Blockade of mTOR signaling potentiates the ability of histone deacetylase inhibitor to induce growth arrest and differentiation of acute myelogenous leukemia cells.

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The abbreviations are: AML, acute myelogenous leukemia; APL, acute promyelocytic leukemia; HDACI, histone deacetylase inhibitor; C/EBPε, CCAAT enhancer binding protein ε.

Abstract

This study found that MS-275, a novel synthetic benzamide histone deacetylase inhibitor (HDACI), blocked Akt/mTOR signaling in acute myelogenous leukemia (AML) HL60 and acute promyelocytic leukemia (APL) NB4 cells, as assessed by decreased levels of the phosphorylated (p)-Akt, p-p70 ribosomal S6 kinase (p70S6K), and p-S6K by Western blot analysis. Interestingly, further inactivation of mTOR by rapamycin analogue RAD001 (everolimus) significantly enhanced MS-275-mediated growth inhibition and apoptosis of these cells in parallel with enhanced upregulation of $p27^{kip1}$ and downregulation of c-Myc. In addition, RAD001 potentiated the ability of MS-275 to induce differentiation of HL60 and NB4 cells, as measured by expression of CD11b cell surface antigens, as well as reduction of nitroblue tetrazolium. Importantly, RAD001 potentiated the ability of MS-275 to induce expression of the myeloid differentiation-related transcription factor CCAAT enhancer binding protein ε in these cells in association with enhanced acetylation of histone H3 on its promoter. Furthermore, RAD001 (5 mg/kg) significantly enhanced the effects of MS-275 (10 mg/kg) to inhibit proliferation of HL60 tumor xenografts in nude mice without adverse effects. Taken together, concomitant administration of a HDACI and a mTOR inhibitor may be a promising treatment strategy for the individuals with a subset of human

leukemia.

Introduction

Acute myelogenous leukemia (AML) is a lethal disease, resulting from the clonal expansion and accumulation of hematopoietic stem cells arrested at various stages of development.[1] Intensification of chemotherapy has led to remissions in 70-85% of individuals with AML; and post-remission relapses occur frequently.[2,3] Recent studies found that the genotype of mutant *nucleophosmin (NPM1)* without *FLT3 internal tandem duplications* and the mutant *CCAAT/enhancer binding protein* α *(CEBPA)* were significantly associated with complete remission in the individuals with cytogenetically normal AML.[4] In addition, these studies found that individuals with wild-type *NPM1* benefited from hematopoietic stem cell transplantation from an HLA-matched related donor[4]; however, regimen-related toxicities and relapse remain serious problems.[5]

Histone deacetylase inhibitors (HDACIs) have emerged as a potentially promising new class of anticancer drugs.[6] These include the hydroxamic acid-derived suberoylanilide hydroxamic acid (SAHA, Vorinostat), LBH 589, tricostatin A (TSA), cyclic depsipeptide FR901228, valproic acid and the benzamide MS-275.[7] HDACIs induce growth arrest and apoptosis of cancer cells by manipulating the transcription of genes involved in regulation of the cell cycle and apoptosis, as well as differentiation.[6]

For example, we previously showed that SAHA and MS-275 induced growth arrest and apoptosis of human mantle cell lymphoma and adult T-cell leukemia cells, respectively, in association with induction of histone acetylation of the p21^{waf1} promoter region, resulting in upregulation of the p21^{waf1} protein.[7,8]

Promyelocytic leukemia/retinoic acid receptor α (PML/RARα) fusion protein, generated by chromosomal translocation in acute promyelocytic leukemia (APL) recruits histone deacetylase (HDAC) and repressor complexes on retinoic acid (RA) target promoters and silences RA signaling, resulting in differentiation block of hematopoietic stem cells.[9] RA, at pharmacological concentration, dissociates HDAC and repressor complexes and recruits co-activators to the promoter and activates RA signaling, resulting in terminal differentiation.[9] HDACI modifies chromatin structure and increases promoter accessibility, leading to transcriptional activation.[10] HDACI increased sensitivity of APL cells to RA-induced differentiation.[11]

Valproic acid (VPA), the antiepileptic drug, induced differentiation and apoptosis of AML cells in a murine model via inhibition of HDAC.[12] The clinical efficacy of VPA as monotherapy or in combination with all-trans-retinoic acid (ATRA) has recently been shown in the individuals with AML and myelodysplastic syndromes.[13]

The CCAAT enhancer binding protein ε (C/EBPε) is a family of transcription

factors that regulates proliferation and differentiation of numerous cell types including granulocytes.[14,15] We and others previously showed that C/EBPε is pivotal to granulocytic differentiation.[16,17] C/EBPε modulates levels of various cell cycle regulating molecules during differentiation; over-expression of C/EBPε down-regulated levels of c-Myc and up-regulated levels of cyclin-dependent kinase inhibitor $p27^{kip1}$ in myeloid cells.[18,19]

The serine/threonine kinase mammalian target of rapamycin (mTOR) is activated by PI3K/Akt signaling and regulates cell proliferation in part by ribosomal protein translation and the initiation of cap-dependent translation.[20,21] mTOR phosphorylates p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP-1) and increases the translation of mRNAs with long, highly structured 5'-untranslated regions, such as cyclin D1 and c-Myc which regulate cell cycle transition from G1 to S phase.[20,21] The mTOR inhibitors, rapamycin or its analog RAD001 (everolimus) have been shown to be active against many types of solid tumors as well as subsets of leukemia, and are now being under evaluation in clinical trials.[22]

This study found that MS-275 induced growth arrest, apoptosis, and differentiation of AML cells in association with blockade of mTOR signaling. Further

inhibition of mTOR signaling by RAD001 significantly potentiated the ability of MS-275 in AML cells in vitro and in vivo. Notably, RAD001 augmented MS-275-mediated acetylation of histone H3 on *C/EBP*ε promoter, resulting in enhanced upregulation of *C/EBP*ε in AML cells. Moreover, RAD001 significantly enhanced MS-275-inducded growth inhibition of HL60 tumor xenografts in nude mice without adverse effects.

Materials and Methods

Reagents. MS-275 and RAD001 were provided by Schering AG (Berlin, Germany) and Novartis (Basel, Switzerland), respectively. These reagents were dissolved in 100% dimethyl sulfoxide (DMSO) to a stock concentration of 10^{-2} M and stored at -80°C. For animal studies, RAD001 and MS-275 were diluted in double-distilled water just before administration by gavage.

Cells. HL60 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). NB4 cell line was a kind gift from M. Lanotte (St Louis Hospital, Paris, France). Cells were grown in RPMI 1640 containing 10% fetal bovine serum.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Cells ($5x10^5$ /mL) were cultured with various concentrations of MS-275 (0.1-1 µM) and/or RAD001 (3-100 nM) for 2 days in 96-well plates. MTT assay was performed as previously described.[23] All experiments were performed in triplicate and repeated at least three times.

Cell cycle analysis by flow cytometry. Cell cycle analysis was performed on HL60 and NB4 cells incubated with various concentrations of either MS-275 and/or RAD001, as previously described.[24]

Apoptosis assays. The ability of MS-275 and/or RAD001 to induce apoptosis in

leukemia cells was measured using the annexin V-FITC apoptosis detection kit (Pharmingen, Inc., San Diego, CA), as previously described.[25]

Assays for differentiation of leukemia cells. The HL60 and NB4 cells ($5x10^5$/ml) were seeded in 12-well plates and were cultured with various concentrations of either MS-275 and/or RAD001. After 2 days, expression of CD11b on their cell surface was measured by staining with a Fluorescein (FITC)-cojugated anti-CD11b mAb (Dako, Glostrup, Denmark) using flow cytometry. For NBT measurements, cells (5×10^5/mL) were incubated in 12-well plates (Corning, Corning, NY) for 2 days as described above. After incubation, each cell suspension was mixed with an equal volume of RPMI 1640 containing 1 mg/ml NBT (Sigma) and 10^{-6} mol/L 12-0-tetradecanoyl-13-acetate (TPA; Sigma) for 20 minutes at 37°C. The cells were washed in phosphate-buffered saline (PBS), cytocentrifuged, fixed in methanol for 5 minutes, and stained with Gram safranin for 10 minutes at room temperature; 300 cells were analyzed for blue dye using light microscopy, as previously described.[26]

Western blot analysis. Western blot analysis was performed as described previously.[27] The membrane was probed sequentially with the antibodies. Anti-Akt (Cell Signaling Technology, Beverly, MA), p-Akt (Cell Signaling Technology), p-ERK (Cell Signaling Technology), ERK (Cell Signaling Technology), $p27^{kip1}$ (Santa Cruz Biotechnology,

Santa Cruz, CA) C/EBPε (Santa Cruz Biotechnology), c-Myc (Santa Cruz Biotechnology), p70S6K (Cell Signaling Technology), p-p70S6K (Cell Signaling Technology), S6K (Cell signaling Technology), p-S6K (Cell Signaling Technology) and -α-tubulin (Santa Cruz Biotechnology) antibodies were used.

