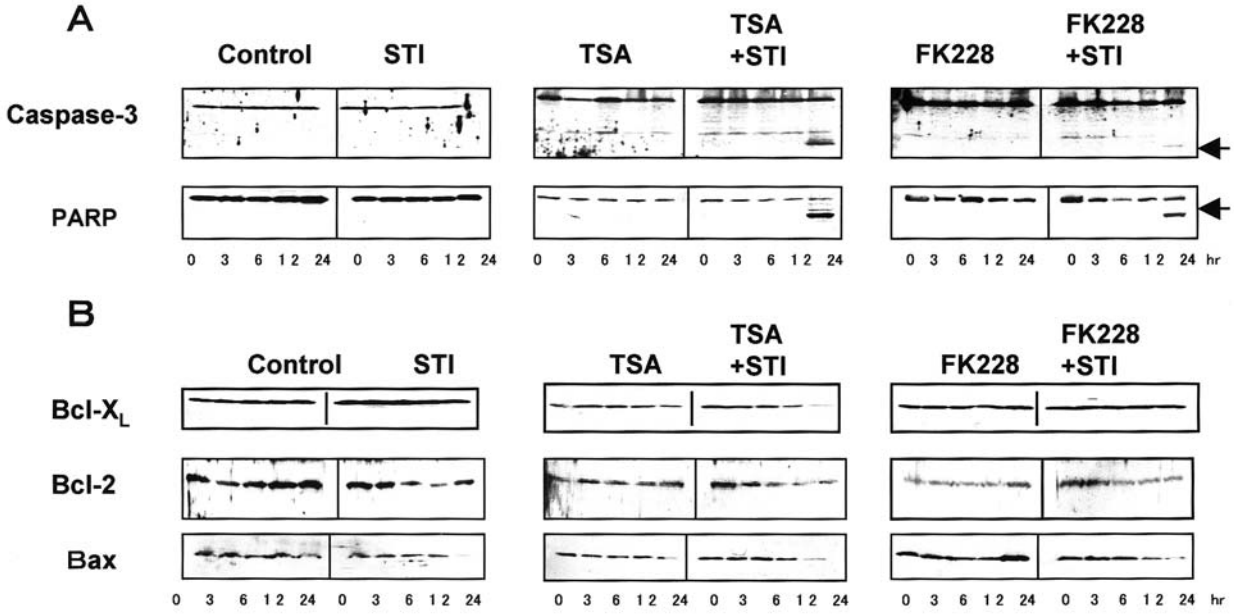

Figure 4. *Changes of caspase-3 and Bcl-2 family genes. K562 cells were treated with each HDACI with or without STI571, and cells were harvested at designated time points. The concentration of each drug was the same as that used in Figure 2. (A) Cleavage of caspase-3 and PARP. Caspase-3 activation was studied by the detection of cleaved fragments of caspase-3 and PARP. Arrows demonstrate the cleaved fragments of caspase-3 and PARP. (B) Changes of Bcl-2 family genes. Changes of Bcl-X_L, Bcl-2 and Bax were studied by immunoblot.*

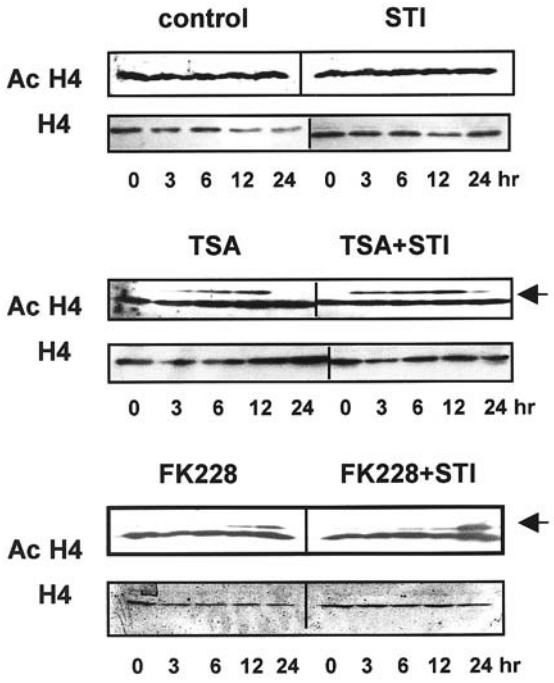

Figure 5. *Histone H4 acetylation by HDACI. Histone H4 acetylation was studied by immunoblotting. K562 cells were treated with each drug as demonstrated in Figure 2. Treated cells were harvested at each indicated time point. Arrows show acetylated histone H4. Ac H4 and H4 demonstrate the blots with anti-acetylated histone H4 and anti-histone H4, respectively.*

