

岩手医科大学  
審査学位論文  
(博士)

**Resveratrol suppresses cell proliferation via inhibition of STAT3 phosphorylation and Mcl-1 and cIAP-2 expression in HTLV-1-infected T-cells**

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**Running Title**

Effects of resveratrol on ATL cells

**Keywords**

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## Summary

Adult T-cell leukemia (ATL) is an aggressive malignancy of peripheral T cells infected with human T-cell leukemia virus type 1 (HTLV-1). The prognosis of aggressive ATL patients remains poor because ATL cells acquire the resistance to conventional cytotoxic agents. Therefore, the development of novel agents is urgently needed. We examined the effects of resveratrol, a well-known polyphenolic compound, on cell proliferation and survival of HTLV-1-infected T-cell line, MT-2 cells. We found that resveratrol suppressed cell proliferation and induced cell death of MT-2 cells. Immunoblot analysis showed the inhibition of myeloid cell leukemia sequence (Mcl)-1 and cellular inhibitor of apoptosis protein (cIAP)-2 expression, and signal transducers and activators of transcription (STAT) 3 phosphorylation at Tyr<sup>705</sup> and Ser<sup>727</sup> in resveratrol-treated cells. We also observed the cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP) in resveratrol-treated cells, indicating that resveratrol induces caspase-dependent apoptosis in MT-2 cells. In addition, a STAT3 inhibitor, S3I-201 induced cell growth arrest and cell death, and activated a caspase-3 in MT-2 cells, indicating STAT3 as a therapeutic target for ATL. These results suggest that resveratrol presents a potent anti-proliferative effect in part via the suppression of STAT3 phosphorylation and Mcl-1 and cIAP-2 expression in HTLV-1-infected T-cells. Resveratrol merits further

investigation as a potential chemotherapeutic agent for ATL.

## **Introduction**

Adult T-cell leukemia (ATL) is a peripheral T lymphocytic malignancy associated with human T-cell leukemia virus type 1 (HTLV-1) [1-3]. Aggressive ATL, such as acute and lymphoma types, and chronic type with poor prognostic factors has a poor prognosis, with a median survival duration of 13 months because of its resistance to conventional chemotherapy [4]; therefore, new therapeutic agents that effectively kill these cells should be developed. The epidemiology of ATL suggests that cumulative genetic defects may be responsible for acquisition of the neoplastic phenotype in a T-cell clone [5]. The proliferation of ATL cells has been shown to be associated with the constitutive activation of Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins [6,7]. We recently showed that activated STAT3 plays a critical role on cell proliferation and survival of HTLV-1-transformed cells, indicating that STAT3 is a novel therapeutic target for ATL [8].

Resveratrol, a polyphenolic compound, has been reported to possess both chemopreventive and chemotherapeutic activities in several cancers [9]. Resveratrol has been shown to suppress a variety of signaling molecules and anti-apoptotic proteins including nuclear factor-kappa B (NF- $\kappa$ B), STAT3, survivin, Bcl-2 and Bcl-xL in cancer cells [9,10]. Recent report demonstrated that resveratrol induces apoptosis via the

suppression of survivin expression in HTLV-1-infected T cells [11]. However, the effect of resveratrol on intracellular signaling molecules in ATL cells remains unknown. We report here that resveratrol induces cell death via the suppression of STAT3 phosphorylation and anti-apoptotic proteins in HTLV-1-infected T cells.

## **Materials and methods**

### *Reagents and antibodies*

Resveratrol was purchased from Calbiochem (Darmstadt, Germany). STAT3 inhibitor, S3I-201 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-specific anti-STAT3 (Tyr<sup>705</sup>), STAT3 (Ser<sup>727</sup>) and STAT5 (Tyr<sup>694</sup>), and anti-STAT3, STAT5, cIAP-1, cIAP-2, Mcl-1, cleaved poly (ADP-ribose) polymerase (PARP), cleaved caspase-3 and  $\beta$ -actin were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against Bcl-2 were purchased from Santa Cruz Biotechnology. Phycoerythrin (PE)-conjugated rabbit antibodies against active caspase-3 and PE-conjugated isotype antibodies were purchased from BD Biosciences (San Diego, CA).

### *Cell lines*

Human HTLV-1-infected cell line MT-2 was kindly provided by Dr. Kibata (Hayasibara Biochemical Laboratories Inc, Okayama, Japan), which is derived from normal human leukocytes transformed by the leukemia T cells of a patient with ATL. MT-2 cells were cultured in RPMI1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin

(Gibco), and 2mM of glutamine (Gibco) in a 5% CO<sub>2</sub> incubator at 37°C.

