MS-275, a novel histone deacetylase inhibitor with selectivity against HDAC1, induces degradation of FLT3 via inhibition of chaperone function of heat shock protein 90 in AML cells

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The abbreviations are: HDACI, histone deacetylase inhibitor; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; PDGFR, platelet derived growth factor receptor; FLT3, fms-like tyrosine kinase 3.

Summary

This study explored the effect of MS-275, a novel histone deacetylase inhibitor (HDACI), against a variety of human leukemia cells with defined genetic alterations. MS-275 profoundly induced growth arrest of acute myelogenous leukemia (AML) MOLM13 and biphenotypic leukemia MV4-11 cells, which possess internal tandem duplication mutation in the *fms-like tyrosine kinase 3* (*FLT3*) gene (*FLT3-ITD*), with IC50s less than 1 μM, as measured by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay on day two of culture. Exposure of these cells to MS-275 decreased levels of total, as well as, phosphorylated forms of FLT3, resulting in inactivation of its downstream signal pathways, including Akt, ERK, and STAT5. Further studies found that MS-275 induced acetylation of heat shock protein 90 (HSP90) in conjunction with ubiquitination of FLT3, leading to degradation of FLT3 proteins in these cells. This was blunted by treatment with the proteasome inhibitor bortezomib, confirming that FLT was degraded via ubiquitin/proteasome pathway. Moreover, we found that further inhibition of MEK/ERK signaling potentiated the action of MS-275 in leukemia cells. Taken together, MS-275 may be useful for treatment of individuals with leukemia possessing activating mutation of *FLT3* gene.

Introduction

The class III receptor tyrosine kinases (RTKs), including fms-like tyrosine kinase 3 (FLT3), c-KIT, and platelet-derived growth factor receptor (PDGFR) are implicated in the pathophysiology of several cancers as well as leukemias. These RTKs all share the same topology, consisting of five extracellular immunoglobulin-like domains, a juxtamembrane (JM) domain, a kinase domain interrupted by a kinase insertion domain, and an intracellular C-terminal domain [1]. Ligand binding to RTKs results in the activation of downstream effectors, including protein kinase B/Akt, signal transducers and activators of transcription (STATs) and extracellular signal-regulated kinases (ERKs) 1/2, leading to cell proliferation, differentiation and/or survival [2,3]. Recent studies have revealed that activating mutations of RTKs frequently occur in acute myelogenous leukemia (AML) patients. For example, internal tandem duplications (ITDs) of the JM domain of *FLT3* (*FLT3-ITD*) are present in 20–30% of *de novo* AML cases [4]; and approximately 7% of AML patients possess the D835 mutation, a point mutation in the activation loop of the second kinase domain of *FLT3* [4]. These mutations result in constitutively activated FLT3 and its downstream signal pathways, and are associated with elevated blast counts, increased relapse rates and poor overall survival in AML [5-8]. Mutations of *c-KIT* and *PDGFR* are also associated with subsets

of AML [9-12]. Therefore, RTK is a therapeutic target and inhibition of activity of RTK may provide a new approach in the treatment of AML patients carrying these mutations.

Heat shock protein 90 (HSP90) is a member of the HSP family and consist of α and β subunits [13]. HSP90 is ubiquitous and functions as chaperone in cytosol, where they stabilize select proteins including hormone receptors, cyclin dependent kinase 4, as well as, RTKs [14]. In addition, HSP90 chaperone complexes prevent degradation of mutated proteins including FLT3-ITD, sustaining the function of these proteins [14]. Recent studies showed that inhibition of HSP90 by 17-allylamino-demethoxy geldanamycin (17-AAG) provoked degradation of FLT3-ITD via ubiquitin/proteasome pathway and inhibited the proliferation of leukemia cells with FLT3-ITD [15,16].

Deacetylation of HSP90 at K294 mediated by HDAC6 is critical for chaperon function; when HDAC6 was genetically knocked down, HSP90 k294 was acetylated and ATP binding to HSP90 was blocked, resulted in impairment of its chaperon function [17]. The hydroxamic acid analogue pan-HDACIs, LAQ824 and LBH589 preferentially inhibit HDAC6 [18]. Both induced acetylation and inhibition of HSP90, resulting in degradation of BCR/ABL oncoprotein and Akt in chronic myeloid leukemia K562 cells [18].

MS-275, a novel and orally available synthetic benzamide HDACI, perferentially

inhibits HDAC1, and does not possess activity against HDAC6 [19,20]. The in vitro enzymatic assay showed that IC50 of MS-275 against HDAC1 was 2.2 μM. On the other hand, even 100 μM of MS-275 was not able to inhibit the enzymatic activity of HDAC6 [21]. MS-275 induced differentiation and/or apoptosis of human leukemia U937, HL-60, K562 and Jurkat cells [21,22]. This study examined the effects of MS-275 on human leukemia cells expressing FLT3-ITD. Also, we investigated the drug interaction of MS-275 and the novel MEK/ERK inhibitor AZD6244 (ARRY-142886) [23] with these cells.

Materials and Methods

Cells. Characteristics of the cell lines utilized in this study have been described [24]. Leukemia cells from patients were freshly isolated with informed consent and institutional review board approval. The informed consent was provided according to the Declaration of Helsinki.

Reagents. MS-275 was provided by Schering AG (Berlin, Germany), and dissolved in 100 % dimethyl sulfoxide (DMSO; Burdick & Jackson, Muskegon, MI) to a stock concentration of 10^{-2} M and stored at -80°C. PS-341 was provided by Millennium Pharmaceuticals (Cambridge, MA) and dissolved in PBS to a stock concentration of 10^{-2} M and stored at -80°C.

Growth inhibition assays. To assess the anti-proliferative effect of MS-275 against various types of leukemia cells, either 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or ^{3}H-thymidine uptake was utilized for leukemia cell lines or freshly isolated leukemia cells from patients, respectively, as previously described [24]. All experiments were performed in triplicate and repeated at least three times.

FLT3 genotyping. *FLT3-ITD* mutation was examined as previously described [24].

Cell cycle analysis by flow cytometry. Cell cycle analysis was performed as previously

described [24].

Apoptosis assays. The ability of MS-275 to induce apoptosis of leukemia cells was measured using the annexin V-FITC apoptosis detection kit (Pharmingen, Inc., San Diego, CA), according to the manufacturer's instructions.

Flow cytometry. The effect of MS-275 on FLT3 and its downstream signals was assessed by flow cytometry. Anti-FLT3/CD135 (Abcam, Cambridge, UK, ab23895), -p-FLT3 (Tyr591, Cell Signaling Technology, Beverly, MA, #3461), -ERK (Cell Signaling Technology, #4374), -p-ERK (Cell Signaling Technology, #9102), -Akt (Cell Signaling Technology, #9272), -p-Akt (Ser473, Cell Signaling Technology, #9271), -Stat5 (C-17; Santa Cruz, Santa Cruz, CA, sc-835), -p-Stat5 (Tyr694, Cell Signaling Technology, #9351), cleaved caspase 3 (Cell Signaling Technology, #9661) and 9 (Cell Signaling Technology, #9501), cleaved PARP (Cell Signaling Technology, #9541) antibodies were used.

The multiparameter flow cytometric analysis was performed to access the effect of MS-275 on acetylation of HSP90 and ubiquitination of FLT3 in leukemia cells. To quantify acetylated HSP90 positive population, cells were simultaneously stained with anti-HSP90α/β (F-8; Santa Cruz, sc-13119) and anti-acetylated lysine antibodies (R&D systems), followed by staining with FITC-conjugated goat anti-rabbit (Caltag

Laboratories, Burlingame, CA) and PE-conjugated goat anti-mouse secondary antibodies (Caltag Laboratories, Burlingame, CA). The ubiquitinated FLT3 expressing cells were quantified using anti-human ubiquitin (R&D systems, CPJ02) and anti-Flt3/CD135 (Abcam) antibodies. Data analysis was performed with the Cell Quest software (Becton Dickinson, San Jose, CA).

Immunoprecipitation of HSP90 or FLT3. To further confirm the effects of MS-275 on acetylation of HSP90 and ubiquitination of FLT3 in leukemia cells, immunoprecipitation followed by Western blot analysis was employed. Lysates from MV4-11 and MOLM13 cells were prepared and immunoprecipitated with the indicated antibody and protein G Sepharose (Pierce, Rockford, IL), as previously described [23]. The precipitated samples were subjected to Western blot analysis, as previously described [24]. The membrane was sequentially probed with the indicated antibodies.

Western blot analysis. Western blot analysis was done as described previously [24]. Anti-Bcl-2 (Santa Cruz), -Bcl-xL (Cell signaling), -Mcl-1 (Santa Cruz), and -α-tubulin (Santa Cruz) antibodies were used.

Data analysis. The combination index (CI) for growth inhibition elicited by MS-275 and AZD6244 in leukemia cells was calculated using the median effect method of Chou and Talalay [25] (Calcusyn Software available from Biosoft, Cambridge, United

Kingdom). CI values <1 indicate synergy, a CI = 1 indicates an additive effect, and a CI >1 indicates antagonism between the two agents.

Statistical analysis. Statistical analyses were carried out using the SPSS software (SPSS Japan, Tokyo, Japan) and the results were considered to be significant when the P-value was < 0.05, and highly significant, when the P-value was < 0.01. To assess the difference between two groups under multiple conditions, one-way ANOVA followed by Boneferroni's multiple comparison tests were performed by using PRISM statistical analysis software (GraphPad Software Inc, San Diego, CA, USA).