FK228 enhanced STI571-induced apoptosis. To study the combined effect of HDACI with STI571, K562 and TCC-S cells were exposed to STI571 with FK228 or TSA. For K562 cells, STI571 was used at 250 nM, while TSA and FK228 were added at the concentrations of 500 nM and 2 nM, respectively. Since TCC-S cells were more sensitive to each drug than K562 cells, STI571 was used at a concentration of 100 nM, while TSA and FK228 were added at 100 nM and 1 nM, respectively. Cells were continuously exposed to the drugs for 48 hours, and apoptosis was studied by double-staining and annexin V analysis. As shown in Figure 2, most K562 cells were viable after single agent treatment. However, apoptotic cells, demonstrated as fragmented nuclei, increased by the combination with STI571 and TSA (Figure 2: K562, upper panel). The combination with STI571 and FK228 also enhanced apoptosis. The increment of apoptosis by these combinations was also confirmed by annexin V analysis. The annexin V-positive fraction was markedly increased in the cells co-treated with the two agents (Figure 2: K562, lower panel). These combination effects were also seen in TCC-S cells. TCC-S cells rarely underwent apoptosis by single treatment, while the combination treatment induced apoptosis in most cells (Figure 2: TCC-S, upper and lower panels). The enhancing effect of the co-treatment was statistically confirmed, as shown in Figure 3. The percentage of apoptosis increased

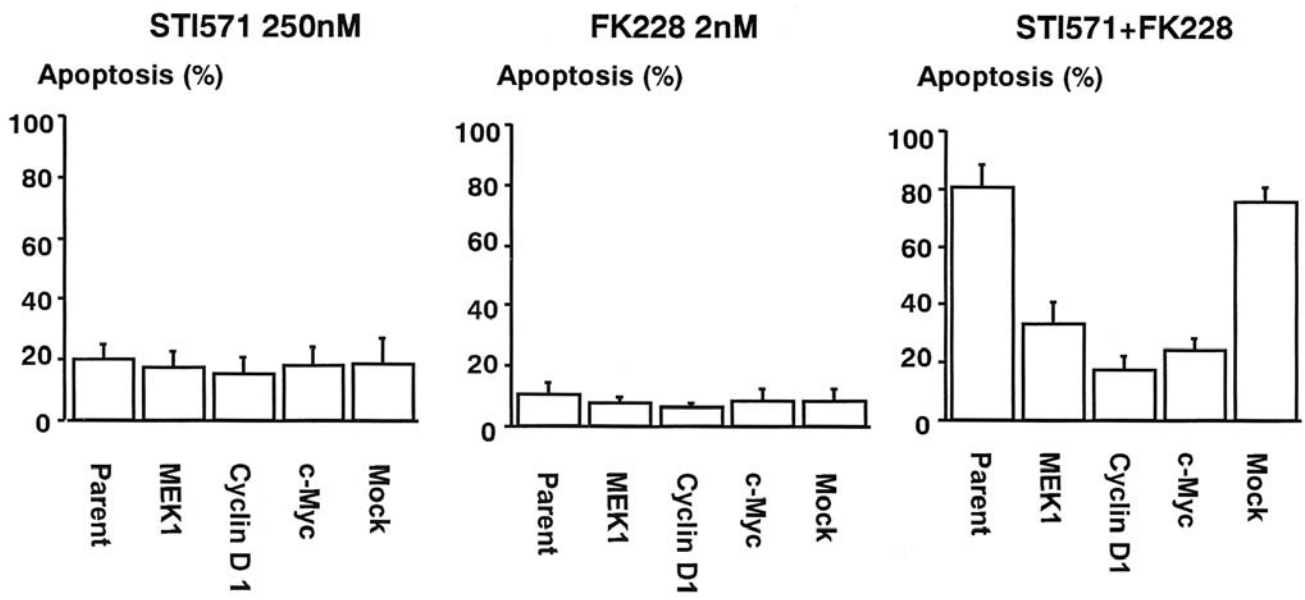

Figure 6. *Apoptosis of each cell line. Parental K562 and each transfectant were treated with 250 nM STI571, 2nM FK228, or combination. Apoptosis was studied by the double-staining method. The enhanced apoptosis by co-treatment was abrogated in each transfected cell line. Experiments were done three times independently and the mean and SD were demonstrated.*

significantly in K562 and TCC-S cells by the co-treatment. Moreover, both TSA and FK228 enhanced apoptosis equally in the combination with STI571.

Immunoblot analysis of apoptosis-related proteins. Since morphological study and annexin V analysis showed the synergistic effect of co-treatments, the changes of apoptosis-related proteins were studied by immunoblot analysis. Because the combined treatment markedly induced apoptosis at 48 hours, the following immunoblot experiments were studied up to 24 hours to avoid the secondary cell-death-related changes. K562 cells were treated with TSA, FK228 and STI571 and the cells were harvested at 0, 3, 6, 12 and 24 hours. Then, cell lysates were prepared to study caspase-3 and poly (ADP-ribose) polymerase (PARP). Treatment with each HDACI or STI571 alone did not induce obvious cleavage of these proteins, but cleaved bands were observed after 24 hours of exposure to each combination (Figure 4A), suggesting that a caspase-3-related apoptosis pathway was activated by the combination treatment.