#### *Cell proliferation and cell death assay*

After cells were cultured in complete RPMI1640 medium in the presence or absence of resveratrol or S3I-201 for 24 or 48 hours, cell proliferation was measured by MTT assay. For the apoptosis assay, one million cells were incubated with or without 50µM resveratrol or 100 µM S3I-201 for 24 or 48 hours. Two hundred thousand cells were stained with annexin V and propidium iodide (PI) according to the manufacturer's instructions (BD Biosciences, San Diego, CA). The percentage of annexin V-positive cells was analyzed by the Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Japan).

#### *Measurement of activated caspase-3*

One hundred thousand cells were incubated with or without 150µM S3I-201 for 48 hours. After washing, cells were fixed and permeabilized using the Cytfix/Cytoperm solution according to manufacturer's instructions (BD Biosciences, CA). Cells then were incubated with PE-conjugated antibodies against activated caspase-3 or PE-conjugated isotype antibodies for 30 minutes at room temperature. After washing,

the frequency of cells with activated caspase-3 was analyzed by the Coulter Epics XL-MCL flow cytometer.

### *Immunoblotting*

After MT-2 cells were treated with resveratrol or S3I-201, they were washed with cold phosphate saline (PBS) twice. Cell lysates then were prepared by ice-cold lysis buffer (0.5% NP-40, 10 mM Tris base, 200 mM NaCl, 10% glycerol, 5 mM NaF, 0.5 mM sodium orthovanadate, pH 7.4) with 1% protease inhibitor cocktail (EMD Biosciences, San Diego, CA), incubated for 30 min at 4°C and then centrifuged at 4066 x g for 10 min, and the supernatants were retained. Cell lysates were eluted by incubation with SDS sample buffer (Pierce, Rockford, IL) for 5 min at 95°C. Samples were loaded in 7-15% Tris-glycine gel (Bio-Rad Laboratories, Hercules, CA) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK). Immunoblotting was then performed according to the antibody manufacturer's instructions. Blots were visualized using HRP-conjugated secondary Abs and an enhanced chemiluminescent detection system (ECL plus; GE Healthcare, Buckinghamshire, UK). To reprobe with other Abs, membranes were incubated in stripping buffer (Pierce, Rockford, IL) according to the manufacturer's instructions.

## **Results**

### *Resveratrol suppresses cell proliferation and survival in MT-2 cells*

We examined whether resveratrol suppresses the proliferation of MT-2 cells. As shown in Fig.1A, resveratrol treatment inhibited the proliferation of MT-2 cells in a dose- and time-dependent manner. We next examined whether resveratrol induces cell death of MT-2 cells. The frequency of cell death was evaluated using flow cytometric analysis with annexin V and PI staining after cells were treated in the presence or absence of 50 $\mu$ M resveratrol for 24 or 48 hours. As shown in Fig. 1B, the frequency of annexin V<sup>+</sup> cells after treatment with resveratrol was significantly higher than in the absence of resveratrol. These results suggested that resveratrol suppressed proliferation and induced cell death in MT-2 cells.

### *Resveratrol inhibits phosphorylation of STAT3 in MT-2 cells*

To better understand the mechanism by which resveratrol suppresses the proliferation and survival of cells, we examined intracellular signaling in resveratrol-treated cells. STAT3 and STAT5 were constitutively phosphorylated in MT-2 cells. As shown in Fig. 2, STAT3 phosphorylation at Tyr<sup>705</sup> and Ser<sup>727</sup> was suppressed 24 hours after treatment of MT-2 cells with resveratrol. In contrast, STAT5 phosphorylation at Tyr<sup>694</sup> was not suppressed, indicating the selectivity of resveratrol for STAT3 in MT-2 cells.

### *Resveratrol suppresses cIAP-2 and Mcl-1 expression*

We next examined the effects of resveratrol on the expression of anti-apoptotic proteins in MT-2 cells. As shown in Fig. 3, cIAP-2 and Mcl-1 expression were suppressed 24 hours after treatment of MT-2 cells with resveratrol, but not cIAP-1 and Bcl-2. In addition, resveratrol activated caspase-3 and PARP in MT-2 cells.