Chromatin immunoprecipitation (Chip) assay. NB4 and HL60 cells ($1x10^6$/ml) were cultured with MS-275 (0.3 μM) and/or RAD001 (10 nM) for 2 days. Untreated cells were used as controls. Formaldehyde was added to the cells to a final concentration of 1%, and the cells were incubated at 37℃ for 10 min. The cells were collected and subjected to chromatin immunoprecipitation utilizing reagents provided by LPBIO (Lake Placid, NY), as previously described.[28] Anti-acetylated histone H3 antibody (Upstate) was used for immunoprecipitation. Immunoprecipitated DNA was recovered and used as a template for real-time-PCR. The primers for C/EBPε promoter were as follows: forward, 5'-GGAAAAGGAAGCAGAGCAGA-3', reverse, 5'-TCGATCTTCTGCCCTAGACC-3'. Real-time PCR was carried out by using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), as previously described.[29] The amplified sequences were normalized to those form cross-linked DNA/protein complexes which were not immunoprecipitated with anti-acetylated histone H3 antibody, as previously described.[30]

Mice. Female immune deficient BALB/c nude mice at 4 weeks of age were purchased from JAPAN SLC. Inc. (Shizuoka, Japan), and were maintained in pathogen-free conditions with irradiated chow. Animals were bilaterally, subcutaneously (s.c.) injected with 2 x 10^6 HL60 cells/tumor in 0.1 ml Matrigel (Collaborative Biomedical Products, Bedford, MA). When HL60 cells formed palpable tumors, mice were divided randomly into four groups receiving control (n=5), or either RAD001 (n=5) or MS-275 (n=5), or combination of both RAD001 and MS-275 (n=5). MS-275 (10mg/kg) was given to mice by oral administration six times during two weeks. RAD001 (5mg/kg) was given to mice by oral administration four times during a week. The dose of these agents was determined by our preliminary studies (data not shown). Control diluent was given to the untreated control mice. Body weight and tumors were measured twice a week. Tumor sizes were calculated by the formula: a x b x c, where “a” is the length and “b” is the width and “c” is the height in millimeters. At the end of the experiment, animals were sacrificed by CO_2 asphyxiation and tumor weights were measured after their careful resection. Tumor tissue was collected for analysis. The experiments were approved by the Review Board of Kochi University.

Statistical Analysis. Statistical analysis was performed to assess the difference between two groups under multiple conditions by one-way ANOVA followed by Boneferroni’s

multiple comparison tests using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

Results.

MS-275 inhibits Akt/mTOR signaling in AML cells. Exposure of HL60 and NB4 cells to MS-275 (0.1-1 μM, 48 hrs) inhibited their proliferation with the concentration that inhibits cell proliferation by 50 % (IC50) values of approximately 0.8 and 0.9 μM, respectively as measured by MTT assay (figure not shown). Western blot analyses showed that both HL60 and NB4 cells constitutively expressed phosphorylated forms of Akt and mTOR substrates p70S6K and S6K (Fig 1). Exposure of these cells to MS-275 (0.1-1 μM, 48 hours) effectively down-regulated levels of the phosphorylated forms of Akt, p70S6K, and S6K without modulating total amount of these proteins, suggesting that MS-275 inhibited the activation of the mTOR signal pathway (Fig 1). These cells also constitutively expressed phosphorylated forms of ERK, which was down-regulated after exposure to MS-275 (0.1-1 μM, 48 hours); however, effects of MS-275 (0.3 or 1 μM) on p-ERK appeared to be less potent compared to those on Akt signaling (Fig 1).

Blockade of mTOR signaling potentiates the ability of HDACI to inhibit proliferation of AML cells. MS-275 blocked Akt/mTOR signaling in AML cells (Fig 1). We, therefore, explored the drug interaction of MS-275 and RAD001, the inhibitor of mTOR in these cells. RAD001 (10 nM) significantly potentiated the ability of MS-275 (0.1-1 μM) to inhibit the proliferation of HL60 and NB4 cells as measured by MTT

assay on the second day of culture (Figs. 2A, B)

Blockade of mTOR signaling potentiates the ability of MS-275 to induce apoptosis of leukemia cells. Cell cycle analysis showed that exposure of HL60 and NB4 cells to MS-275 (1 μM, 48 hrs) induced accumulation of cells in the pre-G1 phase of the cell cycle, a feature characteristic of apoptosis (Figs. 2C, D). RAD001 (10 nM, 48 hrs) alone did not induce accumulation of cells in pre-G1 phase of cell cycle; however, when MS-275 was combined with RAD001, the proportion of cells in pre-G1 phase of the cell cycle dramatically increased compared to either alone ($p<0.01$) with respect to either compound alone; Figs. 2 C, D. For example, either MS-275 (1 μM, 48 hrs) or RAD001 (10 nM, 48 hrs) alone caused an accumulation of 52 ± 10 % and 4 ± 2 % (mean ± SD) of HL60 cells in the pre-G1 phase, respectively. When these cells were exposed simultaneously to both of these compounds at the same concentration, the proportion of cells in the pre-G1 phase of the cell cycle increased to a mean 87 ± 3.8 % (Fig. 2C). The ability of RAD001 to enhance MS-275-induced apoptosis of leukemia cells was further confirmed by Annexin V staining. Exposure of HL60 cells to MS-275 (1 μM, 48 hrs) alone resulted in 68 ± 4 % of HL60 cells becoming annexin V positive. On the other hand, exposure of HL60 cells to RAD001 (10 nM, 48 hrs) alone did not induce apoptosis of HL60 cells. When these cells were exposed to the combination of both at

the same concentration, a mean 89 ± 1 % of cells became annexin V positive ($p<0.01$ with respect to either compound alone; Fig. 2E). Similarly, MS-275-induced apoptosis of NB4 cells was significantly potentiated in the presence of RAD001 (Fig. 2F).

RAD001 enhanced MS-275-induced differentiation of AML cells. We examined the ability of MS-275 to induce differentiation of AML cells by quantifying CD11b expressing population, a marker of myeloid differentiation, by flow cytometry. Exposure of HL60 or NB4 cells to MS-275 (0.1 or 0.3 μM) for 48 hrs induced expression of CD11b antigen on their cell surface in a dose-dependent manner. Effect of RAD001 on the expression of CD11b was negligible; however, when MS-275 (0.1 or 0.3 μM) was combined with RAD001 (10 nM), proportion of cells expressing CD11b antigen was dramatically increased (Figs. 3A, B). The production of superoxide is a marker of granulocyte-like differentiation, which can be measured by the ability to reduce NBT.[25] Using this marker, we examined the ability of MS-275 and RAD001 in combination to induce differentiation of HL-60 and NB4 cells. MS-275 at either 0.1 or 0.3 μM, (48 hrs) induced 11± 2 and 14 ± 2 % of HL-60 cells to reduce NBT, respectively. RAD001 (10 nM, 48 hrs) alone induced 7 ± 1 % of cells to reduce NBT. When both compounds were combined at the same concentration, a mean of 32 % or 39 % of HL-60 cells reduced NBT (Fig. 3C). Similarly, RAD001 (10 nM, 48 hrs)

enhanced the ability of MS-275 (0.1 μM, 48 hrs) to reduce NBT in NB4 cells (Fig. 3D). However, when higher dose of MS-275 (0.3 μM) was combined with RAD001 (10 nM), proportion of cells with reduced NBT decreased (Fig. 3D), mainly because cells underwent apoptosis rather than differentiation (data not shown).

Effect of MS-275 and RAD001 on expression of proteins important in regulating cell cycle and differentiation in leukemia cells. We next examined whether MS-275 and RAD001 modulated levels of C/EBPε, c-Myc, and $p27^{kip1}$ in leukemia cells by Western blot analysis (Fig. 4). Exposure of HL60 and NB4 cells to either MS-275 (0.1 μM, 48 hrs) or RAD001 (10 nM, 48 hrs) alone slightly induced expression of C/EBPε and $p27^{kip1}$. When HL60 and NB4 cells were exposed to the combination of both at the same concentration, levels of these proteins were markedly up-regulated (Fig. 4A). Effect of MS-275 (0.3 μM, 48 hrs) or RAD001 (10 nM, 48 hrs) on levels of c-Myc was negligible in NB4 and HL60 cells; however, combination of both compounds at the same concentration dramatically down-regulated levels of c-Myc in these cells (Fig. 4A). MS-275 in combination with RAD001 completely blocked expression of p-S6K and p-p70S6K in these cells (Fig. 4B), suggesting that mTOR signaling was effectively blocked.

RAD001 enhanced MS-275-induced acetylation of histone H3 on the C/EBPε

promoter in leukemia cells. HL60 and NB4 cells were cultured in the presence of either MS-275 (0.1 or 0.3 μM) and/or RAD001 (10 nM) for 48 hrs. Western blot analyses showed that MS-275, but not RAD001, slightly induced acetyl-histone H3 proteins in NB4 cells (Fig. 5A). Both of MS-275 and RAD001 slightly induced acetyl-histone H3 proteins in HL60 cells (Fig. 5A). When these cells were exposed to both agents at the same concentration, levels of acetyl-histone H3 dramatically increased (Fig. 5A).