Results

MS-275 induces growth arrest of leukemia cells with FLT3-ITD. A variety of types of leukemia cells were exposed to various concentrations of MS-275 (0.1-5 μM) and their viability was determined by MTT assay on day 2 of culture. Results were graphed (data not shown), and the effective dose of MS-275 that inhibited 50% growth (IC_{50s}) of these cell lines was calculated (Table 1). IC_{50} of MS-275 for either MV4-11 or MOLM13 cells was 0.74 and 0.67 μM, respectively. MS-275 was also active against EOL-1 and Kasumi-1 cells which express an activating mutation of PDGFRα and c-KIT, respectively (Table 1). Moreover, MS-275 was able to inhibit the proliferation of freshly-isolated leukemia cells with FLT3-ITD (Table 2).

Effect of MS-275 on the cell cycle of leukemia cells. To investigate the mechanisms by which MS-275 inhibited the growth of MOLM13 and MV4-11 leukemia cells, we explored the effect of MS-275 on cell cycle distribution of these cells by flow cytometry (Fig.1). MS-275 (0.5 or 1μM, 48 hrs) profoundly caused the accumulation of these cells in 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.1). For example, 69 ± 4 % and 77 ± 5 % of either MOLM13 or MV4-11 cells accumulated in the pre-G1 phase of the cell cycle after exposure to MS-275 (1μM, 48 hrs) (Fig. 1A).

Effect of MS-275 on apoptosis of leukemia cells. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic of cells entering apoptosis. Exposure of either MOLM13 or MV4-11 cells to MS-275 (1μM) for 48 hrs markedly increased the proportion of cells positive for Annexin V (42 % of MOLM13, 60 % of MV4-11) (Fig. 1B).

MS-275 decreases levels of Bcl-2 family members in leukemia cells. Bcl-xL plays a pivotal role in FLT3 kinase inhibitor-mediated apoptosis in FLT3-ITD expressing leukemia cells [26]. Exposure of MOLM13 and MV4-11 cells to MS-275 (0.5 or 1μM, 48 hrs) lowered levels of Bcl-xL and Mcl-1, while levels of Bcl-2 did not change (Fig.1C). As levels of anti-apoptotic protein decreased, caspases 3 and 9 were activated, as indicated by the appearance of the cleaved forms of these proteins, leading to cleavage of PARP, a feature characteristic of apoptosis (Fig.1C).

MS-275 inhibits FLT3 and its downstream signal pathways in MOLM13 and MV4 -11 cells. Exposure of MOLM13 and MV4-11 cells to MS-275 (0.5-2 μM) for 24 hrs decreased the percent of the population expressing the phosphorylated form, as well as total amount of FLT3 in a dose-dependent manner (Figs. 2A,B). This occurred in parallel with downregulation of p-Akt, p-ERK, and p-STAT5, which are downstream of FLT3 (Figs. 2A,B).

MS-275 induces acetylation of HSP90 in leukemia cells. FLT3 is a client protein for the chaperone HSP90 [27], which raised the possibility that MS-275 could acetylate HSP90, resulting in impairment of its chaperone function and degradation of FLT3 via ubiquitin/proteasome pathway. To verify this hypothesis, we examined whether MS-275 acetylated HSP90 in MV4-11 and MOLM13 cells by utilizing multiparameter flow cytometric analysis. Approximately 50 % of MOLM13 and MV4-11 cells expressed acetylated HSP90; exposure of these cells to MS-275 (1 μM, 24 hrs) increased the acetylated HSP90 expressing population to approximately 90 % (Fig. 3A). Acetylation of HSP90 is linked to the inactivation of its chaperone activity, leading to ubiquitination of the client proteins [14,15]. We next explored whether MS-275 induced ubiquitination of FLT3 in leukemia cells. As expected, exposure of MV4-11 and MOLM13 cells to MS-275 (1 μM, 24 hrs) markedly induced ubiquitination of FLT3 in these cells (Fig. 3B). We further confirmed MS-275-induced acetylation of HSP90 in MV4-11 and MOLM13 cells by immunoprecipitation followed by Western blot analysis (Fig. 3C). In parallel with this, direct interaction between HSP90 and FLT3 was inhibited, resulting in ubiquitination of FLT3 in these cells (Fig 3D). Polyubiquitination of client proteins results in degradation by 26 S proteasome [28,29]. Pretreatment of MV4-11 and freshly-isolated leukemia cells (#1 and #2 in Table 2) with the proteasome inhibitor

PS-341 (1 nM, 1 hr) blunted MS-275-induced downregulation of FLT3 (Fig. 3E), confirming that the FLT3 protein was degraded via ubiquitin/proteasome pathway.

AZD6244 enhanced the anti-proliferative effect of MS-275 in leukemia cells. As shown above, MS-275 potently inhibited phosphorylation of FLT3 in MOLM13 and MV4-11 cells, but blockade of one of the downstream signals, p-ERK, was only partial. We, therefore, examined the impact of inhibition of MEK/ERK signaling by AZD6244 on the action of MS-275 in these cells. p-ERK was expressed in 96 % and 89 % of control MV4-11 or MOLM13 cells, respectively (Fig.4A); exposure of these cells to MS-275 (0.5 μM, 24 hrs) alone decreased the p-ERK-positive population to approximately 79 % (MV4-11) or 66 % (MOLM13) (Fig. 4A). AZD6244 potently inhibited phosphorylation of ERK with either 10 % or 18 % of MV4-11 and MOLM13 cells having p-ERK positivity after exposure to AZD6244 (0.25 μM, 15 min) (Fig. 4A). When these cells were exposed to a combination of both of these compounds, p-ERK positive population decreased to 2 % in both cell lines (Fig. 4A). We next examined the effect of the combination of MS-275 and AZD6244 on proliferation of MV4-11 and MOLM13 cells. The cells were cultured in the presence of either MS-275 (0.1-1 μM) and/or AZD6244 (0.1–1 μM) for 48 hrs and proliferation was measured. As shown in Fig 4B, the CI value was <1, indicating a synergistic anti-proliferative effect when these

cells were treated with both compounds.

Effect of MS-275 and AZD6244 on the cell cycle in leukemia cells. In addition, we studied the effect of concomitant addition of AZD6244 (0.25 μM) and MS-275 (0.25 or 0.5 μM) on cell cycle distribution of MV4-11 and MOLM13 cells. Accumulation of cells in the sub-G1 phase fraction and decrease in the proportion of cells in the S phase of the cell cycle in both cell lines was significantly enhanced in the presence of both MS-275 and AZD6244 compared to either compound alone ($p<0.01$, Fig. 4C).

Effect of MS-275 and AZD6244 on apoptosis in leukemia cells. In addition, the combination of MS-275 and AZD6244 synergistically enhanced apoptosis of leukemia cells (Fig. 4D). For example, addition of either MS-275 (0.5 μM, 48 hrs) or AZD6244 (0.25 μM, 48 hrs) alone resulted in either 25 ± 5 or 14 ± 3 % of MV4-11 cells becoming annexin V positive, respectively. When these cells were exposed to a combination of both of these compounds, 38 ± 4 % of cells became annexin V positive ($p<0.01$, Fig. 4D).

Discussion

This study found that MS-275, a novel HDACI with selectivity against HDAC1, induced growth arrest and apoptosis of FLT3-ITD expressing MV4-11 and MOLM13 leukemia cells (Fig 1, Table 1). MS-275 induced acetylation of HSP90 and ubiquitination of FLT3, which provoked degradation of FLT3 via the proteasome pathway (Fig 3). Previous studies showed that HDAC6 deacetylased HSP90, stabilized chaperone complexes, and prevented client proteins from proteasome-mediated degradation. This study suggested that HDAC1 was also involved in deacetylation and stabilization of chaperone proteins.

MS-275 potently inhibited phosphorylation of FLT3, although blockade of p-ERK was partial. Inhibition of MEK/ERK signaling by AZD6244 synergistically enhanced MS-275-mediated growth arrest and apoptosis of MV4-11 and MOLM13 cells (Fig 4). Recent studies performed by other investigators also found that inhibition of MEK/ERK signaling enhanced HDACI-induced growth arrest and apoptosis of both colon cancer H-29 and chronic myeloid leukemia K562 and LAMA 84 cells [30,31]. They showed that enhanced growth inhibition and apoptosis mediated by the combination of the MEK inhibitor and HDACI was associated with synergistic induction of reactive oxygen species, mitochondrial damage, and caspase activation

[29,30]. Similarly, we and others have shown that inhibition of MEK/ERK signaling potentiated the action of tyrosine kinase inhibitors, including imatinib, ZD6474, and dasatinib in human leukemia cells [32-34].

In summary, MS-275 effectively induced growth arrest and apoptosis of leukemia cells possessing activating mutations of RTK; and these effects were potentiated by concomitant blockade of signaling by MEK/ERK. Inhibition of chaperone function of HSP90, at least in part, appeared to play an important role in MS-275-mediated growth arrest of leukemia cells. A recent phase 1 clinical trial with MS-275 has evaluated efficacy and safety of MS-275 in 38 individuals with relapsed or refractory AML [34]. MS-275 induced acetylation of histone H3/H4, expression of $p21^{waf1}$ and activation of caspase 3 in bone marrow mononuclear cells, although no responses were observed by classical criteria [35]. Future clinical studies should assess the effect of MS-275 in individuals with AML possessing FLT3-ITD. Concomitant administration of MS-275 and AZD6244 may represent a promising treatment strategy for individuals with AML.

