Histone deacetylase inhibitors induce growth arrest and apoptosis of HTLV-1-infected T-cells via blockade of signaling by nuclear factor κB

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Abbreviations: ATL, adult T-cell leukemia; HTLV-1, human T-cell lymphotropic virus type I; HDACIs, histone deacetylase inhibitors; SAHA, suberoylanilide hydroxamic acid; NF-κB, nuclear factor kappa B; IκBα, inhibitory subunit of NF-κB; XIAP, X-linked inhibitor of apoptosis protein.

Summary

Adult T-cell leukemia/lymphoma (ATL) is a highly aggressive disease with a poor prognosis in which nuclear factor kappa B (NF-κB) is thought to play a role. This study explored the effects of histone deacetylase inhibitors (HDACIs) MS-275, suberoylanilide hydroxamic acid (SAHA), and LBH589 on both human T-cell lymphotropic virus type I (HTLV-1)-infected T cells (MT-1, -2, -4, and HUT102) and freshly isolated ATL cells harvested from patients. HDACIs effectively inhibited the proliferation of these cells. For example, MS-275, SAHA, and LBH589 effectively inhibited the proliferation of MT-1 cells with ED_{50s} of 6 μM, 2.5 μM, and 100 nM, respectively as measured by 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay on day 2 of culture. In addition, HDACIs induced cell cycle arrest at the G2/M phase and apoptosis of HTLV-1-infected T-cells in conjunction with regulation of apoptosis-related proteins. Electrophoretic mobility shift assay showed that exposure of HTLV-1-infected T-cells to HDACIs for 48 h inhibited formation of the NF-κB/DNA binding complex. Moreover, we found that HDACIs accumulated NF-κB and inhibitory subunit of NF-κB in the cytoplasm in conjunction with the down-regulation of NF-κB in the nucleus, suggesting that HDACIs blocked nuclear translocation of NF-κB. Based on these findings, we believe HDACIs can be useful for treating patients with ATL or

other types of cancer in which NF-κB plays a role.

Introduction

Adult T-cell leukemia (ATL) is an aggressive malignancy of $CD4^+$ T lymphocytes for which human T-cell lymphotropic virus type I (HTLV-I) has been recognized as the etiologic agent [1,2]. Despite the development of intensive combination chemotherapy regimens supported by granulocyte colony-stimulating factor, the median survival time of individuals with ATL is less than 13 months [3,4].

Nuclear factor κB (NF-κB) regulates the expression of anti-apoptotic proteins including Bcl-2 family members as well as X-linked inhibitor of apoptosis protein (XIAP). ATL cells aberrantly express these anti-apoptotic proteins via NF-κB signaling, which is associated with the resistance of these cells to apoptosis mediated by anti-cancer agents [5-7].

Histone deacetylase inhibitors (HDACIs) have emerged as a potentially promising new class of anticancer drugs [8]. These include the hydroxamic acid derived suberoylanilide hydroxamic acid (SAHA), LBH 589, and tricostatin A (TSA), cyclic depsipeptide FR901228, and benzamide MS-275 [8]. HDACIs induce the growth arrest and apoptosis of cancer cells by manipulating the transcription of genes involved in regulation of the cell cycle, apoptosis, as well as, differentiation [8]. For example, we previously showed that SAHA induces growth arrest and apoptosis of human mantle

cell lymphoma cells in association with induction of the histone acetylation of $P21^{waf1}$ promoter region, resulting in the up-regulation of $P21^{waf1}$ protein [9].

Recently, a new mode of action for HDACIs has been identified in which TSA and FR901228 inhibit NF-κB/DNA binding activity in HTLV-1-infected T-cells and murine epidermal skin JB6, respectively [5,10]. However, the precise mechanism by which HDACIs inhibit NF-κB remains to be fully elucidated.

This study explored the effects of the HDACIs MS-275, SAHA, and LBH589 on NF-κB signaling in HTLV-1-infected T-cells. Exposure of these cells to HDACIs increased their levels of inhibitory subunit of NF-κB (IκB) and NF-κB in the cytoplasm in conjunction with the down-regulation of NF-κB in the nucleus, resulting in the inhibition of NF-κB signaling and induction of apoptosis of these cells.

Materials and Methods.

Cells. HTLV-1-infected T-cell lines MT-1, MT-2, and MT-4 were the kind gifts of I. Miyoshi (Kochi Medical School, Kochi, Japan). MT-1 is a leukemia T-cell line established from the leukemia cells of a ATL patient with the disease [1]. MT-2 and -4 are HTLV-1-transformed cell lines established using an in vitro co-culture protocol [11]. The HUT102 cells were generously provided by Y. Maeda (Kinki University School of Medicine, Osaka, Japan). Cells were suspended in standard RPMI 1640 medium (Sigma, St. Louis, Missouri) supplemented with 10% heat inactivated fetal bovine serum. ATL cells were freshly isolated from patients with acute-type ATL once informed consent was obtained. $CD4^+$ T lymphocytes were isolated from healthy volunteers by magnetic cell sorting utilizing CD4 MicroBeads as the manufacturer recommended (Miltenyi Biotec GmbH, Germany).

Reagents. MS-275 and LBH589 were provided by Schering AG (Berlin, Germany) and Novartis (Basel, Switzerland), respectively. SAHA was kindly provided by Dr. V.M. Richon (Merk, New Jersey, USA). All reagents were dissolved in 100 % dimethyl sulfoxide (DMSO; Burdick & Jackson, Muskegon, MI) to a stock concentration of 10^{-2} M and stored at -80°C.