We next examined the effect of MS-275 and RAD001 in combination on acetylation of histone H3 around the *C/EBPε* gene by ChIP assay. To quantify acetylated DNA precisely, we employed real-time PCR. As shown in Fig. 5B, exposure of HL60 and NB4 cells to RAD001 (10 nM) and MS-275 (0.1 μM) in combination for 48 hrs prominently enhanced acetylation of histone H3 on the *C/EBPε* promoter in these two cell lines.

RAD001 enhanced the ability of MS-275 to inhibit the proliferation of human HL60 leukemic xenografts *in vivo*. We further evaluated the anti-proliferative activity of MS-275 combined with RAD001 *in vivo* (Fig. 6). MS-275 (10 mg/kg) or RAD001 (5 mg/kg) alone inhibited the proliferation of HL60 xenografts by approximately 40 % or 50 %, respectively compared to control tumors (Fig. 6A). When mice were treated with

both compounds, tumor growth was inhibited by nearly 75 % (Fig. 5A), resulting in decreased weights of HL60 tumors at autopsy (Fig. 6B). None of the treated mice show signs of illness or significant weight loss (Fig. 6C).

Discussion

This study, for the first time, showed that inhibition of mTOR dramatically potentiated the ability of HDACI to acetylate histone H3 in leukemia cells (Fig 5). RAD001 markedly increased MS-275-stimulated acetylation of histone H3 on C/EBPε promoter and enhanced its expression in HL60 and NB4 cells (Figs 4, 5, 7), which was associated with increased differentiation of these cells (Fig 3). In parallel, MS-275 in combination with RAD001 upregulated levels of $p27^{kip1}$ and downregulated levels of c-Myc proteins (Figs 4, 7).

MS-275-induced growth arrest, apoptosis, and differentiation of AML cells appeared to be dose-dependent. Low dose of MS-275 (0.1 or 0.3 μM) induced differentiation of these cells in association with increased levels of C/EBPε (Figs 3, 4). When these cells were exposed to higher dose of MS-275 (1 μM), induction of apoptosis was prominent in parallel with blockade of Akt/mTOR signaling (Figs 1, 2). Similarly, previous studies showed that MS-275 exerted dose-dependent effects in human leukemia cells (U937, HL60, K562 and Jurkat); it induced growth arrest and differentiation of leukemia cells at low concentrations. On the other hand, higher dose of MS-275 caused marked apoptosis of leukemia cells.[31] These investigators showed that high dose of MS-275 generated reactive oxygen species (ROS) in leukemia cells

followed by the loss of mitochondrial membrane potential and cytosolic release of cytochrome c, resulting in activation of the caspase cascade and apoptosis.[31] Another study also found that MS-275 produced ROS and activated caspases, leading to apoptosis of chronic lymphocytic leukemia cells.[32] We have recently shown that MS-275 inhibited the nuclear factor κ B, which stimulated a variety of anti-apoptotic molecules such as bcl-2 family members, and induced growth arrest and apoptosis of adult T-cell leukemia cells.[8]

We have recently shown that SAHA (vorinostat), one of the HDACI derived from hydroxamic acid, also inhibited Akt/mTOR signaling, resulting in downregulation of cyclin D1 in mantle cell lymphoma cells.[33] In addition, other investigators found that SAHA, as well as, TSA inhibited Akt activity in prostate cancer cells.[34] These observations suggested that Akt signal pathway may be a common target of HDACI.

The Akt/mTOR signal pathway negatively regulates granulocytic differentiation; all-*trans* retinoic acid (ATRA) induced myeloid differentiation of APL cells in conjunction with inactivation of the Akt/mTOR signal pathway.[35,36] We have recently identified RTP801, the stress responsive gene, as one of the ATRA-target genes responsive to ATRA-mediated differentiation of AML cells. RTP801 is a negative regulator of the mTOR pathway.[37] It is unlikely that MS-275 induced differentiation of

AML cells and inhibited mTOR signaling via RTP801. MS-275 inhibited both Akt and mTOR (Fig 1); however, RTP801 was shown to inhibit mTOR but not Akt.[38]

The MEK/ERK signal pathway is another prosurvival signal pathway activated in AML. We have recently shown that MS-275 also inhibited MEK/ERK signaling in AML cells. Interestingly, further inactivation of MEK/ERK signaling by AZD6244 enhanced MS-275-mediated acetylation of histone H3 on p21^{waf1} promoter, resulting in marked upregulation of p21^{waf1} protein and induction of apoptosis of AML cells.[28] Contrary to this study, inhibition of MEK/ERK signaling blunted MS-275-induced differentiation of AML cells.[28]

Taken together, the combination of MS-275 and RAD001 may be useful for treatment of individuals with a subset of AML. Further studies are required to verify the molecular mechanisms by which blockade of mTOR signaling affects chromatin remodeling.

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References

1. Lowenberg B, Downing JR, Burnet A. Acute myeloid leukemia. N. Engl. J. Med. 1999; 341: 1051-1062.
2. Wolff SN, Herzig RH, Fay JW, LeMaistre CF, Brown RA, Frei-Lahr D, et al. High-dose cytarabine and daunorubicin as consolidation therapy for acute myeloid leukemia in first remission: long-term follow-up and results. J Clin Oncol 1989; 7: 1260-1267.
3. Wells RJ, Woods WG, Buckley JD, Odom LF, Benjamin D, Bernstein I, et al. Treatment of newly diagnosed children and adolescents with acute myeloid leukemia: a Childrens Cancer Group study. J Clin Oncol 1994 ;12: 2367-2377.
4. Harousseau JL, Cahn JY, Pignon B, Witz F, Milpied N, Delain M, et al. Comparison of autologous bone marrow transplantation and intensive chemotherapy as postremission therapy in adult acute myeloid leukemia. Blood 1997; 90: 2978-2986.
5. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, et al. German-Austrian Acute Myeloid Leukemia Study Group. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358:1909-18.

6. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 2006;6:38-51.
7. Sakajiri S, Kumagai T, Kawamata N, Saitoh T, Said JW, Koeffler HP. Histone deacetylase inhibitors profoundly decrease proliferation of human lymphoid cancer cell lines. Exp Hematol 2005;33:53-61.
8. Nishioka C, Ikezoe T, Yang J, Komatsu N, Bandobashi K, Taniguchi A, et al. Histone deacetylase inhibitors induce growth arrest and apoptosis of HTLV-1-infected T-cells via blockade of signaling by nuclear factor kappaB. Leuk Res. 2008;32:287-96.
9. Grignani F, De Matteis S, Nervi C, Tomassoni L, Gelmetti V, Cioce M, et al. Fusion proteins of the retinoic acid receptor-α recruit histone deacetylase in promyelocytic leukaemia. Nature 1998;391:815–8.
10. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature. 1997;389:349-352.
11. He LZ, Tolentino T, Grayson P, Zhong S, Warrell RP Jr, Rifkind RA, et al. Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J Clin Invest. 2001;108:1321-30.
12. Gottlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S,et al. Valproic

acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969–78.

13. Kuendgen A, Gattermann N. Valproic acid for the treatment of myeloid malignancies. Cancer. 2007;110:943-54.
14. Lekstrom-Himes J, Xanthopoulos KG. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem. 1998;273: 28545-28548.
15. Chumakov AM, Grillier I, Chumakova E, Chih D, Slater J, Koeffler HP. Cloning of the novel human myeloid-cell-specific C/EBP-epsilon transcription factor. Mol Cell Biol. 1997;17:1375-86.
16. Morosetti R, Park DJ, Chumakov AM, Grillier I, Shiohara M, Gombart AF, et al. A novel, myeloid transcription factor, C/EBP epsilon, is upregulated during granulocytic, but not monocytic, differentiation. Blood. 1997;90:2591-600.
17. Yamanaka R, Barlow C, Lekstrom-Himes J, Liu PP, Eckhaus M, Decker T, et al. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. Proc Natl Acad Sci U S A. 1997;94:13187-13192.
18. Nakajima H, Watanabe N, Shibata F, Kitamura T, Ikeda Y, Handa M. N-terminal

region of CCAAT/enhancer-binding protein epsilon is critical for cell cycle arrest, apoptosis, and functional maturation during myeloid differentiation. J Biol Chem. 2006;281:14494-502.

19. Gery S, Gombart AF, Fung YK, Koeffler HP. C/EBPepsilon interacts with retinoblastoma and E2F1 during granulopoiesis. Blood. 2004;103:828-35.
20. Bjornsti MA, Houghton PJ. Lost in translation: dysregulation of cap-dependent translation and cancer. Cancer Cell 2004;5:519-23.
21. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004;18:1926-45.
22. Yee KW, Zeng Z, Konopleva M, Verstovsek S, Ravandi F, Ferrajoli A, et al. Phase I/II study of the mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory hematologic malignancies. Clin Cancer Res. 2006;12:5165-73.
23. Nishioka C, Ikezoe T, Yang J, Koeffler HP, Taguchi H. Fludarabine induces apoptosis of human T-cell leukemia virus type 1-infected T cells via inhibition of the nuclear factor-kappaB signal pathway. Leukemia. 2007;21:1044-9.
24. Ikezoe T, Nishioka C, Tasaka T, Yang Y, Komatsu N, Togitani K, et al. The antitumor effects of sunitinib (formerly SU11248) against a variety of human

hematologic malignancies: enhancement of growth inhibition via inhibition of mammalian target of rapamycin signaling. Mol Cancer Ther. 2006; 5: 2522-2530.