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Author contribution: Takayuki Ikezoe contributed to the concept and design, interpreted and analyzed the data, and wrote the article. Chie Nishioka performed the experiments and wrote the article. Jing Yang performed the experiments. Seisho Taeuchi and H.Phillip Koeffler provided critical revision and intellectual content. Akihito Yokoyama provided important intellectual content and gave final approval.

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Figure legends

Fig. 1. Anti-proliferative effects of MS-275 against leukemia cells. (Panel A), Cell cycle analysis. MOLM13 and MV4-11 cells were cultured with MS-275 (0.1-1μM). After 48 hrs, cell cycle distribution of these cells was analyzed. Statistical significance was determined by paired *t*-test. Results represent the mean ± SD of 3 experiments performed in duplicate; *, $p < 0.01$, with respect to control. **(Panel B), Annexin V staining.** MOLM13 and MV4-11 cells were cultured with MS-275 (0.1-1μM). After 48 hrs, cells were stained with annexin-V/ propidium iodide, and analyzed by flow cytometry. Lower left quadrants, viable cells. Lower right quadrants, early apoptotic cells (Annexin V+, PI -). Upper right quadrants, nonviable, late apoptotic/necrotic cells (Annexin V+ and PI +). The numerical results represent the mean of triplicate plates, and a representative experiment is shown. **(Panel C), Western blot analysis.** MOLM13 and MV4-11 cells were cultured with MS-275 (0.5 or 1μM). After 48 hrs, cells were harvested and subjected to Western blot analysis. The membranes were sequentially probed with anti-Mcl-1, -Bcl-2, -Bcl-xL, -cleaved caspase 3 and 9, -cleaved PARP, and -α-tubulin antibodies.

Fig. 2. Effect of MS-275 on FLT3 and its downstream signals. MV4-11 **(Panel A)** and MOLM13 **(Panel B)** cells were cultured with various concentrations of MS-275

(0.5-2 μM). After 24 hrs, cells were harvested and the phosphorylated forms, as well as total amount of FLT3, Akt, ERK, and STAT5 expressing populations were measured by flow cytometry. The figure is representative of three similar experiments.

Fig. 3. (Panel A), MS-275 induces acetylation of HSP90 in leukemia cells. MV4-11 and MOLM13 cells were cultured with MS-275 (1μM). After 24 hrs, cells were harvested and percent of cells expressing the acetylated versus total HSP90 were analyzed by flow cytometry. Lower right quadrants (acetylated lysine -, HSP90 +). Upper right quadrants, (acetylated lysine +, HSP90 +). **(Panel B), MS-275 induces ubiquitination of FLT3 in leukemia cells.** MV4-11 and MOLM13 cells were cultured with MS-275 (1μM). After 24 hrs, cells were harvested and percent of cells expressing the ubiquitinated FLT3 versus total FLT3 were analyzed by flow cytometry. Upper left quadrants (FLT3 +, ubiquitin -). Upper right quadrants (FLT3 +, ubiquitin +). **(Panel C, D),** MV4-11 and MOLM13 cells were cultured with MS-275 (1μM). After 24 hrs, cells were harvested and lysates were prepared. The lysates were immunoprecipitated with anti-HSP90 **(Panel C)** or -FLT3 **(Panel D)** antibody. The precipitated samples were subjected to Western blot analysis and the membranes were sequentially probed with the indicated antibodies. **Immunoprecipitation. (Panel E), PS-341 blocks MS-275-mediated downregulation of FLT3.** MV4-11 and freshly-isolated leukemia

cells (#1 and #2 in Table 2) were pre-incubated with either proteasome inhibitor PS-341(1nM) or control diluent. After 1hr, cells were exposed to either MS-275 (1μM) or control diluent for 24hrs. Cells were harvested and the percent of FLT3 expressing population was measured by flow cytometry. Results represent one of the experiments performed independently three times.

Fig. 4. AZD6244 enhanced the action of MS-275 in leukemia cells. (Panel A) Analysis of p-ERK by FACS. MV4-11 and MOLM13 cells were cultured with either MS-275 (0.5 μM) or control diluent. After 24 hours, cells were exposed to either AZD6244 (0.25 μM) or control diluent for 15 minutes. Cells were harvested, incubated with anti-p-ERK antibody for 30 minutes at room temperature, and analyzed by flow cytometry. Results are representative of two experiments performed in duplicate plates. p-ERK-positive population was quantified using the CellQuest software package. **(Panel B) MTT assay.** MV4-11 and MOLM13 cells were cultured in the presence of either MS-275 (0.1–1 μM) and/or AZD6244 (0.1–1 μM). After 2 days, cell proliferation was measured by MTT assay, and concentration of each compound that induced 25, 50, or 75 % growth inhibition (IC_{25}, IC_{50}, IC_{75}) was determined (data not shown). The combination index (CI) of MS-275 and AZD6244 was calculated using the median effect method. CI values less than 1 indicate synergy, a CI = 1 indicates an additive

effect, and a CI greater than 1 indicates antagonism between the two agents. **(Panel C) Cell cycle analysis.** MV4-11 and MOLM13 cells were cultured with either MS-275 (0.25 or 0.5 μM) and/or AZD6244 (0.25 μM). After 2 days, the cell cycle distribution of these cells was analyzed. The statistical significance of difference between populations in either sub-G1 or S phase of cell cycle induced by either MS-275 and/or AZD6244 was determined by one-way analysis of variance (ANOVA) followed by Boneferroni's multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate; *, $p< 0.01$, with respect to control. **(Panel D), Annexin V staining.** MV4-11 and MOLM13 cells were cultured with either MS-275 (0.25 or 0.5 μM) and/or AZD6244 (0.25 μM). After 2 days, cells were stained with annexin V/propidium iodide, and analyzed by flow cytometry. The statistical significance of difference between annexin V positive populations produced by MS-275 and/or AZD6244 was determined by ANOVA followed by Boneferroni's multiple comparison tests. Results represent the mean ± SD of 3 experiments performed in triplicate; *, $p< 0.01$, with respect to control.

Table 1. Inhibition of the proliferation of leukemia cells by MS-275

Cell Line	Gene Alterations	(μM)*
Eosinophilic leukemia		
EOL-1	FIP1L1/PDGFRα	0.74
Biphenotypic leukemia		
MV4-11	FLT3-ITD	0.74
MOLM13	FLT3-ITD	0.67
Myeloid leukemia		
Kasumi-1	c-KIT (Asn822Lys)	0.70
U937		4.6
THP-1		2.0
PL21		1.3
Kcl-22	BCR/ABL	not achieved
K562	BCR/ABL	3.4
KU812	BCR/ABL	1.7
Lymphoblastic leukemia		
PALL-2	BCR/ABL	0.40

*Concentration of MS-275 that induced 50 % growth inhibition (IC50) was calculated from dose-response curves.

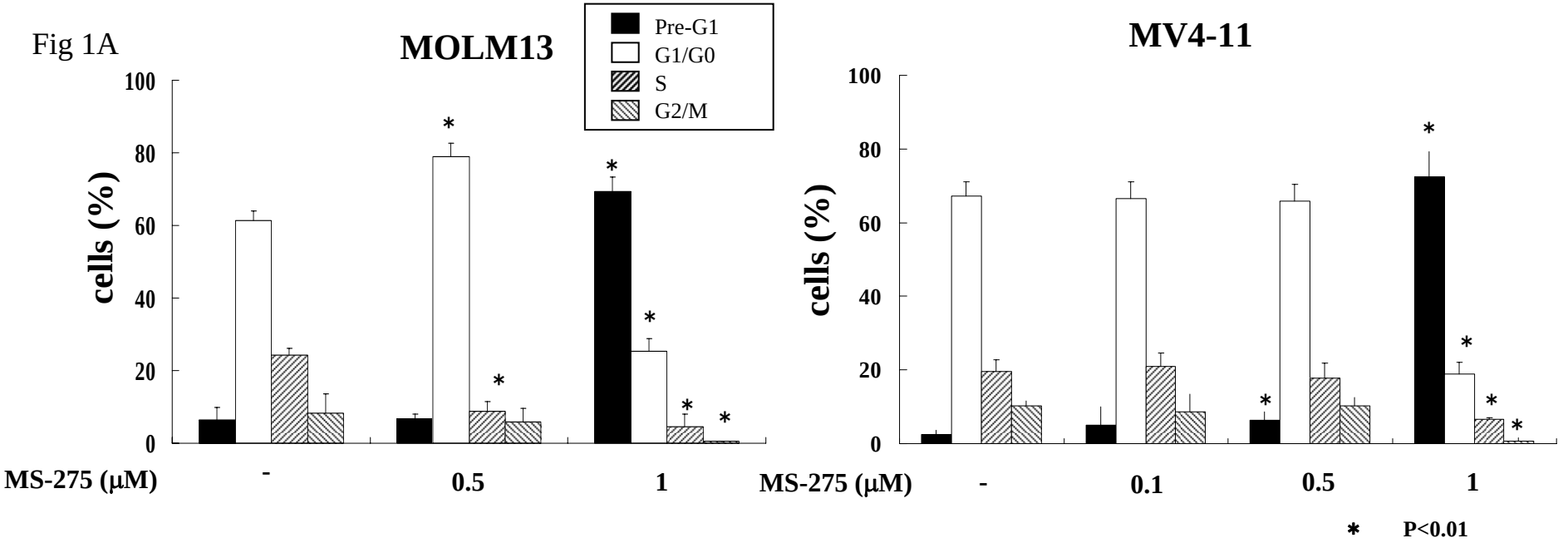
Table 2. Patient Clinical Characteristics

Pt. #	Age/Sex	FAB	WBC $x10^6$/L	% Blast	FLT3 mutation	Previous treatment	IC50 (μM)
1	61/M	M1	200,000	99%	ITD	No	0.3
2	55/F	M1	188,400	99%	ITD	No	0.1

The freshly isolated leukemia cells were cultured in the presence of various concentrations of MS-275 (0.01-1μM). After 2 days, the proliferation of cells was measured by ^{3}[H]-thymidine-uptake. The concentration of MS-275 that induced 50 % growth inhibition (IC50) was calculated from the dose-response curves.