MTT Assays. HTLV-1-infected cells ($5x10^5$/ml) were cultured with various

concentrations of HDACIs for 2 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell number and viability were evaluated by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylithiazol-2-yl)-2,5-diphenyl tetrazolium salt (MTT) (Sigma) to a colored formazan product.

Cell Cycle analysis by Flow Cytometry. Cell cycle analysis was performed as previously described [12].

Apoptosis Assays. The ability of HDACIs to induce apoptosis of HTLV-1-infected T-cells was measured using an annexin V-FITC apoptosis detection kit according to the manufacturer's instructions (Pharmingen, Inc., San Diego, CA).

Electrophoretic Mobility Shift Assay (EMSA). Electrophoretic mobility shift assay (EMSA) was done as previously described [13]. Briefly, 4 µg of nuclear extract was incubated with 16 fmol ^{32}P-end labeled NF-κB binding probe. The DNA-protein complex was separated from the free oligonucleotide on a 5% polyacrylamide gel. Gels were dried and exposed to Kodak XAR film (Eastman Kodak, New Haven, CT).

Western Blot Analysis. Western blot analysis was performed as described previously [12]. Protein concentrations were quantitated using a Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved on a 10% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Corp.,

Arlington Heights, IL), and probed sequentially with antibodies. Anti-IκBα (Imgenex, San Diego, CA), anti-p65 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA), anti-XIAP (Cell Signaling Technology Inc., Beverly, MA), anti-Bcl-2 (Santa Cruz), anti-Bcl-xL (Santa Cruz), and anti-α-tubulin (Santa Cruz Biotechnology) antibodies were used.

Statistical analysis. Statistical analyses were carried out by paired *t*-test using SPSS software (SPSS Japan, Tokyo, Japan). The results were considered to be significant when the P-value was < 0.05, and when the P-value was < 0.01, highly significant.

Results.

Effect of HDACIs on HTLV-1-infected T-cells.

To examine the effects of HDACIs on the growth of HTLV-1-infected T-cells, we cultured these cells in the presence of various concentrations of either MS-275 (0.1-6 μM), SAHA (0.1-5 μM) or LBH589 (10-1000 nM). Cell viability was assessed using the MTT assay on day 2 of culture, and the results were graphed and the effective dose that inhibited 50 % growth (ED50) of these cells was calculated (Fig 1) (Table 1). MS-275 inhibited the growth of MT-1, -2, and -4 cells with an ED50 of approximately 6 μM (Fig 1A, Table 1). MS-275 (4 μM, 48 h) inhibited the growth of HUT102 cells by 30 %, although ED50 was not reached (Fig 1A, Table 1). LBH589 potently inhibited the growth of MT-1 and -4 cells (ED50 of 100 and 58 nM, respectively) (Fig 1B, Table 1). SAHA also effectively inhibited growth of the HTLV-1-infected T-cells (ED50 ranging from 2.7 to 8.8 μM) (Fig 1C, Table 1).

Effect of MS-275 on the cell cycle distribution of HTLV-1-infected T-cells.

To investigate the mechanisms by which MS-275 inhibited the growth of HTLV-1-infected T-cells, we analyzed the cell cycle distribution after exposure of these cells to MS-275 (Fig 2). MS-275 (3 or 6 μM, 48h) prominently induced the accumulation of HTLV-1-infected T-cells at the pre-G1 phase of the cell cycle, a feature

characteristic of apoptosis, with a concomitant decrease in the proportion of cells in the S phase (Fig 2). In addition, MS-275 increased the percent of cells in the G2/M phase (Fig 2). For example, exposure of MT-1 or -2 cells to MS-275 (3 μM, 48 h) caused the accumulation of the mean 19 ± 7 and 32 ± 7 % cells in G2/M phase of the cell cycle versus a mean 8 ± 3 and 19 ± 6 % in the diluent treated control cells, respectively (Fig 2).

MS-275 induced apoptosis of HTLV-1-infected T-cells.

To confirm further the ability of MS-275 to induce apoptosis of HTLV-1-infected T-cells, annexin V staining was utilized (Fig 3). Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic of cells entering apoptosis [14]. Exposure of HTLV-1-infected T-cells to MS-275 (6 μM, 48h) profoundly increased the population of cells that became positive for Annexin V (32-67 %) (Fig 3).

Effect of HDACIs on cell cycle and apoptosis-related proteins in HTLV-1-infected T-cells. We next examined whether HDACIs modulated the cell cycle and the level of apoptosis-related proteins in HTLV-1-infected T-cells by Western blot analysis (Fig 4). HTLV-1-infected T-cells aberrantly expressed XIAP, which was consistent with previous studies [6], and exposure of these cells to MS-275 (3 or 6 μM, 48 h)