25. Nishioka C, Ikezoe T, Takeshita A, Yang J, Tasaka T, Yang Y, et al. ZD6474 induces growth arrest and apoptosis of human leukemia cells, which is enhanced by concomitant use of a novel MEK inhibitor, AZD6244. Leukemia. 2007;21:1308-10.
26. Ikezoe T, Daar ES, Hisatake J, Taguchi H, Koeffler HP. HIV-1 protease inhibitors decrease proliferation and induce differentiation of human myelocytic leukemia cells. Blood. 2000; 96: 3553-3559.
27. Ikezoe T, Hisatake Y, Takeuchi T, Ohtsuki Y, Yang Y, Said JW et al. HIV-1 protease inhibitor, ritonavir: a potent inhibitor of CYP3A4, enhanced the anticancer effects of docetaxel in androgen-independent prostate cancer cells in vitro and in vivo. Cancer Res. 2004;64:7426-31.
28. Nishioka C, Ikezoe T, Yang J, Koeffler HP, Yokoyama A. Inhibition of MEK/ERK signaling synergistically potentiates histone deacetylase inhibitor-induced growth arrest, apoptosis and acetylation of histone H3 on p21(waf1) promoter in acute myelogenous leukemia cell. Leukemia (in press).
29. Ikezoe T, Tanosaki S, Krug U, Liu B, Cohen P, Taguchi H , et al. Insulin-like growth factor binding protein-3 antagonizes the effects of retinoids in myeloid

leukemia cells. Blood. 2004;104:237-42.

30. Nagaki K, Talbert PB, Zhong CX, Dawe RK, Henikoff S, Jiang J. Chromatin immunoprecipitation reveals that the 180-bp satellite repeat is the key functional DNA element of Arabidopsis thaliana centromeres. Genetics. 2003;163:1221-25.
31. Rosato RR, Almenara JA, Grant S.The histone deacetylase inhibitor MS-275 promotes differentiation or apoptosis in human leukemia cells through a process regulated by generation of reactive oxygen species and induction of p21$^{CIP1/WAF1 1}$. Cancer Res. 2003; 63: 3637-3645.
32. Lucas DM, Davis ME, Parthun MR, Mone AP, Kitada S, Cunningham KD, et al. The histone deacetylase inhibitor MS-275 induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells. Leukemia. 2004;18:1207-14.
33. Kawamata N, Chen J, Koeffler HP. Suberoylanilide hydroxamic acid (SAHA; vorinostat) suppresses translation of cyclin D1 in mantle cell lymphoma cells. Blood. 2007;110:2667-73.
34. Chen CS, Weng SC, Tseng PH, Lin HP, Chen CS. Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes. J Biol Chem. 2005;280:38879-87.
35. Ishida S, Shigemoto-Mogami Y, Shinozaki Y, Kagechika H, Shudo K, Ozawa S, et

al. Differential modulation of PI3-kinase/Akt pathway during all-trans retinoic acid- and Am80-induced HL-60 cell differentiation revealed by DNA microarray analysis. Biochem Pharmacol. 2004;68:2177-86.

36. Ozpolat B, Akar U, Steiner M, Zorrilla-Calancha I, Tirado-Gomez M, Colburn N, et al. Programmed cell death-4 tumor suppressor protein contributes to retinoic acid-induced terminal granulocytic differentiation of human myeloid leukemia cells. Mol Cancer Res. 2007;5:95-108.

37. Gery S, Park DJ, Vuong PT, Virk RK, Muller CI, Hofmann WK , et al. RTP801 is a novel retinoic acid-responsive gene associated with myeloid differentiation. Exp Hematol. 2007;35:572-8.

38. Corradetti MN, Inoki K, Guan KL. The stress-inducted proteins RTP801 and RTP801L are negative regulators of the mammalian target of rapamycin pathway. J Biol Chem. 2005;280:9769-72.

Figure Legend

Fig.1. Western blot Analysis. HL60 and NB4 cells were cultured with MS-275 (0.1-1 μM) for 3 hrs. Western blot analysis was utilized to monitor the levels of Akt, p-Akt, S6K, p-S6K, p70S6K, p-p70S6K and α-tubulin. Each lane was loaded with 30 μg of protein. The figure represents one of the three experiments performed independently with similar results.

Fig.2. RAD001 potentiates the action of MS-275 in leukemia cells. MTT assay. **(A)** HL60 and **(B)** NB4 cells were cultured with either MS-275 (0.1-1 μM) and/or RAD001 (10 nM). After 2 days, cell proliferation was measured by MTT assay. The statistical significance of difference between growth inhibition induced by either MS-275 or RAD001 alone and those induced by a combination of both 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. **Cell cycle analysis. (C)** HL60 and **(D)** NB4 cells were cultured with MS-275 or RAD001 either alone or in combination. After 2 days, the cell cycle distribution of these cells was analyzed. Results represent one of the three experiments performed in duplicate. **RAD001 enhanced MS-275-inducing apoptosis. (E)** HL60 and **(F)** NB4 cells were cultured with MS-275 (0.1-1 μM) or RAD001 (10 nM) either

alone or in combination. 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 induced by either MS-275 or RAD001 alone and those induced by combination of both 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.

Fig.3. The effect of RAD001 on MS-275-induced expression of CD11b in leukemia cells. HL60 (A) and NB4 (B) cells were cultured with either MS-275 (0.1 or 0.3 μM) and/or RAD001 (10 nM). After 2 days, CD11b expressing population was measured by FACScan. Results represent one of the experiments performed twice in duplicate plate. **The effect of RAD001 on MS-275-induced NBT reduction in leukemia cells.** HL60 **(C)** and NB4 **(D)** cells were cultured with either MS-275 (0.1 or 0.3 μM) and/or RAD001 (10 nM) for 2 days, and differentiation was determined by NBT reduction. The statistical significance of difference between NBT reduction induced by either MS-275 and/or RAD001 was determined by ANOVA followed by Boneferroni's multiple comparison tests. Results represent the mean of 3 experiments performed in triplicate; *, p< 0.01, with respect to control.

Fig.4. Western blot Analysis. HL60 and NB4 cells were cultured with either MS-275

(0.1 or 0.3 μM) and/or RAD001 (10 nM) for 48 hrs **(A)** or 3 hrs **(B)**. Western blot analysis was performed to monitor the levels of **(A)** C/EBPε, c-Myc, $p27^{kip1}$, **(B)** S6K, p-S6K, p70S6K, p-p70S6K and α-tubulin. Each lane was loaded with 30 μg of proteins.

Fig.5. RAD001 enhanced MS-275-induced acetylation of histone H3. (A) HL60 and NB4 cells were cultured in the presence of either MS-275 (0.1 or 0.3 μM) and/or RAD001(10 nM). After 48 hrs, cells were harvested and nuclear proteins were prepared and subjected to Western blot analysis. The membranes were sequentially probed with anti-acetyl-histone H3 and anti-histone H1 antibodies. Ac-histone H3, acetylated-histone H3. **Chromatin immunoprecipitation assay. (B)** Acetylation of histone H3 in the *C/EBPε* promoter was analyzed by chromatin immunoprecipitation assay. HL60 and NB4 cells were cultured in the presence of either MS-275 (0.1 μM) and/or RAD001 (10 nM). After 48 hrs, cells were harvested and subjected to chromatin immunoprecipitation followed by real-time PCR. The amplified sequences were normalized to those form input (the cross-linked DNA/protein complexes which were not immunoprecipitated with anti-acetylated histone H3 antibody). The statistical significance of difference between acetylation of histone H3 induced by either MS-275 and/or RAD001 was determined by ANOVA followed by Boneferroni's multiple comparison tests. Results represent the mean of 4 experiments performed in duplicate; *,

$p < 0.01$, with respect to control.

Fig 6. Effect of co-administration of MS-275 and RAD001 on the proliferation of HL60 cells in murine xenograft model. (A), HL60 cells were injected bilaterally s.c. into BALB/c nude mice, forming two tumors/mouse. When HL60 tumors were palpable (approximately 50 mm^3), mice were randomized into four groups (n=5) and treatment was initiated. MS-275 (10 mg/kg) was administered to mice by oral administration six times every another day. RAD001 (5 mg/kg) was given four times every another day. Tumor volumes were measured twice a week. Each point represents the mean ± SD of 10 tumors. **(B), Tumor weights at autopsy.** After 2 weeks of treatment, tumors were removed and weighed. Results represent mean ± SD of tumor weights. Statistical significance was determined by one-way ANOVA followed by Boneferroni's multiple comparison tests. Bars, SD. **(C), Body weight.** Body weight of mice was measured twice a week during treatment. cont, diluent control.