Pt, patient; M, male; F, female; FAB, French-Amercan-British (leukaemia classification); ITD, internal tandem duplication; WBC, white blood cells.

Fig 1A


MOLM13
Pre-G1
G1/G0
S
G2/M
cells (%)
100
80
60
40
20
0
MS-275 (μM)
-
0.5
1
MV4-11
cells (%)
100
80
60
40
20
0
MS-275 (μM)
-
0.1
0.5
1
*
P<0.01

B

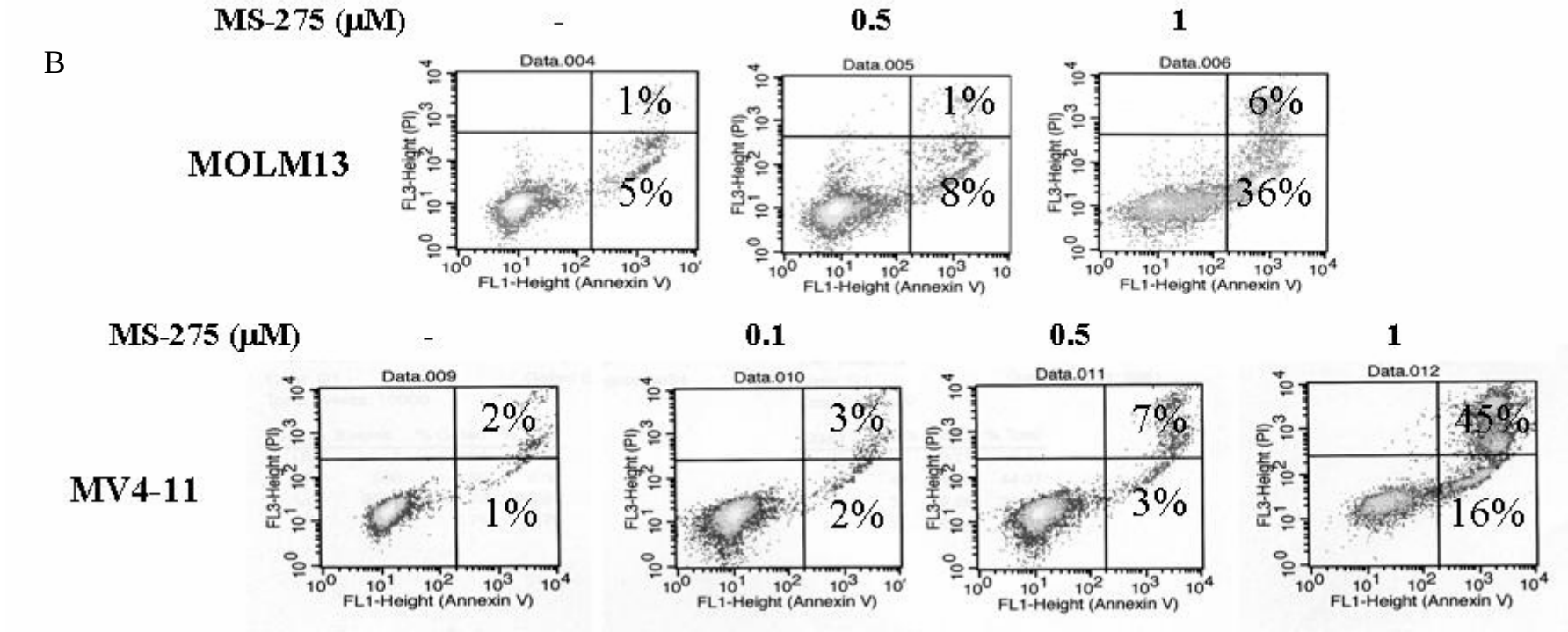

MS-275 (μM)
-
0.5
1
MOLM13
Data.004
1%
5%
Data.005
1%
8%
Data.006
6%
36%
FL3-Height (PI)
FL1-Height (Annexin V)
MS-275 (μM)
-
0.1
0.5
1
MV4-11
Data.009
2%
1%
Data.010
3%
2%
Data.011
7%
3%
Data.012
45%
16%

Fig 1C

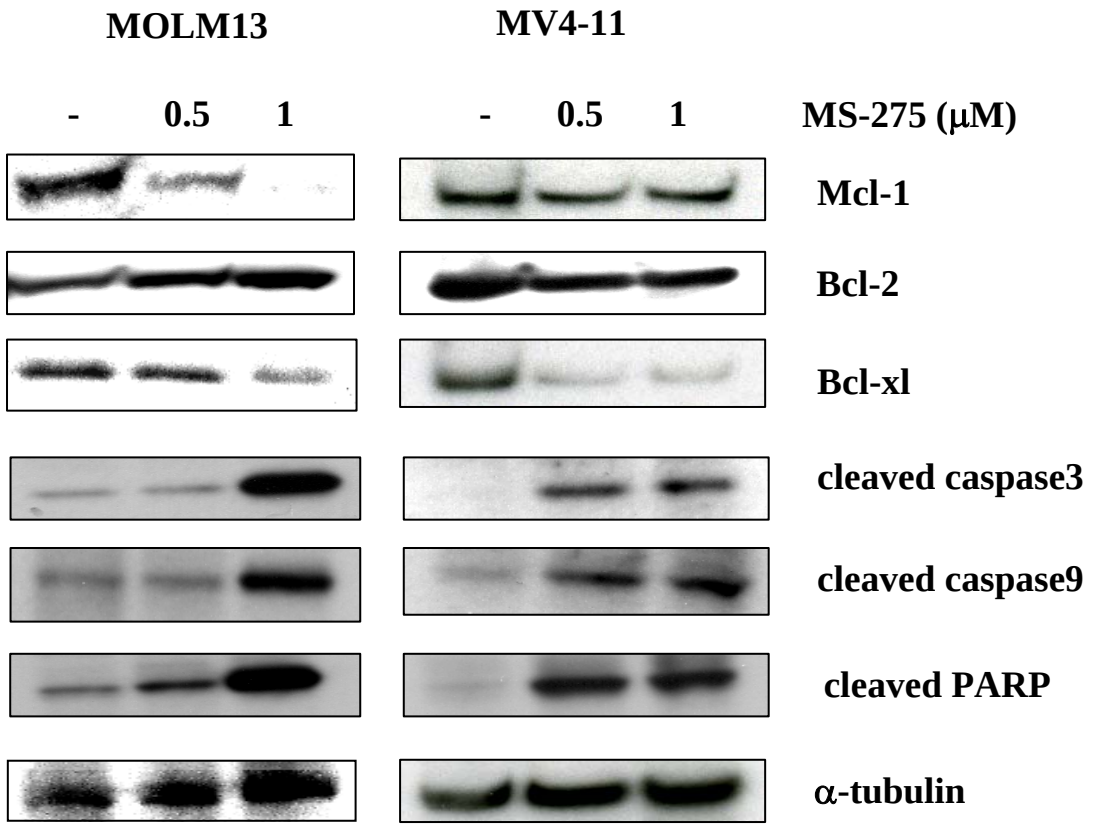

MOLM13
MV4-11
- 0.5 1
- 0.5 1
MS-275 (μM)
Mcl-1
Bcl-2
Bcl-xl
cleaved caspase3
cleaved caspase9
cleaved PARP
α-tubulin