prominently decreased levels of this anti-apoptotic protein (Fig 4A). Expression of $p21^{waf1}$ was not detectable in MT-1 and HUT102 cells (Fig 4A); exposure of these cells to MS-275 (3 or 6 μM, 48 h) dramatically induced $p21^{waf1}$ levels (Fig 4A). MT-2 and -4 cells slightly expressed $p21^{waf1}$ protein, which dramatically increased after exposure to MS-275 (3 or 6 μM, 48 h) (Fig 4A). Similarly, LBH589 or SAHA decreased levels of XIAP in conjunction with the up-regulation of $p21^{waf1}$ in MT-1 and -4 cells (Fig 4B). XIAP is a key member of the apoptosis protein family inhibitors, which block apoptosis by blocking the activity of caspase-3, -7, and -9 [14]. We, therefore, examined whether down-regulation of XIAP correlate with activation of caspases. As expected, cleavage of caspase 3, indicating activation of this cysteine protease, was apparently induced after exposure to MS-275 (3 or 6 μM, 48 h) (Fig 4A). Modulation of the levels of Bcl-2 family members in HTLV-1-infected T-cells after exposure to MS-275 was cell-type specific (Fig 4A). MS-275 (3 or 6 μM, 48 h) increased levels of Bcl-2 and Bcl-xL in MT-1 and -2 cells, while these proteins were down-regulated in HUT102 cells (Fig 4A). Exposure of MT-4 cells to MS-275 (3 or 6 μM, 48 h) decreased levels of Bcl-xL, while levels of Bcl-2 were not modulated in these cells (Fig 4A).

Effect of HDACIs on NF-κB activity in HTLV-1-infected T-cells.

XIAP is one of the NF-κB target genes [14]. Thus, we examined whether HDACIs

affected NF-κB activity in HTLV-1-infected T-cells by utilizing EMSA (Fig 5). Exposure of MT-1 cells to either MS-275 (6 μM, 48 h), LBH589 (100 nM, 48h), or SAHA (5 μM, 48h) almost completely disrupted formation of the NF-κB/DNA binding complex (Fig 5). Similarly, MS-275 (6 μM, 48 h) completely inhibited NF-κB/DNA binding formation in MT-4 cells (Fig 5). MS-275 (6 μM, 48 h) also interfered with formation of the NF-κB/DNA binding complex in HUT102 and MT-2 cells, although the effect was less dramatic compared to that occurred with the other cell lines (Fig 5). The specificity of the NF-κB band was confirmed by competing with 100-times molar excess of unlabeled wild type oligonucleotides, but not mutated oligonucleotides (Fig. 5).

Activation of NF-κB involves two important steps: First, the phosphorylation and subsequent degradation of IκBα caused by IκB kinase, resulting in the release of NF-κB; and second, the nuclear translocation of the activated NF-κB. To elucidate the effect of MS-275 on these steps, we measured the levels of NF-κB proteins in the cytoplasm and nucleus of the HTLV-1-infected T-cells after their exposure to MS-275 (Fig 6). IκBα and NF-κB accumulated in the cytoplasm (Fig 6A). Concomitantly, levels of NF-κB prominently decreased in the nucleus (Fig 6B), suggesting that MS-275 blocked translocation of NF-κB from the cytoplasm to the nucleus.

MS-275 induced growth arrest and apoptosis of ATL cells freshly isolated from patients. We explored the effect of MS-275 on ATL cells freshly isolated from patients with acute-type ATL (Table 2). ATL cells were cultured in the presence of various concentrations of MS-275 (0.5-6 μM). After 48 h, MTT activity and the proportion of cells positive for annexin V staining were measured; exposure of these cells to MS-275 induced growth arrest and apoptosis in a dose-dependent manner (Fig 7). On the other hand, MS-275 (0.5-6 μM) did not affect the viability of $CD4^+$ T lymphocytes from healthy volunteers (n=3, data not shown).

Discussion.

This study shows that the MS-275, SAHA, and LBH589 HDACIs induced growth arrest and apoptosis of ATL cells in association with the blockade of signaling by NF-κB. Previous study has shown that the blockade of NF-κB by either the diterpenoid oridonin [15], the proteasome inhibitor Velcade (Bortezomib) [16], or the IκB kinase inhibitor Bay 11-7082 [5] effectively induces apoptosis of ATL cells. Thus, NF-κB may be intimately involved in the regulation of pro-survival signals in ATL cells and can hence act as an attractive molecular target for treatment of this lethal disease.

MS-275 was shown to induce apoptosis of B-chronic lymphocytic leukemia cells and Jurkat lymphoblastic T-cells via the generation of reactive oxygen species (ROS) [17,18]. Since LAQ824, a hydroxamic acid derivative, was found to induce apoptosis of leukemia cells in association with the down-regulation of XIAP, which is mediated by ROS production [19], and NF-κB negatively regulates ROS production [20]. Hence, HDACIs might induce ROS generation via NF-κB inhibition, resulting in the induction of apoptosis of leukemia cells.

We demonstrated the likely mechanism by which HDACIs inhibited NF-κB signaling in HTLV-1-infected T-cells; MS-275 increased levels of the p65 subunit of NF-κB and IκBα in the cytoplasm in conjunction with the down-regulation of NF-κB in

the nucleus in the MT-1 cells (Fig 6), suggesting that MS-275 blocked nuclear translocation of NF-κB in these cells. Recently, other investigators have shown that SAHA inhibited both the cytokine-inducible and constitutive NF-κB activity in leukemia or lung cancer cells by blocking degradation of IκBα [21].