### *S3I-201 suppresses proliferation and survival of MT-2 cells*

To determine if STAT3 is essential for cell proliferation and survival in MT-2 cells, we next examined the effects of STAT3 inhibitor, S3I-201 on MT-2 cells. S3I-201 suppressed cell proliferation in a dose-dependent manner and induced apoptosis (Fig. 4A and B). Immunoblot analysis showed that S3I-201 suppressed STAT3 phosphorylation at Tyr<sup>705</sup> in a dose-dependent manner in MT-2 cells (Fig. 4C). Flow cytometric analysis revealed that S3I-201 induced activation of caspase-3 in MT-2 cells (Fig. 4D).

## **Discussion**

Resveratrol has been shown to inhibit the growth of a wide variety of tumor cells including hematological malignancies [12]. We showed that resveratrol suppressed cell proliferation in a time- and dose-dependent manner, and induced cell death in HTLV-1-infected cell line, MT-2 cells. These findings suggest that resveratrol has a potent anti-leukemia effect. To better understand the action mechanisms of resveratrol, we first examined intracellular signaling in MT-2 cells. The cells presented the constitutive phosphorylation of STAT3 and STAT5. Resveratrol has been shown to inhibit activation of signaling molecules such as Akt, NF- $\kappa$ B, mitogen-activated protein kinase (MAPK) and STAT in cell cultures and in animal models [13-15]. Bhardwaj et al. reported that resveratrol down-regulated the constitutive activation of STAT3 and NF- $\kappa$ B in multiple myeloma cells [15]. In the present study, resveratrol suppressed constitutive phosphorylation of STAT3 at Tyr<sup>705</sup> and Ser<sup>727</sup> but not STAT5 in MT-2 cells. We next examined whether STAT3 plays a role in cell growth and survival of MT-2 cells using STAT3 specific inhibitor, S3I-201. The compound is a chemical probe inhibitor of STAT3 activity, which inhibits STAT3-STAT3 complex formation and STAT3 DNA-binding and transcriptional activities [16]. In the present study, S3I-201 inhibited cell proliferation and induced caspase-dependent apoptosis, which were associated with suppression of STAT3 phosphorylation. We previously reported that deguelin, a

naturally occurring rotenoid, suppressed constitutively phosphorylated STAT3 but not STAT5 and induced a caspase-dependent apoptosis in MT-2 cells [8]. These observations suggest that STAT3 may play a role in cell proliferation and survival of MT-2 cells. In response to cytokine stimulation, JAKs (Janus kinases) activate STAT3 through phosphorylation of a single tyrosine residue (Tyr<sup>705</sup>) resulting in dimerization and translocation of STAT3 to the nucleus where it binds to defined target sequences and activates transcription [17,18]. Additional phosphorylation of STAT3 at Ser<sup>727</sup> has been predicted to be responsible for increased transcriptional activity. However, it has not been elucidated the mechanism by which resveratrol inhibited STAT3 phosphorylation in this study. Madan et al. demonstrated that resveratrol inhibited cellular proliferation of human epidermoid carcinoma A341 cells via suppression of JAK and STAT1 phosphorylation [19]. Migone et al. demonstrated that JAK1 and JAK3, and STAT3 and STAT5 were constitutively activated in HTLV-1-transformed cells [6]. In addition, Takemoto et al. [7] reported that proliferation of ATL cells was associated with the constitutive activation of JAK/STAT proteins. The authors demonstrated that JAK3 was phosphorylated and associated with STAT3 in primary ATL cells. These observations raise the possibility that resveratrol may inhibit the phosphorylation of STAT3 through JAK3 inactivation in ATL cells. Future work will refine our

understanding of how resveratrol suppresses STAT3 phosphorylation in ATL cells.

We found that resveratrol suppressed the expression of Mcl-1 and cIAP-2 but not Bcl-2 and cIAP-1 in MT-2 cells. Hayashibara et al. [11] showed that resveratrol induced downregulation in survivin expression and apoptosis in HTLV-1-infected cell lines. Interestingly, Bcl-2 and cIAP-1 expression were not downregulated in resveratrol-treated cells. This finding is compatible with our results. However, the mechanism by which resveratrol inhibits Mcl-1 and cIAP-2 expression has not been elucidated. STAT3 activation results in the upregulation of various genes involved in cell survival and proliferation, such as those encoding Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, cyclin D1, and c-Myc [20-22]. Epling-Burnette et al. [23] showed that inhibition of STAT3 signaling leads to apoptosis in lymphocytes through decreased Mcl-1 expression. Mcl-1 belongs to the pro-survival Bcl-2 family proteins and is regulated at the transcriptional and post-transcriptional levels resulting in alternative splicing [24]. In addition, Liu et al. [25] reported that serine phosphorylation of STAT3 is essential for Mcl-1 expression and macrophage survival. Thus, resveratrol may suppress Mcl-1 expression at least in part via STAT3 dephosphorylation in MT-2 cells. cIAP-2 belongs to IAP family proteins which modulate apoptotic cell death and function by preventing activation of caspases [26,27]. Bhattacharya et al. [28] showed that inhibition of STAT3 by dominant