Fig 7. RAD001 potentiates the action of MS-275. (A), RAD001 enhances MS-275-induced acetylation of histone H3 on the promoter of C/EBPε, resulting in enhanced up-regulation of this transcription factor and induction of differentiation of AML cells. **(B),** Both RAD001 and MS-275 targets Akt/mTOR signaling in AML cells. Combination of both down-regulated levels of c-Myc, resulting in growth inhibiton. Ac,

acetylation, H3, histone H3.

Fig.1

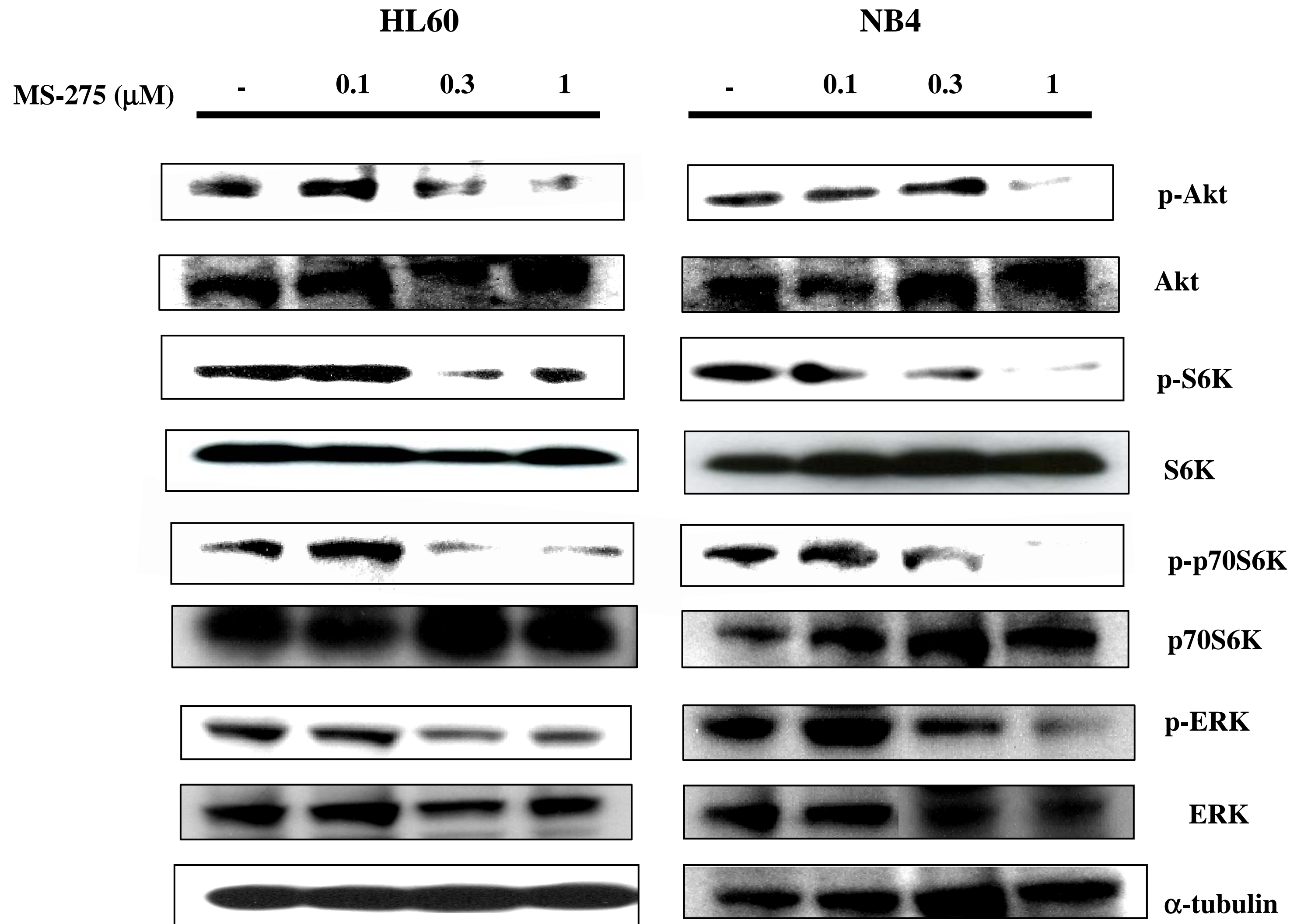
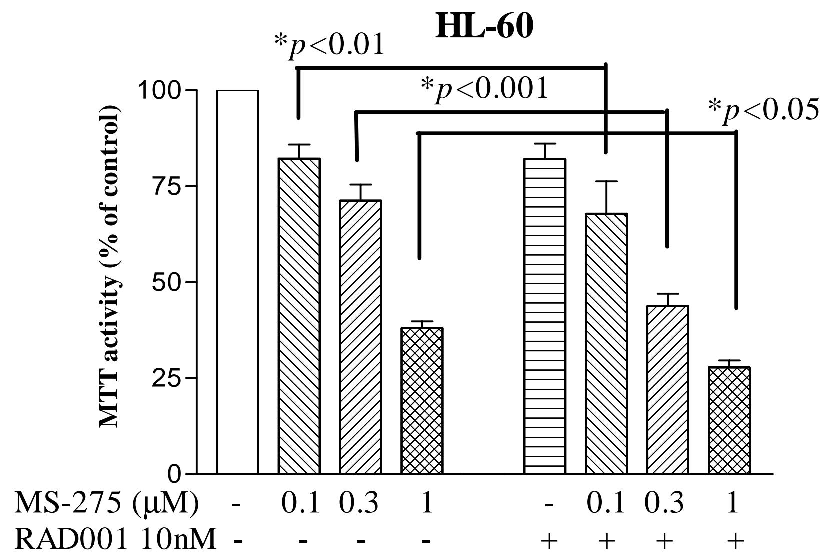


Fig.2

A



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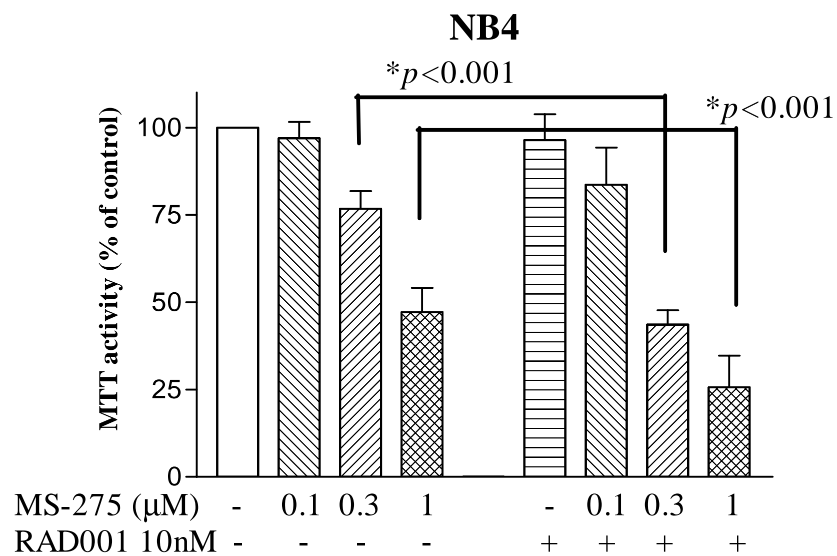
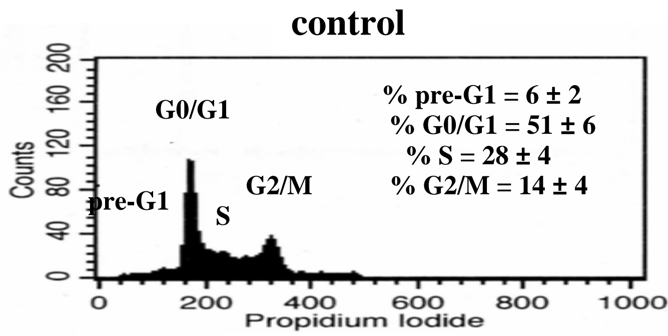
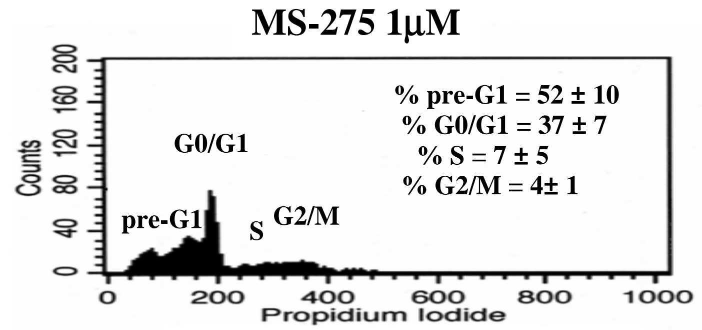