NF-κB is involved in producing proinflammatory cytokines. Targeting this transcriptional factor may be an attractive strategy for treating inflammatory diseases. For example, we were able to rescue mice from lipopolysaccharide (LPS)-induced septic shock by blocking NF-κB signaling by the eight herbal mixture PC-SPES [13]. Recent preclinical studies have raised the possibility that HDACIs may be used for inflammatory diseases since SAHA decreased the LPS-stimulated production of proinflammatory cytokines in murine macrophages [22]. In a murine lupus erythematosus model, SAHA decreased production of proinflammatory cytokines such as interleukin-6 (IL-6) and -10 and decreased glomerulonephritis [23]. SAHA also prevented graft-versus-host disease in a murine bone marrow transplantation model by reducing the production of proinflammatory cytokines [24]. Interestingly, SAHA preserved the reactivity of donor-lymphocytes against host antigens [24]. We expect that HDACIs can block exaggerated cytokine production in lymphocytes and macrophages by inhibiting NF-κB. Nevertheless, additional studies are required to clarify all of the

molecular mechanisms by which SAHA decreases cytokine production in the above-mentioned model systems.

In summary, HDACIs may be useful in the treatment of patients with ATL by targeting NF-κB. Similarly, this group of drugs may be effective against inflammatory diseases. Further studies are warranted to evaluate the therapeutic efficacy in this class of agents.

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Figure legends.

Fig 1. HDACIs inhibited growth of HTLV-1-infected T-cells.

HTLV-1-infected MT-1, -2, -4, and HUT102 cells were plated in a 96-well plate and cultured in the presence of various concentrations of either MS-275 **(A)**, LBH589 **(B)**, or SAHA **(C)**. Their viability was assed by MTT assay on day 2 of culture. Results represent the mean ± SD of 3 experiments performed in triplicate.

Fig 2. Effect of MS-275 on cell cycle distribution of HTLV-1-infected T-cells.

MT-1, -2, -4, and HUT102 were plated in a 24-well plate and cultured with either MS-275 (3 or 6 μM) or diluent control. After 48 h, cell cycle distribution was analyzed by flow cytometry by staining with propidium iodide. Results represent the mean ± SD of 3 experiments performed in duplicate. Statistical significance was analyzed by paired *t*-test. MS, MS-275.

Fig 3. MS-275 induced apoptosis of HTLV-1-infected T-cells.

Cells were plated in a 24-well plate and cultured with either MS-275 (6 μM) or diluent control. After 48 h, annexin binding and propidium iodide staining were analyzed by FACscan. Lower left quadrants, viable cells. Lower right quadrants, early apoptotic cells. Upper right quadrants, nonviable late apoptotic/necrotic cells. These results represent one of three experiments performed independently.

Fig 4. HDACIs modulated levels of cell cycle and apoptosis-regulating proteins in HTLV-1-infected T-cells. (Panel A), MT-1, -2, -4, and HUT102 cells were cultured with either MS-275 (6 μM) or diluent control. After 48 h, cells were harvested and proteins were extracted and subjected to Western blot analyses. The membrane was sequentially probed with anti-XIAP, Bcl-2, Bcl-xL, cleaved caspase3, caspase3, $p21^{waf1}$ and α-tubulin antibodies. Two repeated experiments yielded similar results. **(Panel B).** MT-1 and -4 cells were cultured with either LBH589 (100 or 500 nM) or SAHA (2.5 or 5 μM). After 48 h, cells were harvested and proteins were extracted and subjected to Western blot analyses. The membrane was sequentially probed with anti-XIAP, $p21^{waf1}$ and α-tubulin antibodies. Two repeated experiments yielded similar results. LBH, LBH589.

Fig 5. Effect of HDACIs on NF-κB DNA binding activity in HTLV-1-infected T-cells. MT-1, -2, -4, and HUT102 cells were cultured with either MS-275 (6 μM), LBH589 (100 nM), or SAHA (5 μM). After 48 h, nuclear proteins were extracted and subjected to EMSA. The arrow indicates the gel location of NF-κB bound to DNA. Three repeated experiments yielded similar results. WT, wild type and mt, mutant oligonucleotides (100 x) were used to compete the cellular lysate NF-κB binding to the labeled oligonucleotides.

Fig 6. MS-275 caused accumulation of NF-κB and IκBα in the cytoplasm (A) and down-regulation of levels of NF-κB in the nucleus (B). MT-1, -2, -4, and HUT102 cells were cultured with MS-275 (3 or 6 μM). After 48 h, cytoplasmic (A) and nuclear extracts (B) of these cells were prepared and subjected to Western blot analysis to measure the levels of IκBα, the p65 subunit of NF-κB, and α-tubulin as loading control.

Fig 7. Effect of MS-275 on ATL cells freshly isolated from patients. ATL cells were freshly isolated from patients and cultured in the presence of various concentrations of MS-275 (0.5-6 μM). After 48 h, MTT activity **(A)** and the proportion of cells positive for annexin V staining **(B)** were measured.

Table 1. Inhibition of proliferation (ED50) of HTLV-1-infected T-cell lines by MS-275, SAHA, and LBH589.

		MS-275	SAHA	LBH589
HTLV-1- infected T-cell	MT-1	6.0 μM	2.7 μM	100 nM
	MT-2	6.0 μM	4.6 μM	N.R.
	MT-4	5.4 μM	5.0 μM	58 nM
	HUT102	N.R.	8.8 μM	N.R.