Fig 2A **MV4-11**

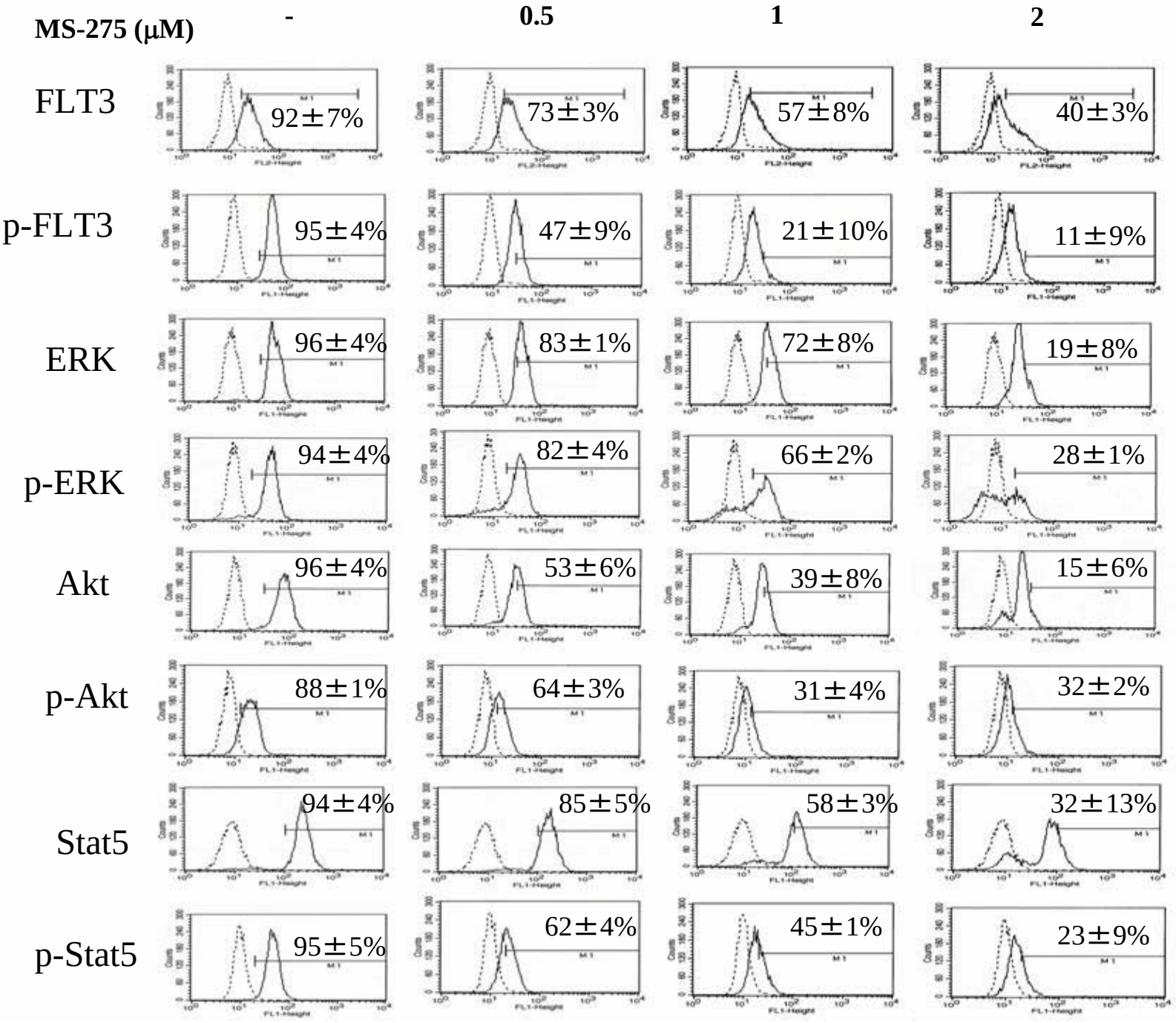

MS-275 (μM)
-
0.5
1
2
FLT3
92±7%
73±3%
57±8%
40±3%
p-FLT3
95±4%
47±9%
21±10%
11±9%
ERK
96±4%
83±1%
72±8%
19±8%
p-ERK
94±4%
82±4%
66±2%
28±1%
Akt
96±4%
53±6%
39±8%
15±6%
p-Akt
88±1%
64±3%
31±4%
32±2%
Stat5
94±4%
85±5%
58±3%
32±13%
p-Stat5
95±5%
62±4%
45±1%
23±9%

Fig 2B

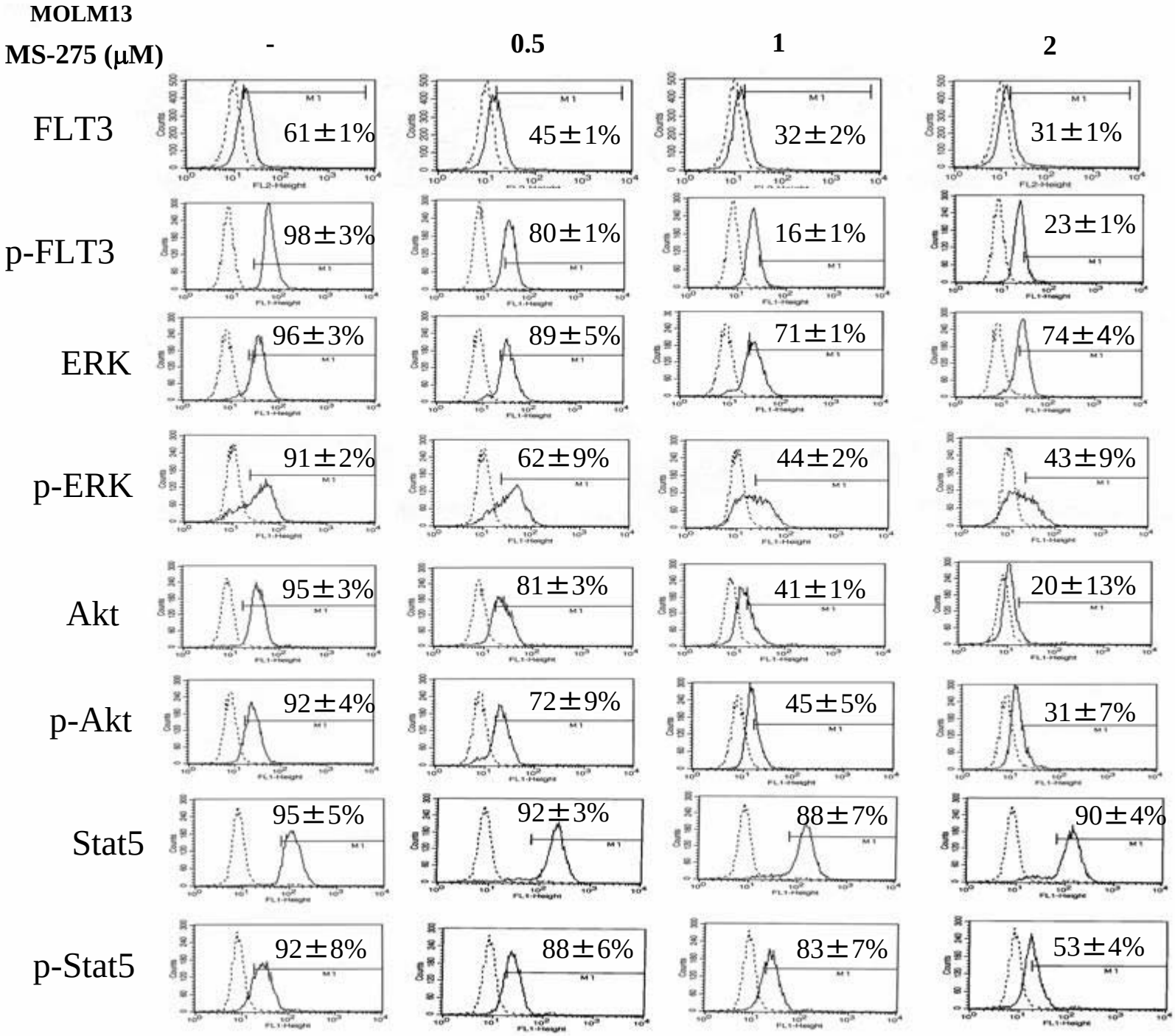

MOLM13
MS-275 (μM)
-
0.5
1
2
FLT3
61±1%
45±1%
32±2%
31±1%
p-FLT3
98±3%
80±1%
16±1%
23±1%
ERK
96±3%
89±5%
71±1%
74±4%
p-ERK
91±2%
62±9%
44±2%
43±9%
Akt
95±3%
81±3%
41±1%
20±13%
p-Akt
92±4%
72±9%
45±5%
31±7%
Stat5
95±5%
92±3%
88±7%
90±4%
p-Stat5
92±8%
88±6%
83±7%
53±4%