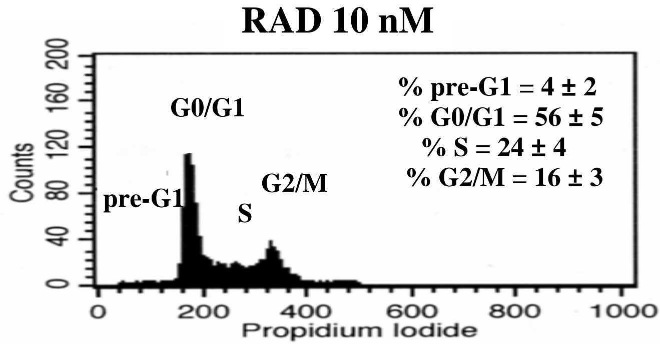
Fig.2

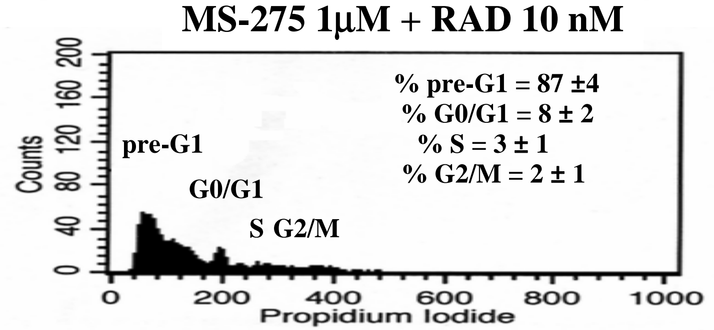
C

HL60



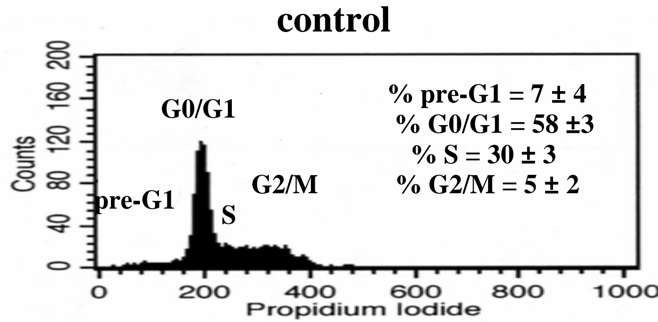


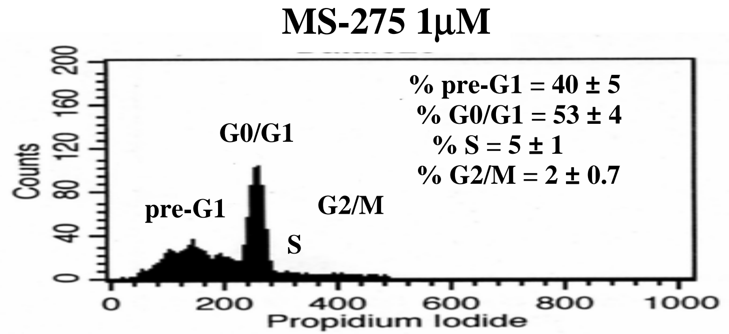


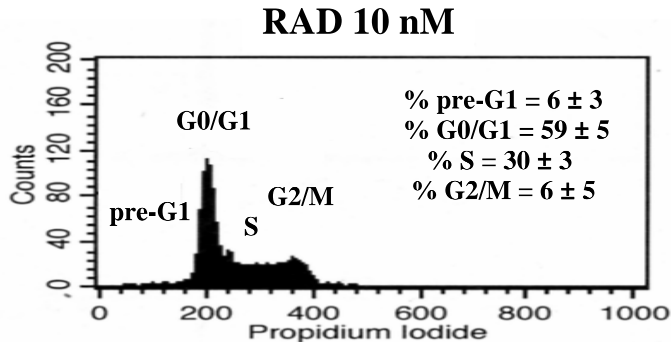


D

NB4







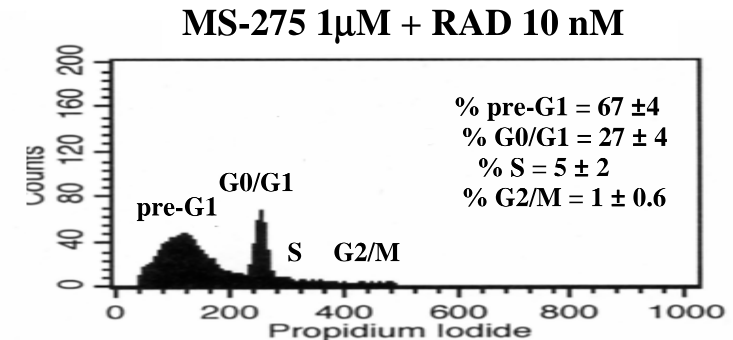
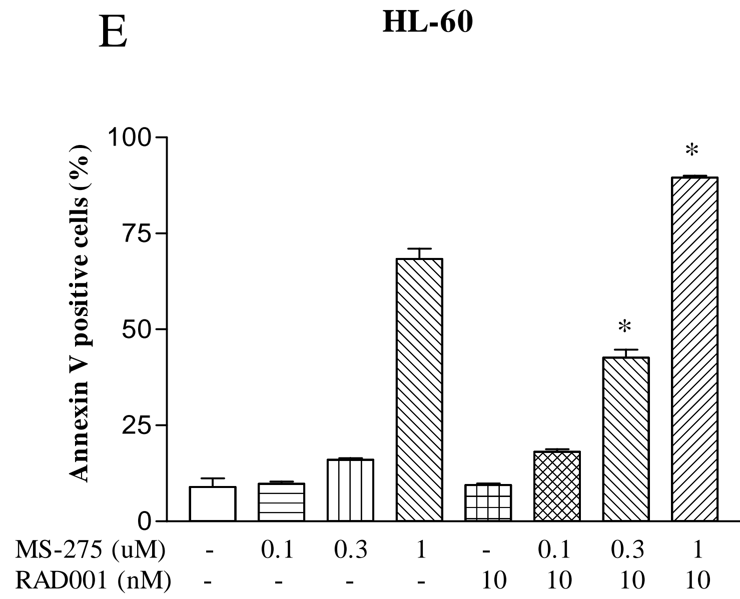