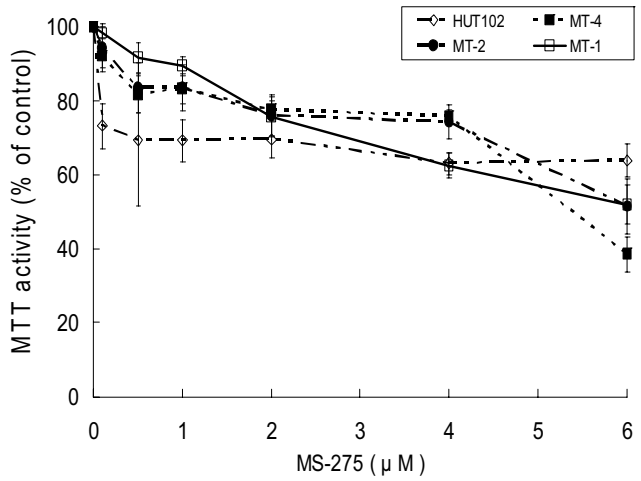
Concentrations of HDACIs that produced 50% growth inhibition (ED50) of HTLV-1-infected T-cells. ED50 was determined by plotting the inhibition of cell proliferation (MTT assays) in the presence of increasing concentrations of MS-275 (0.1-6 uM, 48 h), SAHA (0.1-5 uM, 48 h), or LB589 (10-1000 nM, 48 h). SAHA, suberoylanilide hydroxamic acid; N.R., not reached.

Table 2. Patient Clinical Characteristics

Patient	Age	Sex	WBC ($x10^9$/L)	ATL cells (%)	Type	Disease Status
#1	60	F	56,600	73 %	Acute	Newly diagnosed
#2	72	F	47,700	90 %	Acute	Newly diagnosed

Figure

Fig.1 A



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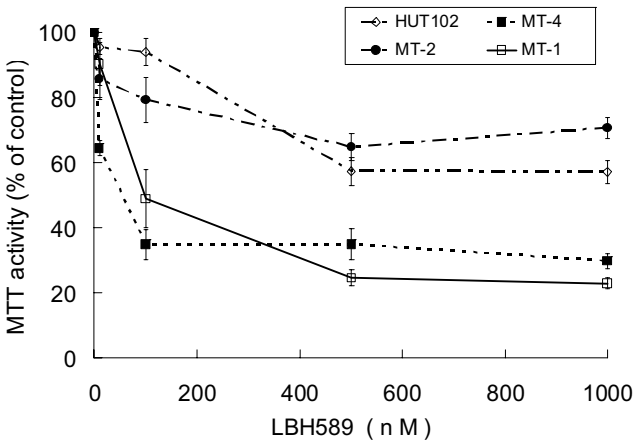

C

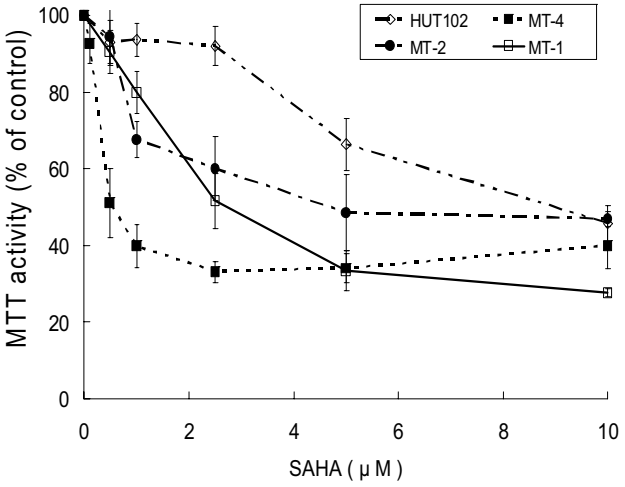

Figure2

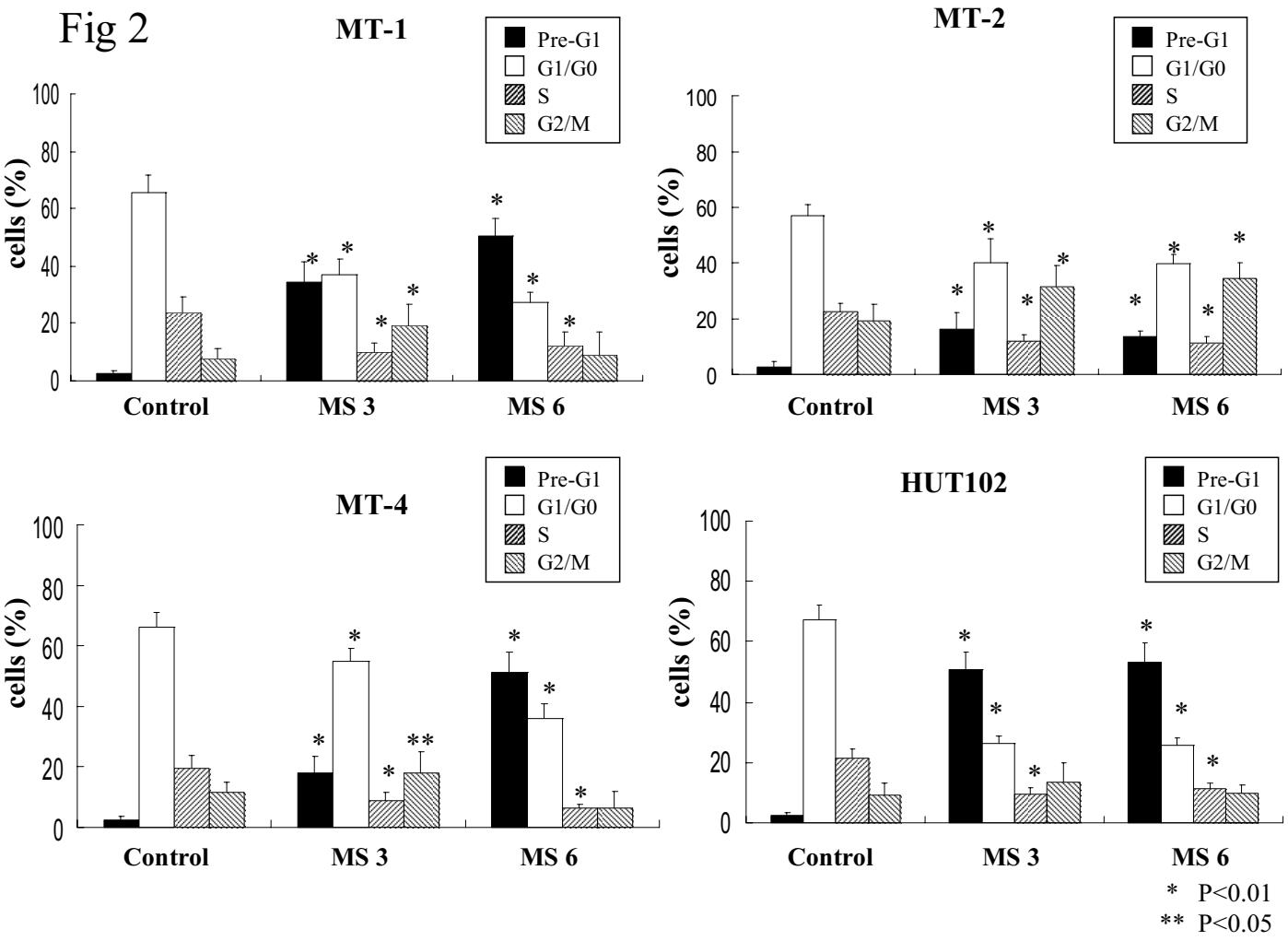
Fig 2
MT-1
MT-2
MT-4
HUT102
Pre-G1
G1/G0
S
G2/M
cells (%)
0
20
40
60
80
100
Control
MS 3
MS 6
* P<0.01
** P<0.05