negative-STAT3 decreased mRNA and protein levels for Bcl-2, Mcl-1 and cIAP-2 in polyamine-depleted cells. These findings indicate that Mcl-1 and cIAP-2 are regulated in part by STAT3. Thus, resveratrol might suppress the survival of MT-2 cells at least in part via the transcriptional downregulation of Mcl-1 and cIAP-2 by STAT3 dephosphorylation. We also found that resveratrol activated caspase-3 and PARP in MT-2 cells, indicating that resveratrol induces caspase-dependent apoptosis. Taken together, JAK/STAT3-Mcl-1/cIAP-2 axis might be an attractive therapeutic target in ATL.

In conclusion, resveratrol appears to inhibit the proliferation and induce apoptosis of HTLV-1-infected T-cells at least in part via the suppression of STAT3 activation followed by downregulation of Mcl-1 and cIAP-2. Early stage clinical studies in humans are underway to assess the safety and potential of resveratrol specific to cancer treatment and prevention [29]. The results from these studies will help us understand how we can target its anti-tumor activities to prevent and treat ATL. Thus, resveratrol merits further investigation as a potential therapeutic agent for ATL.

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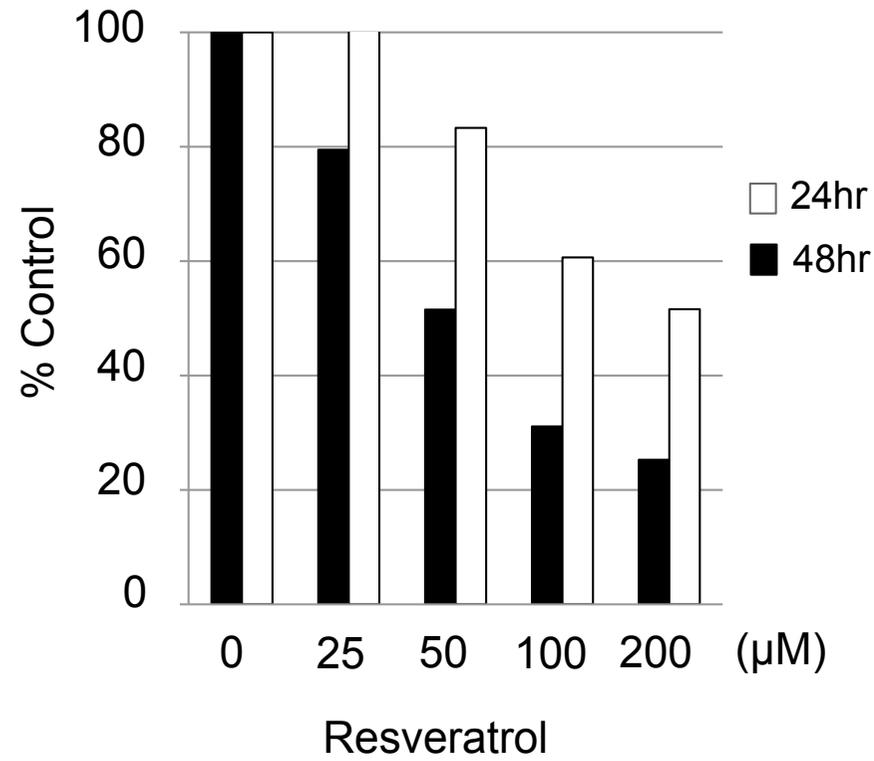
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A