Fig 3A

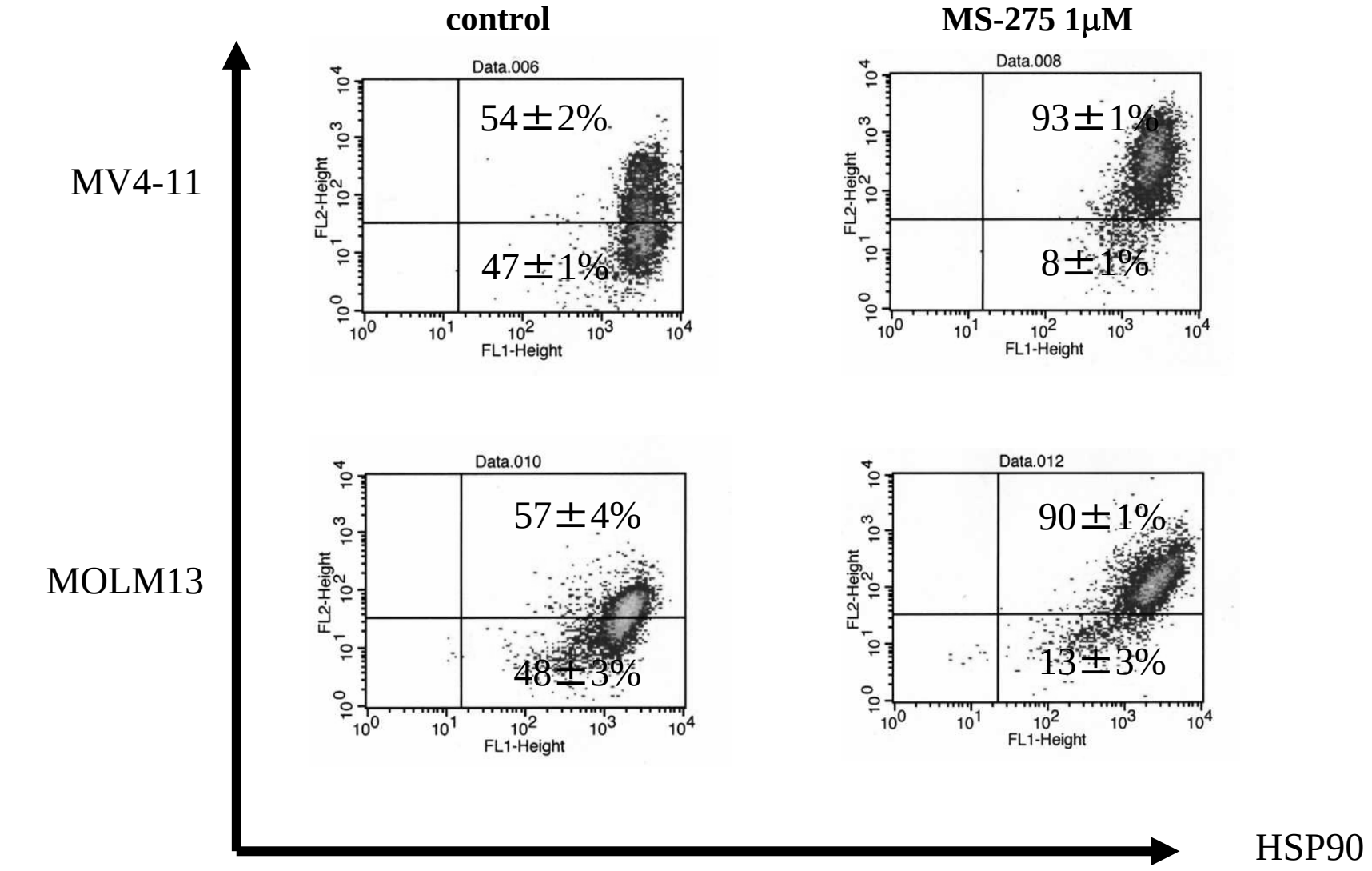

Acetylated-HSP90
control
MS-275 1μM
MV4-11
MOLM13
HSP90
Data.006
54±2%
47±1%
Data.008
93±1%
8±1%
Data.010
57±4%
48±3%
Data.012
90±1%
13±3%
FL1-Height
FL2-Height

Fig 3B

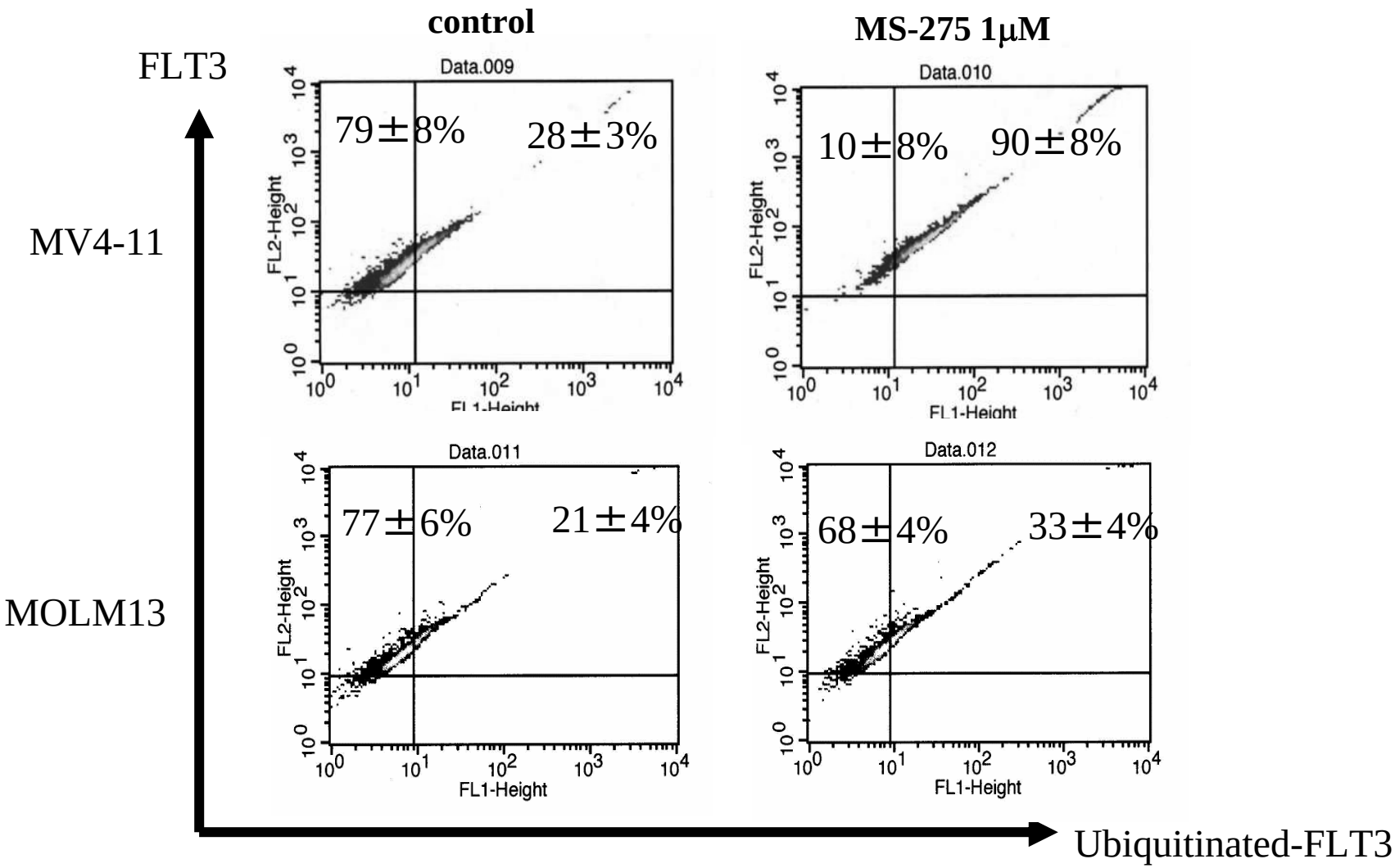

control
MS-275 1μM
FLT3
MV4-11
MOLM13
Data.009
79±8%
28±3%
Data.010
10±8%
90±8%
Data.011
77±6%
21±4%
Data.012
68±4%
33±4%
FL2-Height
FL1-Height
Ubiquitinated-FLT3

Fig 3C

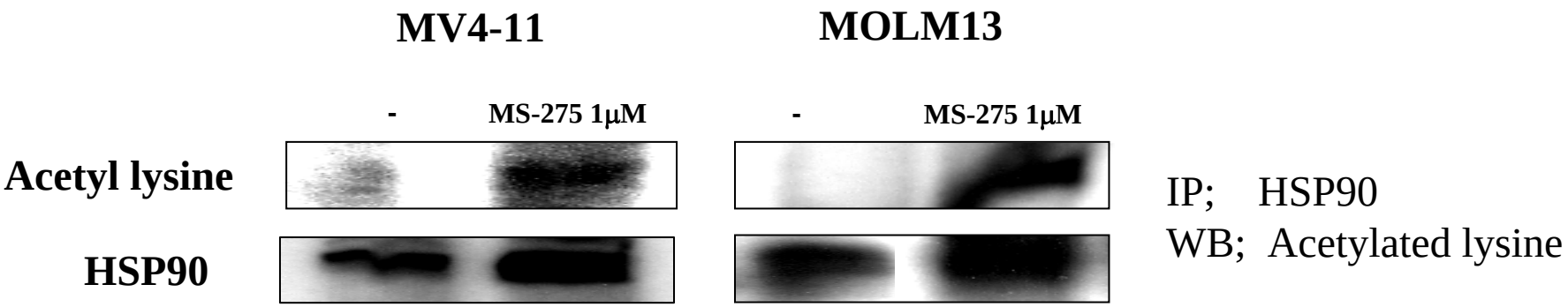

MV4-11
MOLM13
-
MS-275 1μM
-
MS-275 1μM
Acetyl lysine
HSP90
IP; HSP90
WB; Acetylated lysine

Fig 3D

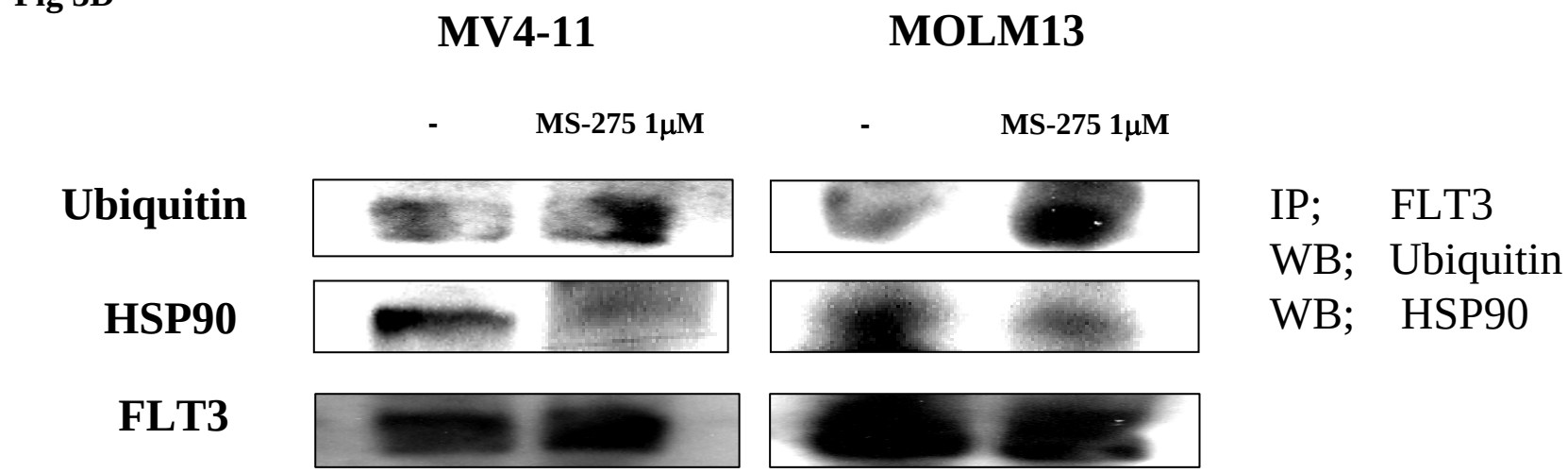

MV4-11
MOLM13
-
MS-275 1μM
-
MS-275 1μM
Ubiquitin
HSP90
FLT3
IP; FLT3
WB; Ubiquitin
WB; HSP90