Figure3
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Fig 3

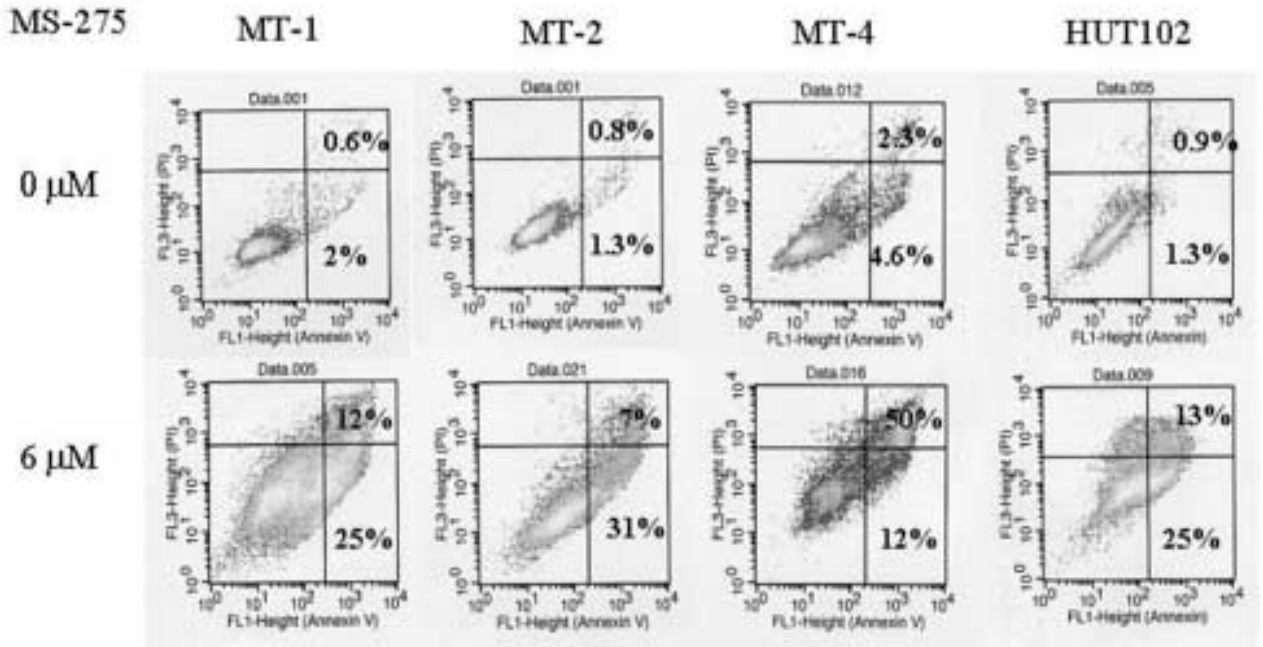

Figure4A
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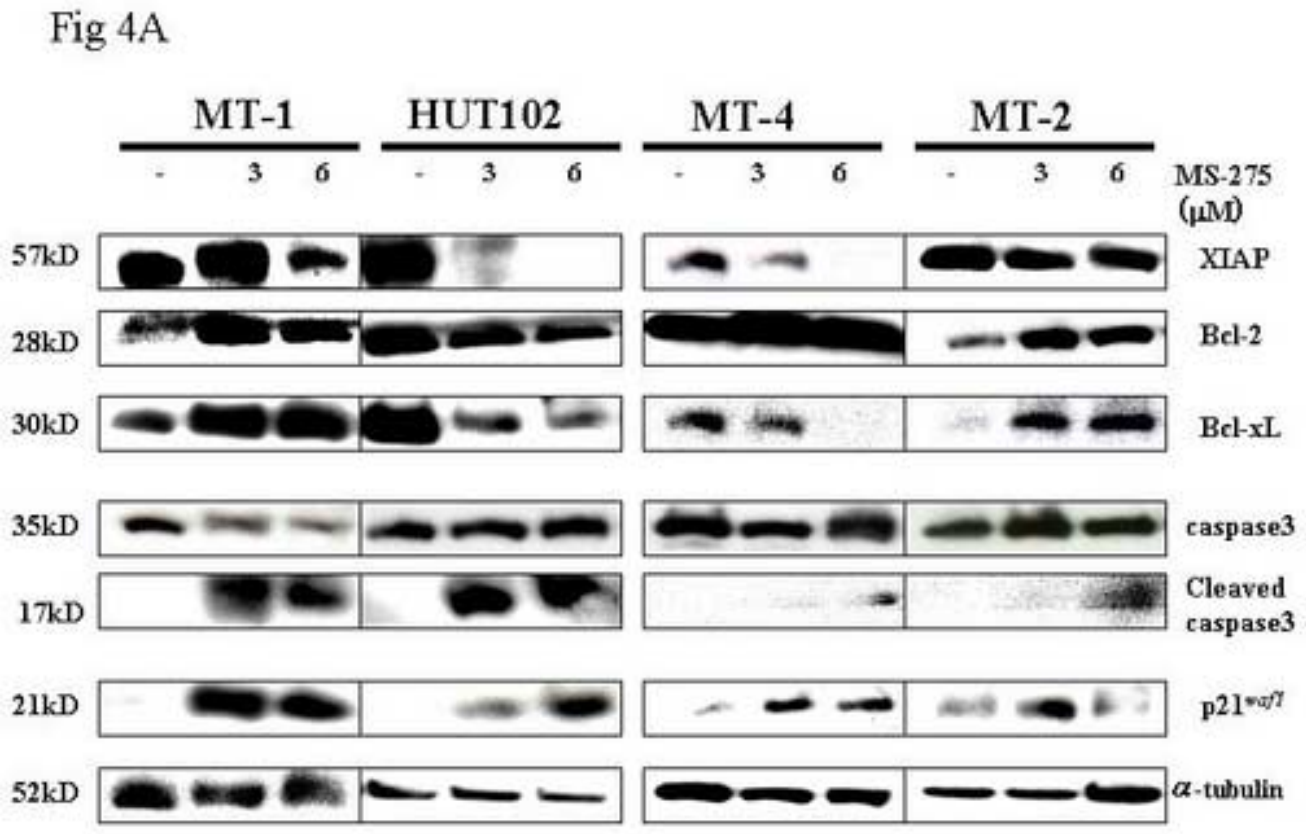