B

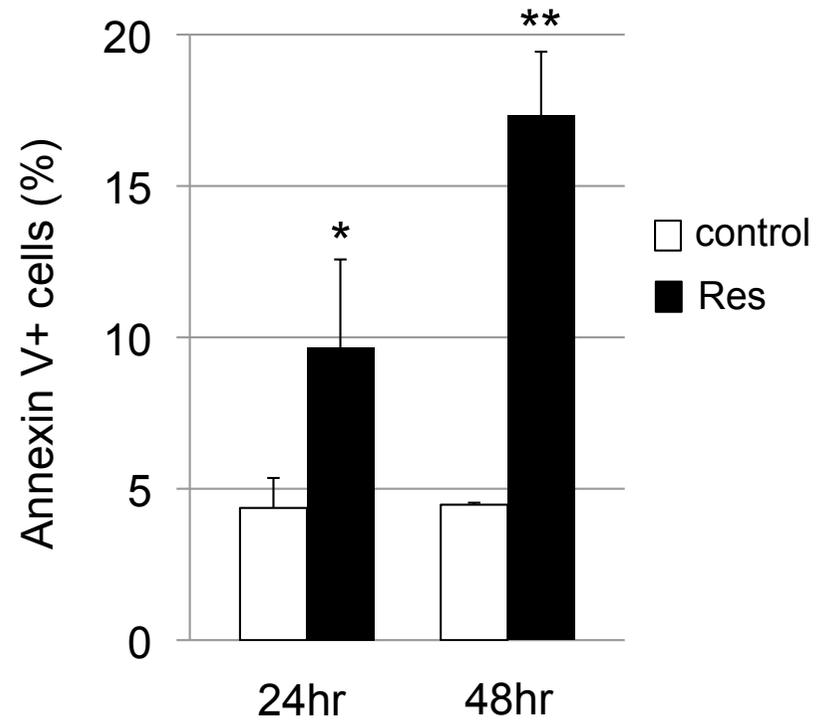
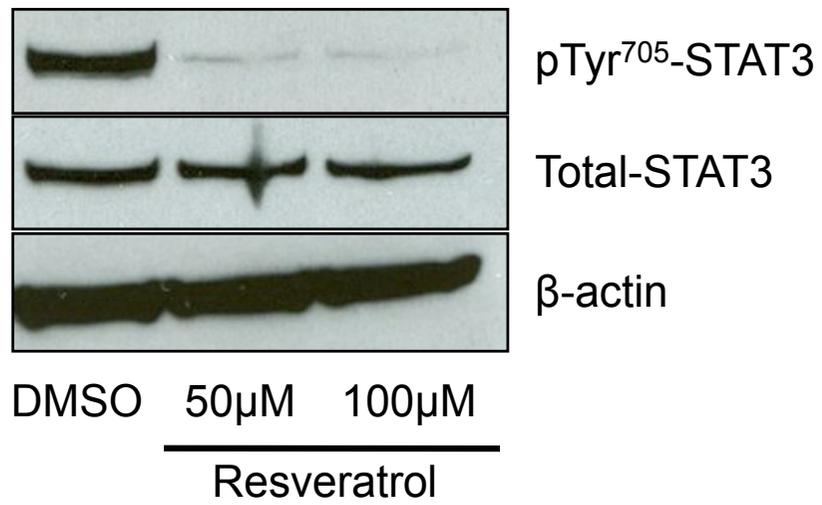


Fig.1

A



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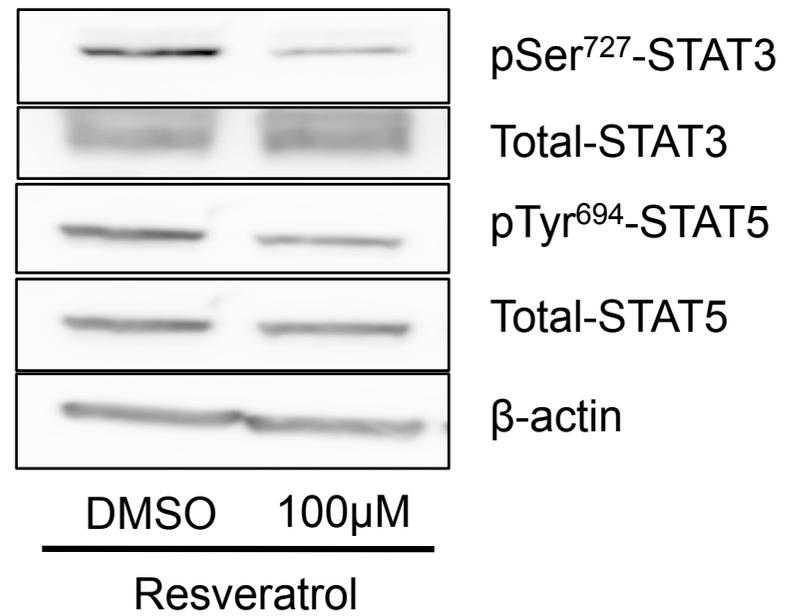
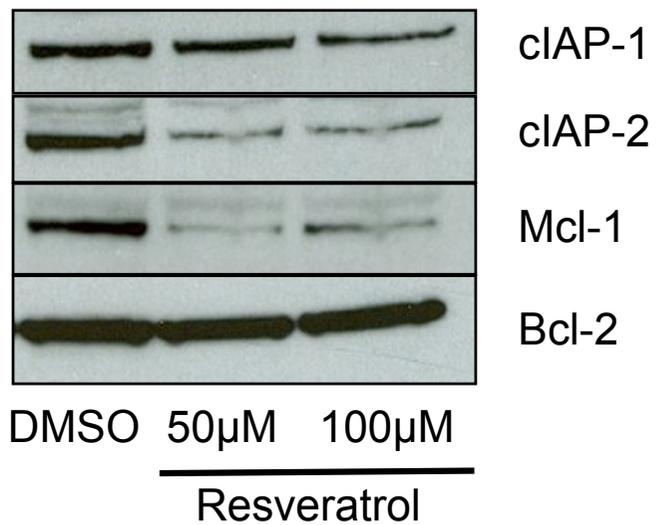