Fig.2



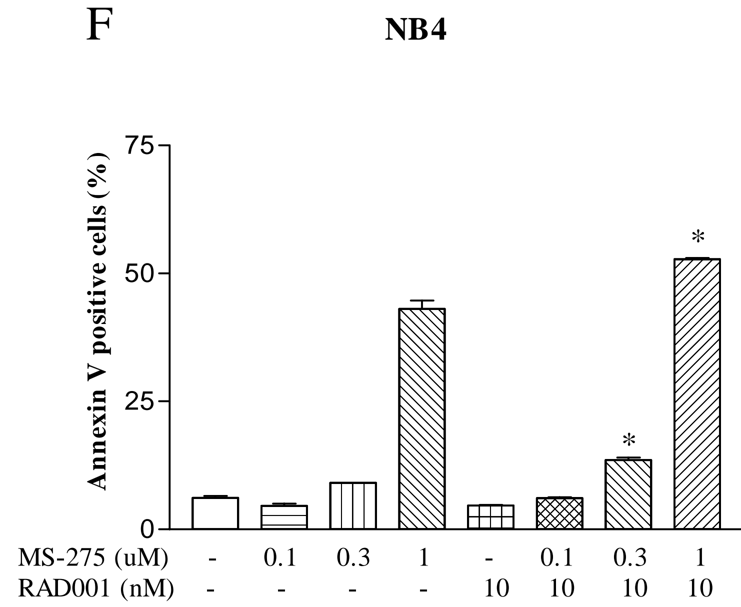


Fig.3

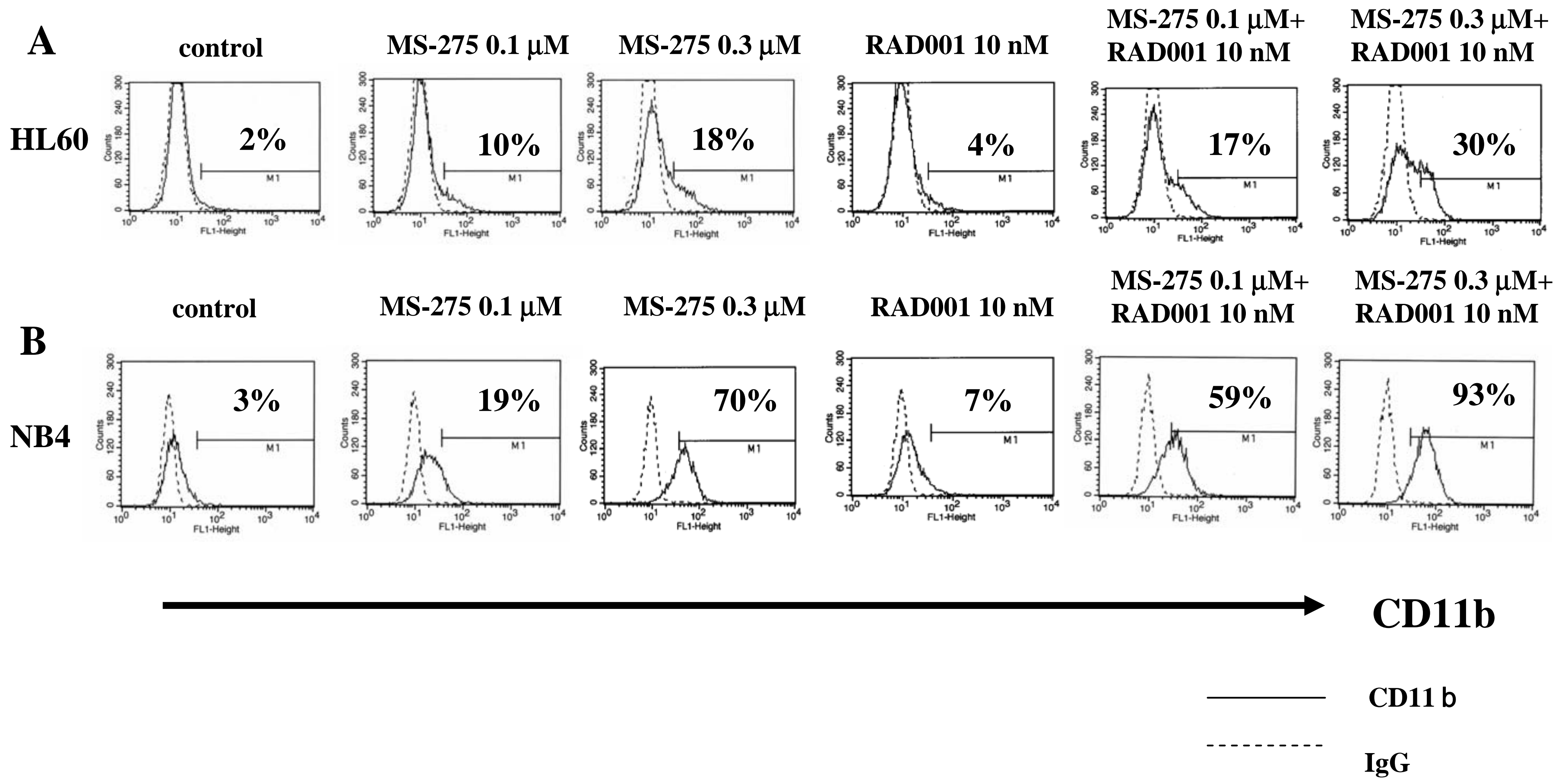
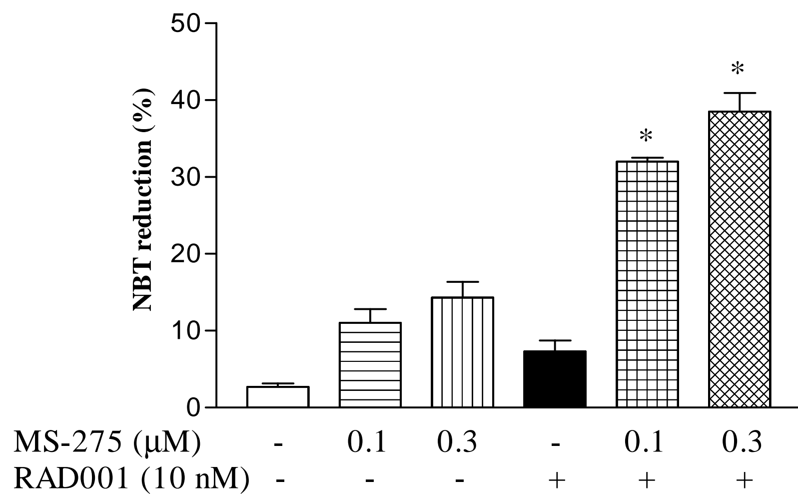


Fig.3



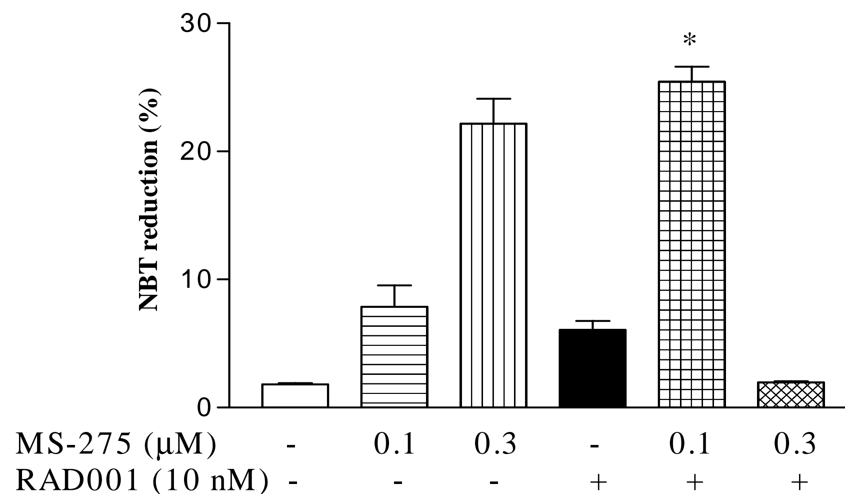
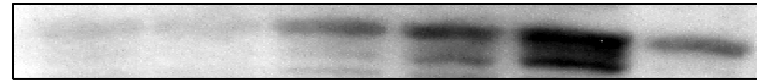


Fig.4 A

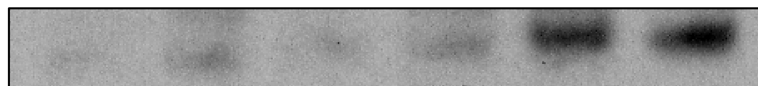
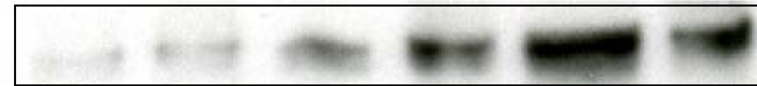
HL60 NB4

MS-275 (μM) - 0.1 0.3 - 0.1 0.3 - 0.1 0.3 - 0.1 0.3

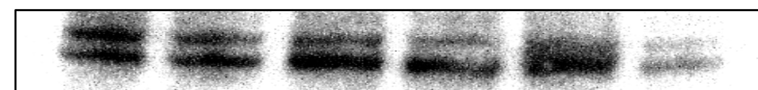
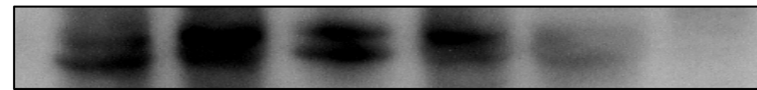
RAD001 (nM) - - - 10 10 10 - - - 10 10 10



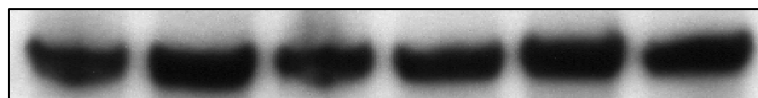
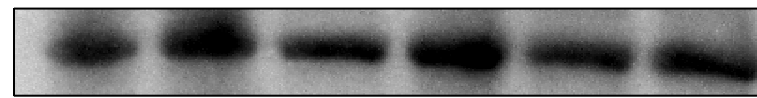
C/EBPε

p27^{kip}

c-Myc

α-tubulin

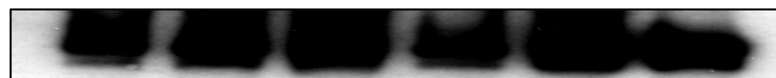
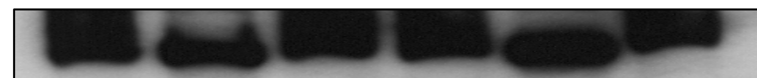
B

HL60 NB4

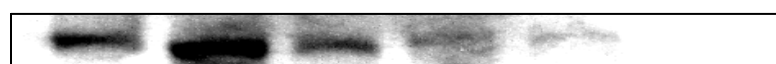
MS-275 (μM) - 0.1 0.3 - 0.1 0.3 - 0.1 0.3 - 0.1 0.3

RAD001 (nM) - - - 10 10 10 - - - 10 10 10

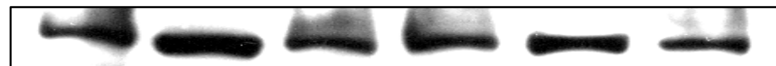
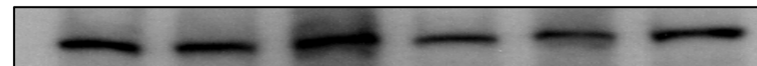
p-S6K