Figure4B
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Fig 4B

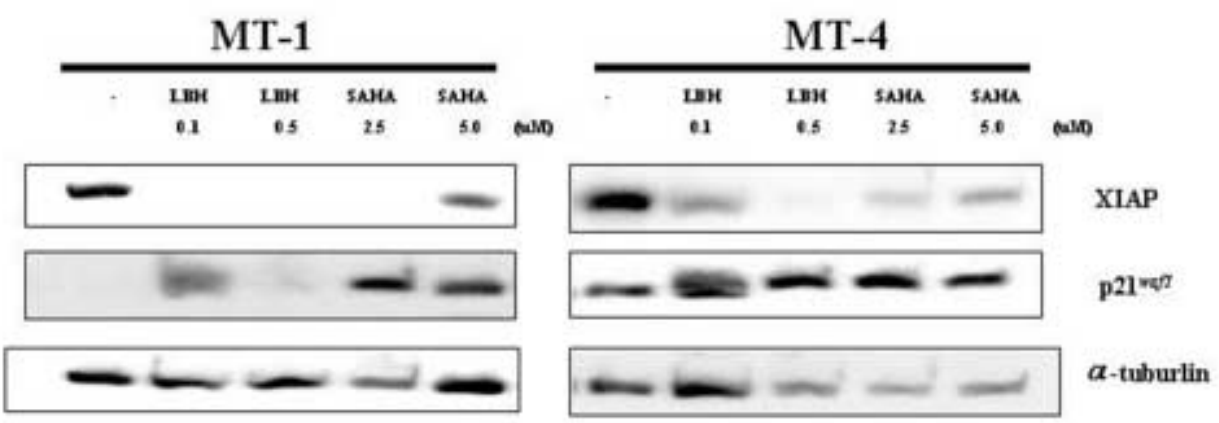

Figure5

Fig 5

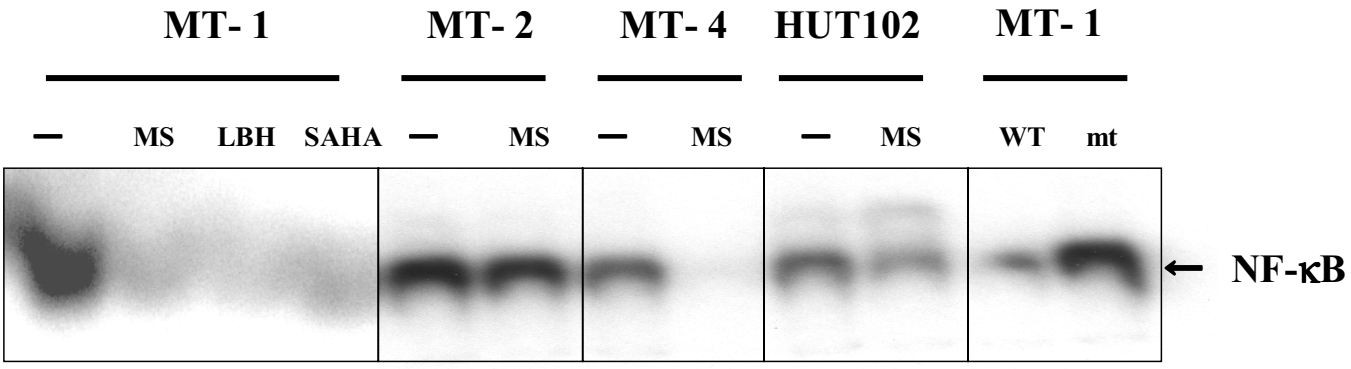

Figure6A