Fig 3E

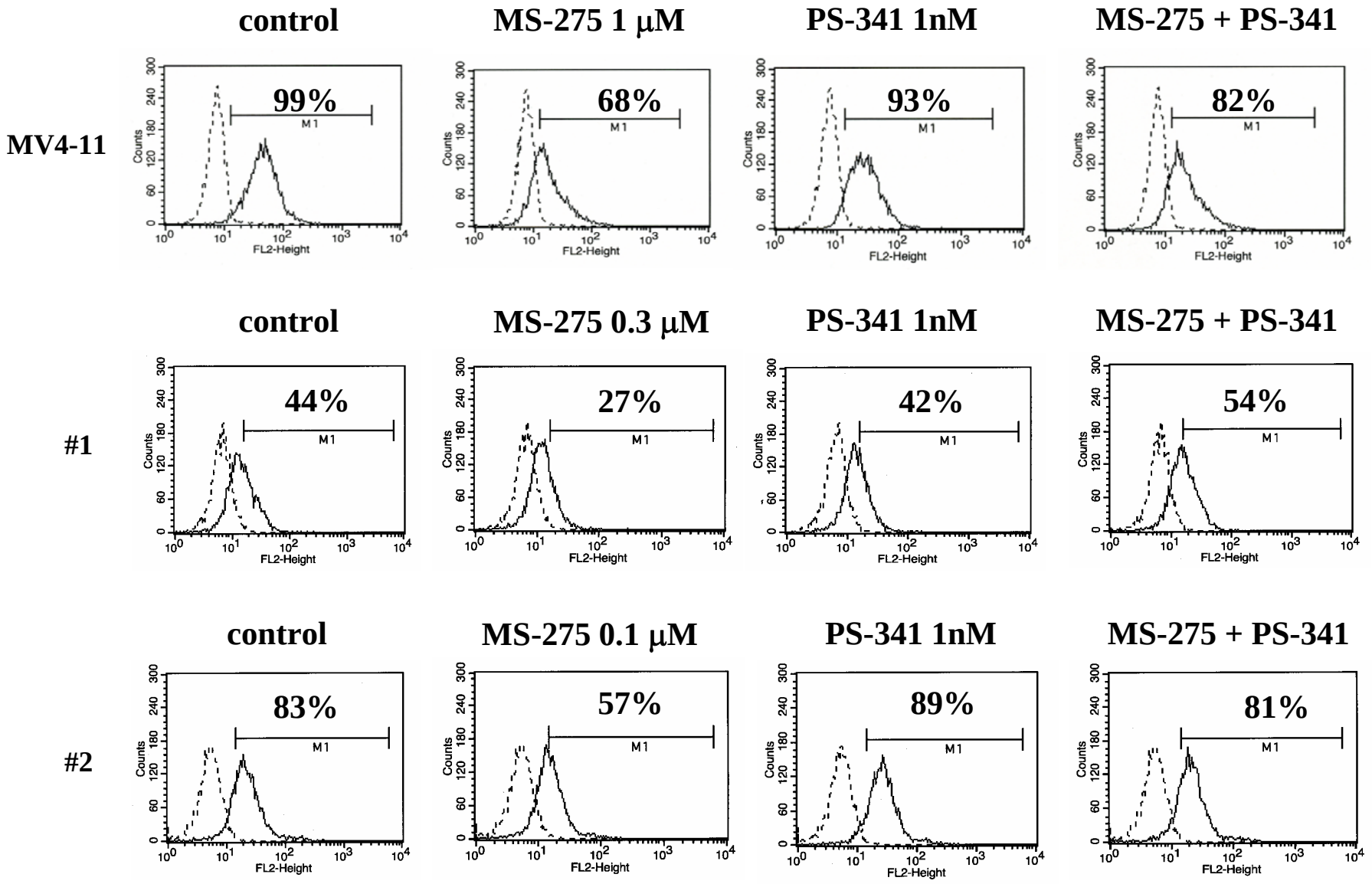

control
MS-275 1 μM
PS-341 1nM
MS-275 + PS-341
MV4-11
99%
68%
93%
82%
control
MS-275 0.3 μM
PS-341 1nM
MS-275 + PS-341
#1
44%
27%
42%
54%
control
MS-275 0.1 μM
PS-341 1nM
MS-275 + PS-341
#2
83%
57%
89%
81%
M1
Counts
FL2-Height
FLT3

Fig 4A

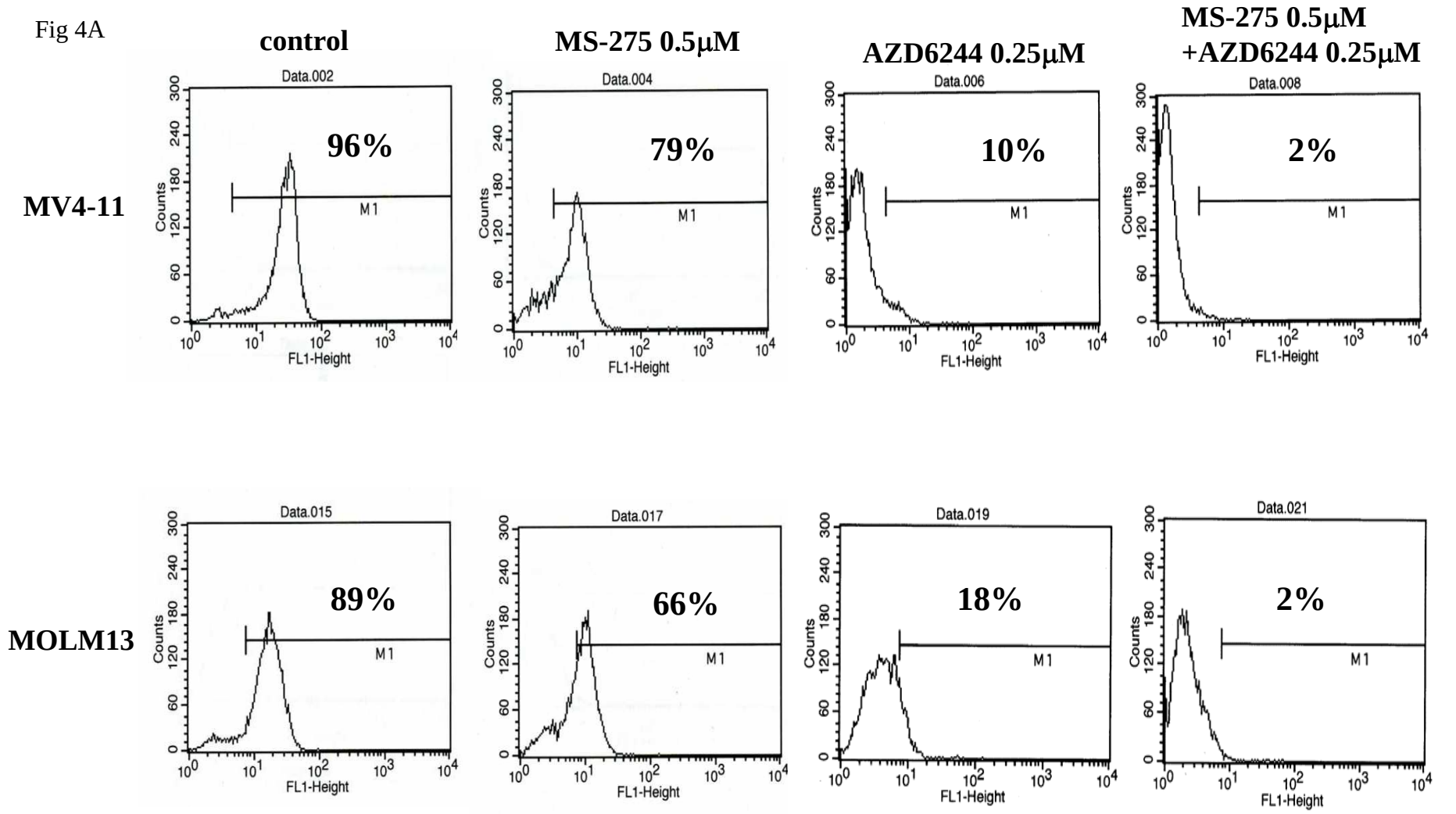

control
MS-275 0.5μM
AZD6244 0.25μM
MS-275 0.5μM
+AZD6244 0.25μM
MV4-11
MOLM13
Data.002
Data.004
Data.006
Data.008
Data.015
Data.017
Data.019
Data.021
96%
79%
10%
2%
89%
66%
18%
2%
M1
Counts
FL1-Height
p-ERK