The apoptosis-regulating Bcl-2 family genes (Bcl-X_L, Bcl-2 and Bax) were also studied (Figure 4B). STI571 attenuated Bax at 24 hours and Bcl-2 in the latter half of the treatment, while TSA and FK228 failed to alter these genes by single treatment. The co-treatment of TSA and STI571 attenuated Bcl-X_L expression at 24 hours, but it also reduced Bax. Bcl-2 expression was also attenuated a little by the co-treatment. However, the degree of Bcl-2 change was not remarkable compared of Bcl-X_L and Bax. The combination with STI571 and FK228 did not induce any change except for the down-regulation of Bax in the latter half of the co-treatment.

Acetylation of histone H4. We investigated whether the enhancement of apoptosis depended on the acetylation of histones. As demonstrated in Figure 5, TSA induced histone acetylation early in the treatment of K562 cells. Acetylation was observed at 3 hours and lasted until 12 hours. Unlike TSA, the induction of histone acetylation by FK228 occurred in the latter half of treatment. Acetylated histone H4 was observed after 12 hours of incubation and acetylation was maximum at 24 hours. These acetylation patterns were not altered by co-treatment with STI571.

Effects of ectopic expression of cyclin D1, c-Myc, or active MEK genes on the co-treatment. Since the erythroid differentiation of K562 cells induced by STI571 was markedly affected by the activation of the MEK/ERK pathway and cyclin D1 (21, 22), we studied whether cyclin D1, c-Myc and active MEK could modulate the induction of apoptosis by the combination treatment (Figure 6). Either 250 nM STI571 or 2 nM FK228 induced a low level of apoptosis, and no significant differences were observed among cell lines. In contrast, each cell line co-treated with

FK228 and STI571 showed high sensitivity. Eighty percent of parental K562 cells underwent apoptosis by the co-treatment. However, the co-treatment caused apoptosis only in 33%, 17% and 24% of active MEK-, cyclin D1- and c-Myc-transfected cells, respectively. Cyclin D1-transfected K562 cells showed the strongest resistance to the combination treatment.

Discussion

We studied the effects of the combination treatment with STI571 and HDACI (TSA or FK228). Although the effective concentration of TSA and FK228 differed, both drugs markedly enhanced the induction of apoptosis in Bcr-Abl-positive cells by STI571. Previous reports have demonstrated that SAHA and butylate synergistically enhance STI571-induced apoptosis (19, 20). TSA belongs to the same class of HDACI as SAHA, but FK228 belongs to a different class (8). Therefore, most HDACIs may enhance the induction of apoptosis by STI571 in CML cells.

The dose-response effects of HDACI and STI571 were interesting. STI571 induced apoptosis of both K562 and TCC-S cells in a linear dose-dependent manner. But the cytotoxicity of HDACIs (especially FK228) increased exponentially. This indicates that the subtle change in HDACI concentration brings a significant increment of apoptosis in treated cells. The growth of CML cells is mainly supported by the signals originated from a Bcr-Abl fusion protein. Inhibition of those signals by STI571 may elicit sufficient changes in the sensitivity of K562 and TCC-S cells to HDACIs and, in consequence, treated cells may undergo apoptosis. To study the mechanisms of this combination effect, we examined the acetylation of histone H4. As demonstrated in Figure 5, the extent of histone acetylation by TSA and FK228 was not influenced by co-treatment with STI571.

The apoptosis induced by the combination treatment was associated with cleavage of caspase-3 and PARP, so the main pathway to apoptosis caused by STI571 plus an HDACI is mediated through caspase-3, as previously demonstrated (19, 20). Bcl-2, Bcl-X_L and Bax showed little change during the first half of co-treatment in our study (Figure 4B). Yu *et al.* demonstrated that the co-treatment of SAHA and STI571 elicited the release of apoptotic factors from mitochondria, and they also described the down-regulation of Mcl-1 (20). Peart *et al.* showed Bid activation in response to FK228 treatment (23). Therefore, other Bcl-2 family genes may be involved in apoptosis during the co-treatment.

We next focused on the MEK/ERK pathway and its downstream target genes, cyclin D1 and c-Myc. Our previous studies on erythroid differentiation induced by STI571 clearly demonstrated that the activation of the MEK/ERK pathway inhibits STI571-induced differentiation. On the other hand, the inhibition of the MEK/ERK pathway promotes differentiation (21). Cyclin D1, one of the downstream targets of the MEK/ERK pathway, also inhibits the differentiation by STI571 (22). Therefore, we studied whether active MEK, cyclin D1 and c-Myc could modulate apoptosis induced by the co-treatment. As shown in Figure 6, all of the genetically-modified K562 cells were resistant to apoptosis by the combination treatment. STI571 inhibits the MEK/ERK pathway (22) and the raf/MEK/ERK pathway is down-regulated by FK228 in transformed cells (15, 24). The addition of HDACI to STI571 treatment may further modulate the activation of the MEK/ERK pathway. As FK228 treatment was demonstrated to down-regulate cyclin D1 (25), cyclin D1-overexpressing K562 cells might show the highest resistance to the co-treatment. Moreover, our previous data demonstrated that overexpression of cyclin D1 confers resistance on K562 cells for ERK-inactivation induced by STI571 (22).

In conclusion, co-treatment with STI571 and FK228 enhances apoptosis and the activation of the MEK/ERK pathway and its downstream target genes may directly affect sensitivity to the co-treatment.

Acknowledgements

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