Fig 6A

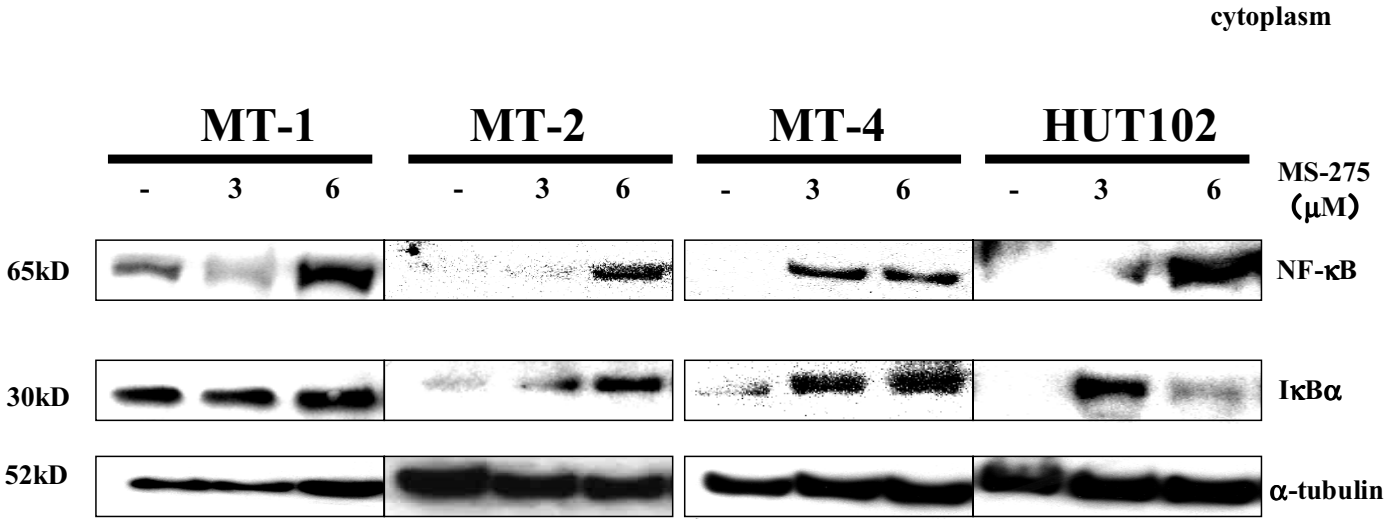

Figure6B

Fig 6B

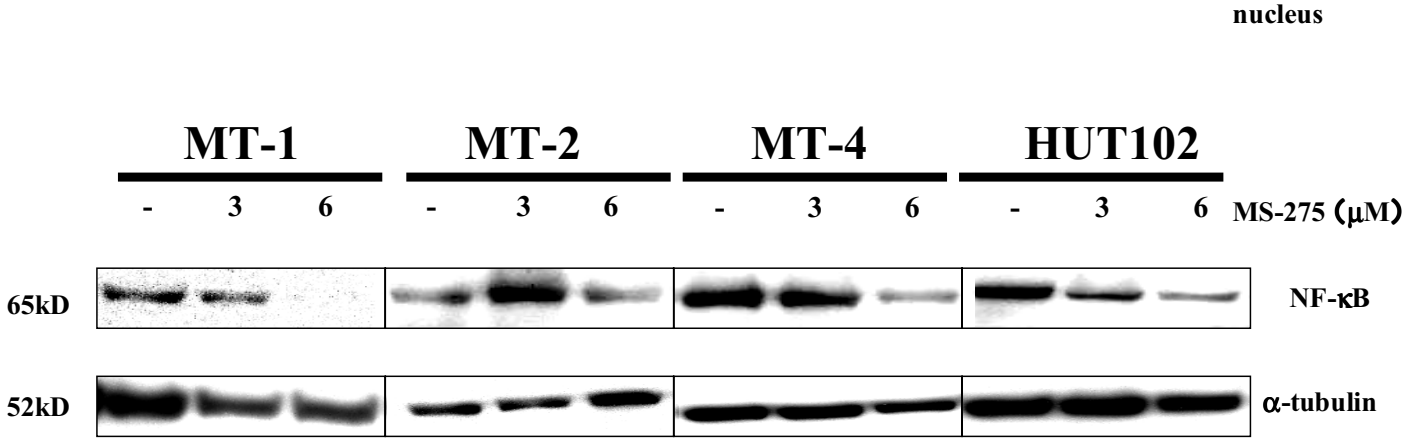

Figure7
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