Fig.2

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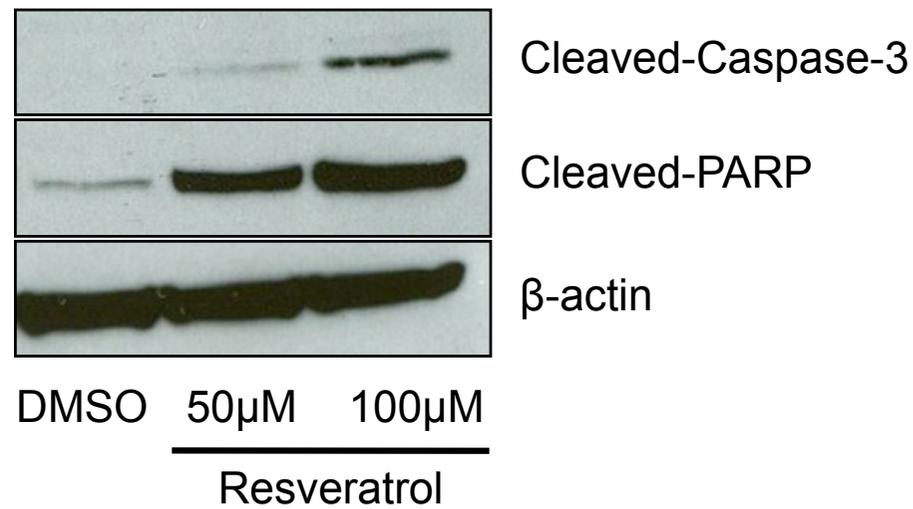
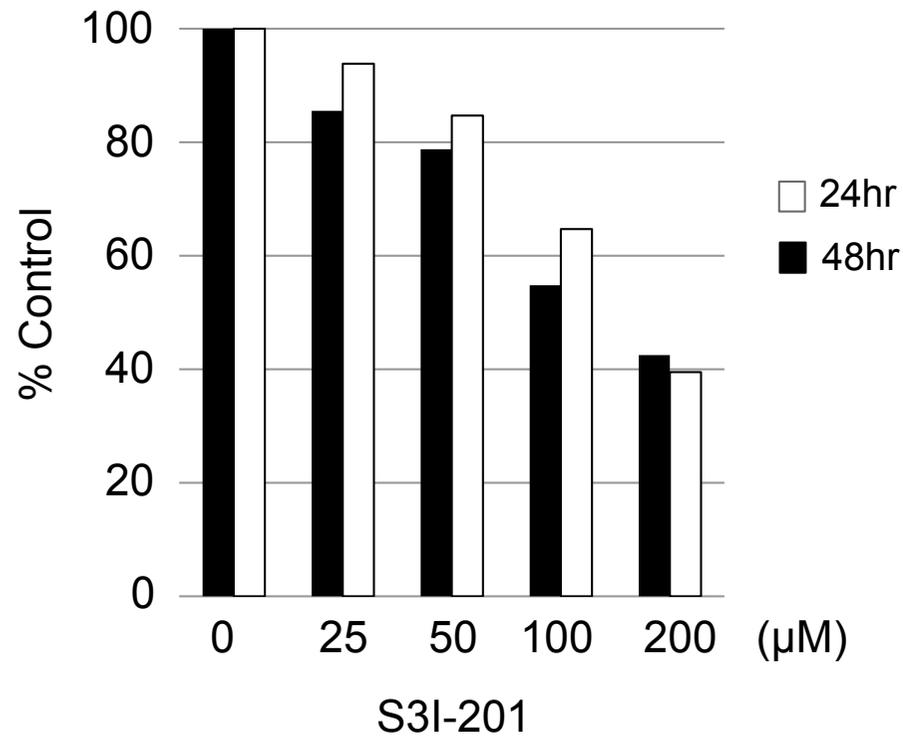