Fig 4B

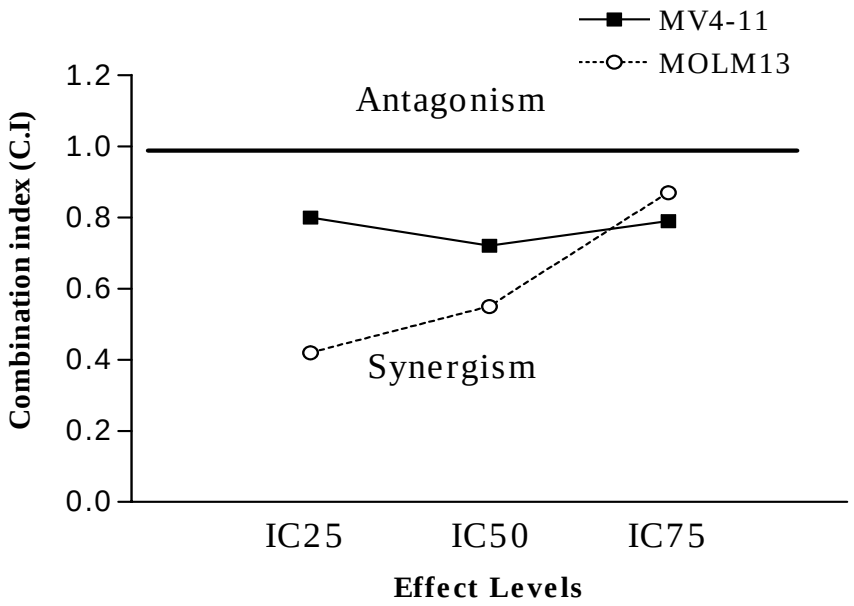

MV4-11
MOLM13
Antagonism
Synergism
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Combination index (C.I)
IC25
IC50
IC75
Effect Levels

Fig 4C

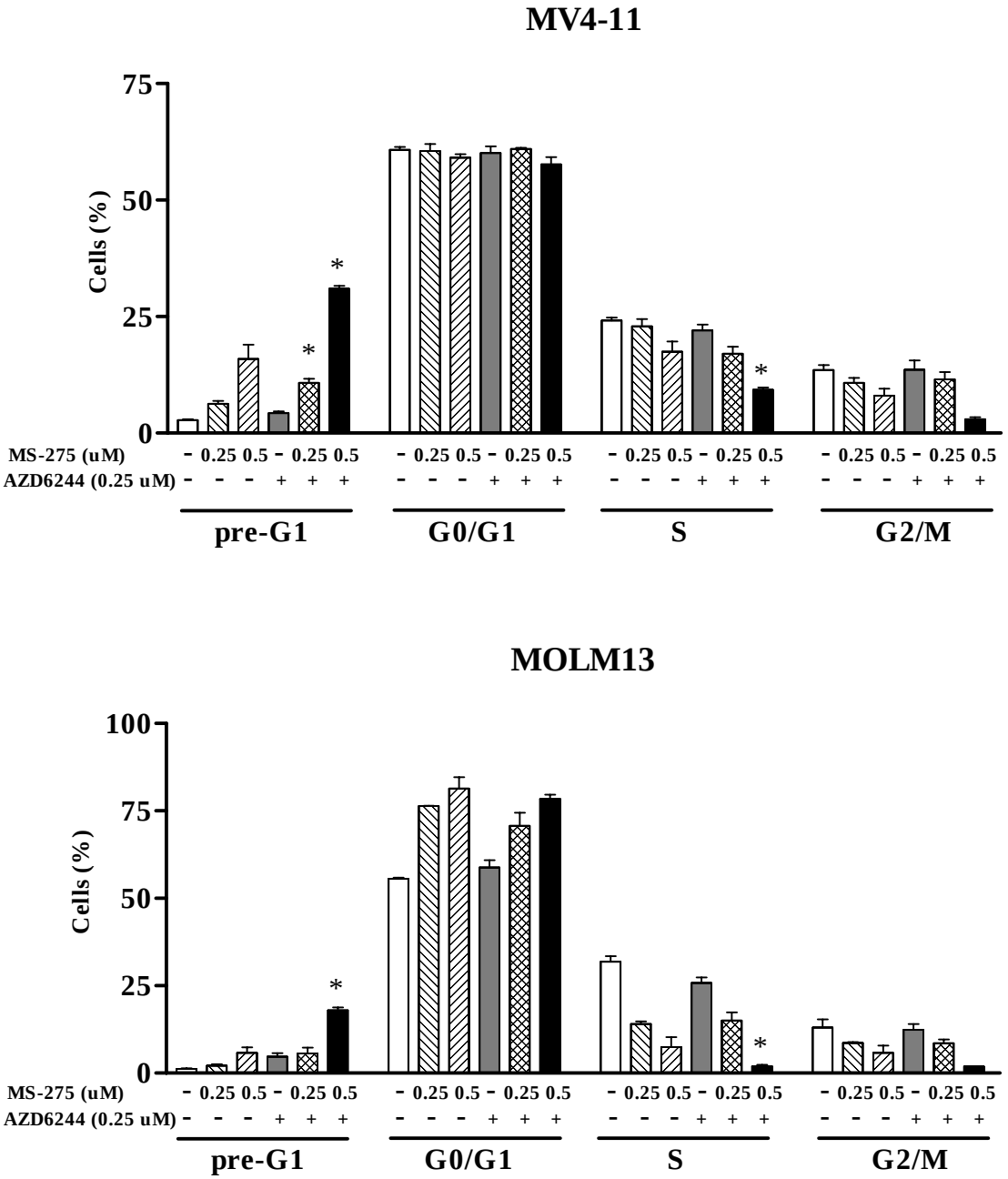
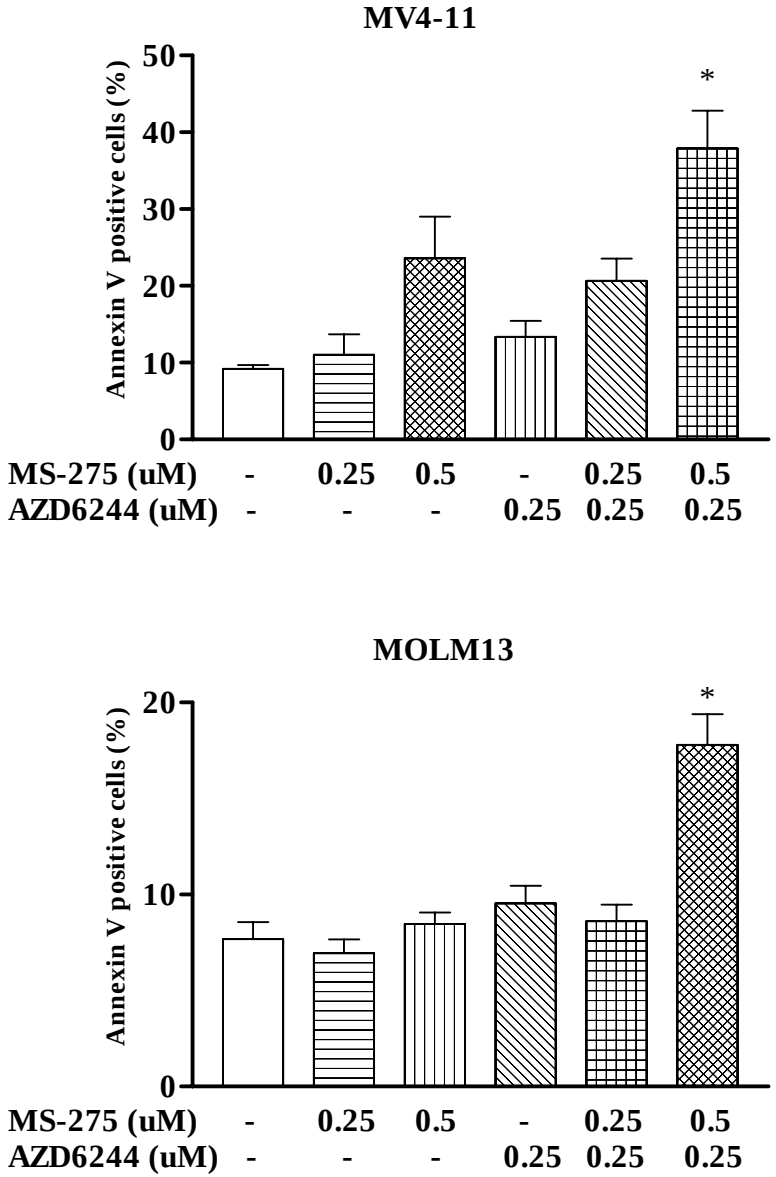

MV4-11
Cells (%)
75
50
25
0
MS-275 (uM) - 0.25 0.5 - 0.25 0.5 - 0.25 0.5 - 0.25 0.5 - 0.25 0.5 - 0.25 0.5
AZD6244 (0.25 uM) - - - + + + - - - + + + - - - + + + - - - + + +
pre-G1
G0/G1
S
G2/M
MOLM13
Cells (%)
100
75
50
25
0
MS-275 (uM) - 0.25 0.5 - 0.25 0.5 - 0.25 0.5 - 0.25 0.5 - 0.25 0.5 - 0.25 0.5
AZD6244 (0.25 uM) - - - + + + - - - + + + - - - + + + - - - + + +
pre-G1
G0/G1
S
G2/M

Fig 4D


MV4-11
Annexin V positive cells (%)
50
40
30
20
10
0
*
MS-275 (uM) - 0.25 0.5 - 0.25 0.5
AZD6244 (uM) - - - 0.25 0.25 0.25
MOLM13
Annexin V positive cells (%)
20
10
0
*
MS-275 (uM) - 0.25 0.5 - 0.25 0.5
AZD6244 (uM) - - - 0.25 0.25 0.25