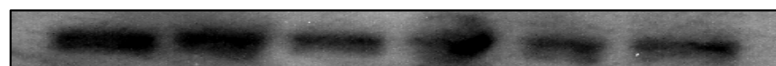
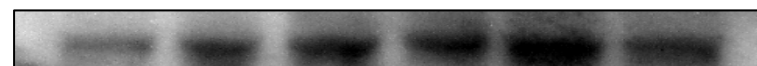
S6K



p-p70S6K

p70S6K

α-tubulin

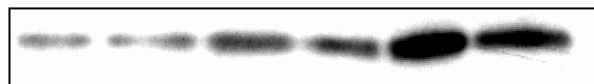
Fig 5

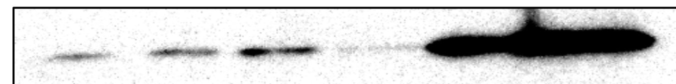
A

mucleus HL60 NB4

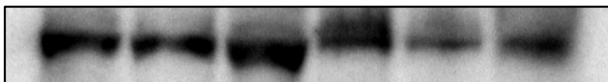
MS-275 (μM) - 0.1 0.3 - 0.1 0.3 - 0.1 0.3 - 0.1 0.3

RAD001 (10 nM) - - - + + + - - - + + +





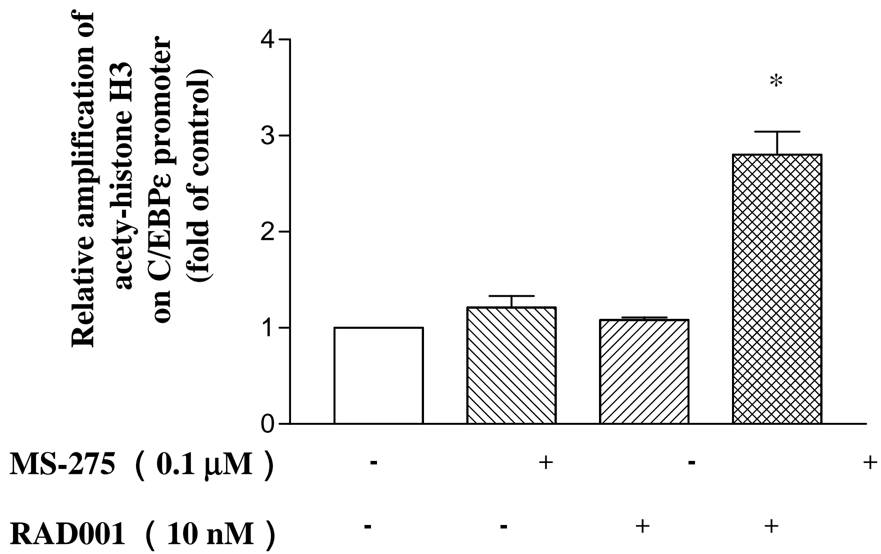
Ac-HistoneH3



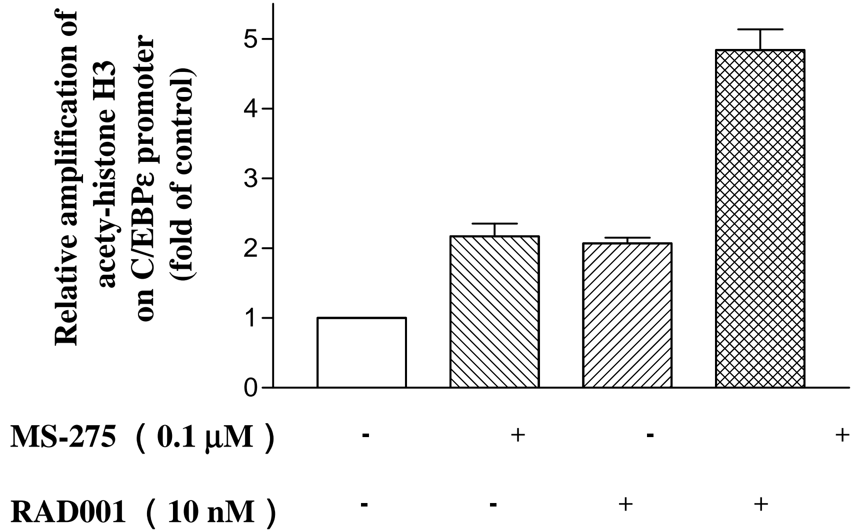
HistoneH1

Fig 5

B

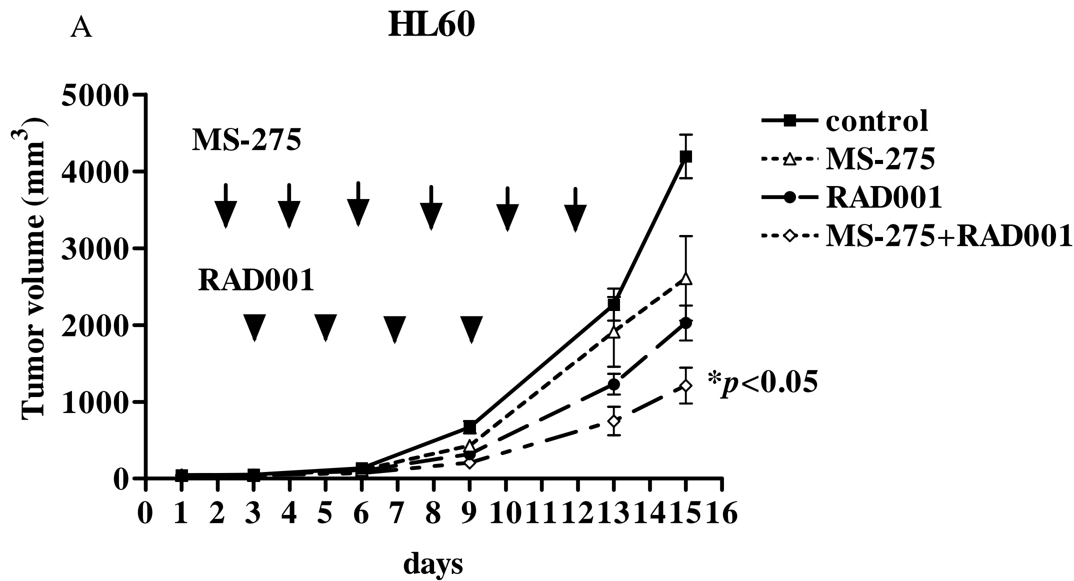


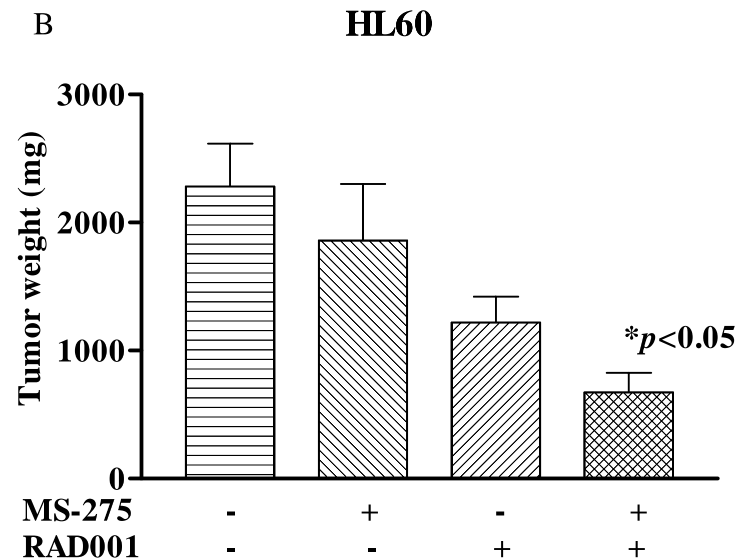
C



*$p<0.01$

Fig.6





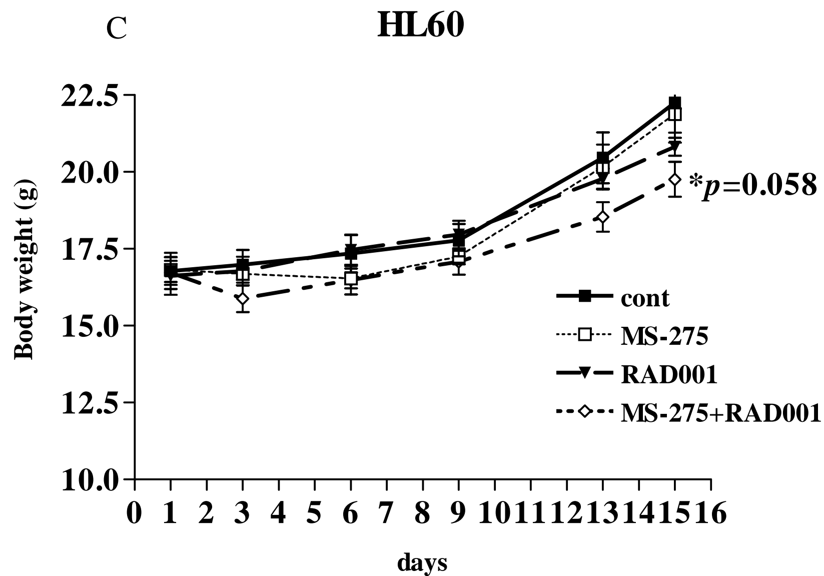


Fig.7