Fig.3

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B

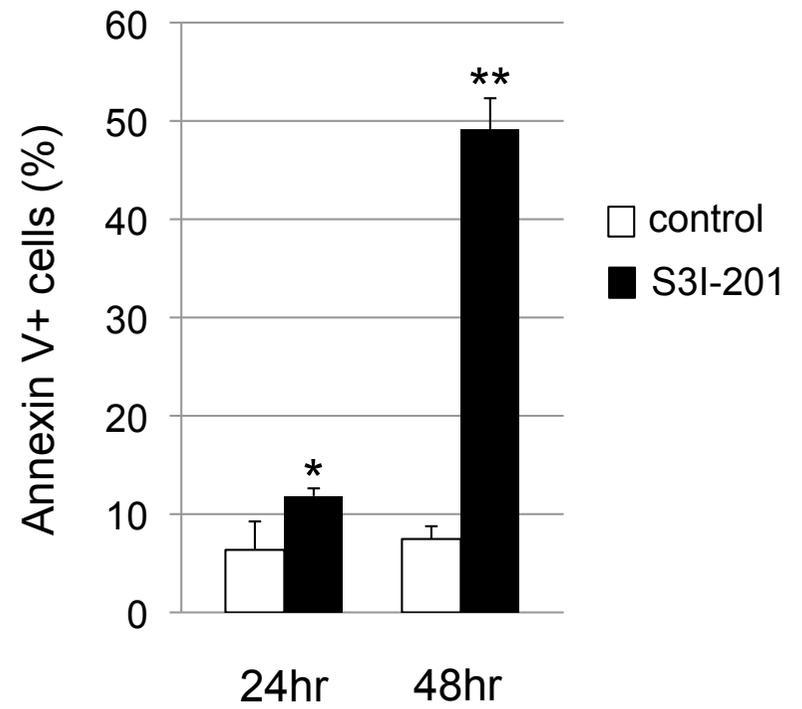
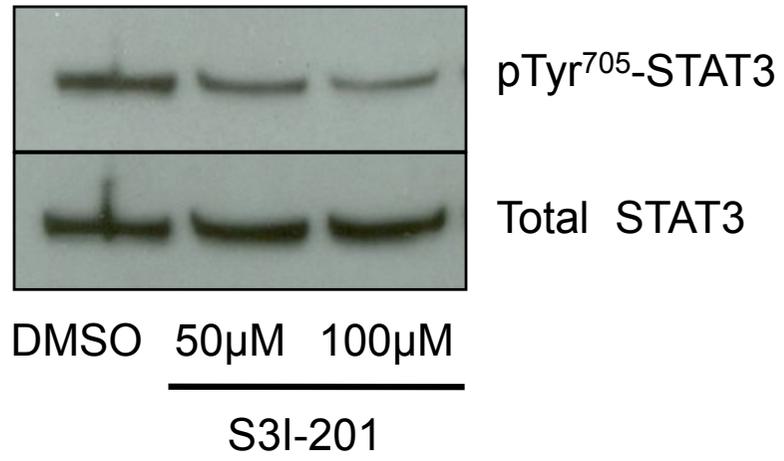


Fig.4

C



D

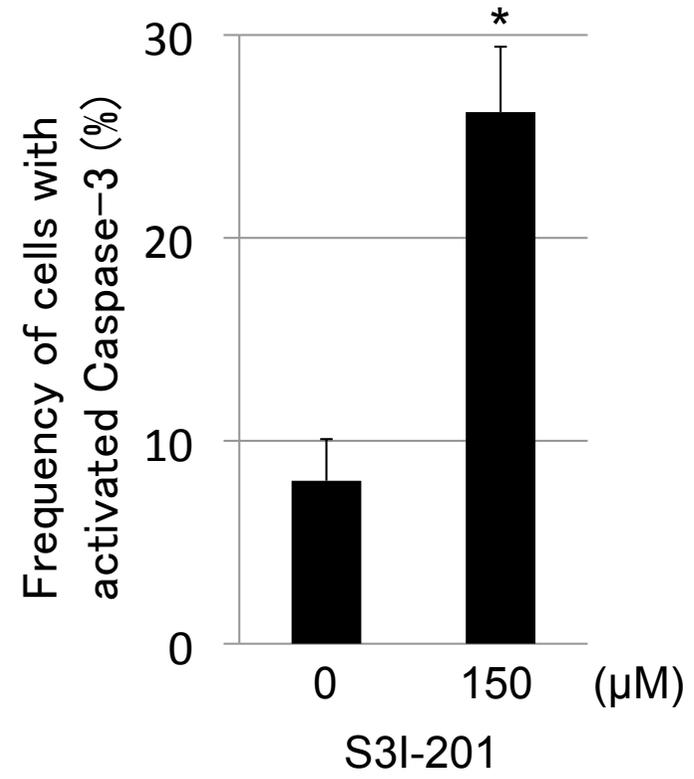


Fig.4

## Figure legends

### **Figure 1. Growth inhibition and cell death of MT-2 cells by resveratrol.**

(A) MT-2 cells were cultured in a 96-well tissue culture plate in the absence or presence of resveratrol at the indicated concentration for 24h or 48 h. Viable cells were measured using MTT assay. Data are expressed at the mean percentage of the control (untreated cells) and are the average of triplicate samples. Results are representative of 3 independent experiments. (B) MT-2 cells ( $1 \times 10^6$ /ml) were cultured in a 6-well tissue-culture plate in the absence or presence of resveratrol (50 $\mu$ M) for 24h or 48h. Data represent the average of triplicate samples, and error bars represent 1 SD from the mean for triplicate wells. Results are representative of 3 independent experiments. Res; resveratrol. \* $P < 0.05$ , \*\* $P < 0.001$  (control versus resveratrol).

### **Figure 2. Resvratrol inhibited constitutive phosphorylation of STAT3 but not STAT5 in MT-2 cells.**

MT-2 cells were treated with resveratrol (50 $\mu$ M or 100 $\mu$ M) for 24h. Immunoblots were performed with specific antibodies for (A) STAT3, p-STAT3 (Tyr<sup>705</sup>) and  $\beta$ -actin, and (B) STAT3, p-STAT3 (Ser<sup>727</sup>), STAT5, p-STAT5 (Tyr<sup>694</sup>) and  $\beta$ -actin. Immunoblot shown is representative of 2 independent experiments.

### **Figure 3. Resveratrol inhibited cIAP-2 and Mcl-1, and activated caspase-3 and PARP in MT-2 cells.**

MT-2 cells were treated with resveratrol (50 $\mu$ M or 100 $\mu$ M) for 24h. Immunoblots were performed with specific antibodies for (A) cIAP-1, cIAP-2, Mcl-1 and Bcl-2, and (B) cleaved caspase-3, cleaved PARP and  $\beta$ -actin. Immunoblot shown is representative of 2 independent experiments.

### **Figure 4. Growth inhibition and cell death of MT-2 cells via the caspase-3 activation by S3I-201.**

(A) MT-2 cells were cultured in a 96-well tissue culture plate in the absence or presence of STAT3 inhibitor, S3I-201 at the indicated concentration for 24h or 48 h. Viable cells were measured using MTT assay. Data are expressed at the mean percentage of the control (untreated cells) and are the average of triplicate samples. Results are representative of 3 independent experiments. (B) MT-2 cells ( $1 \times 10^6$ /ml) were cultured

in a 6-well tissue-culture plate in the absence or presence of S3I-201 (100 $\mu$ M) for 24h or 48h. Data represent the average of triplicate samples, and error bars represent 1 SD from the mean for triplicate wells. Results are representative of 3 independent experiments. \* $P$ <0.05, \*\* $P$ <0.0001 (control versus S3I-201). (C) MT-2 cells were treated with S3I-201 (50 $\mu$ M or 100 $\mu$ M) for 24h. Immunoblots were performed with specific antibodies for STAT3 and p-STAT3 (Tyr<sup>705</sup>). Immunoblot shown is representative of 2 independent experiments. (D) MT-2 cells (1 x 10<sup>5</sup>/ml) were cultured in a 12-well tissue-culture plate in the absence or presence of S3I-201 (150 $\mu$ M) for 48h. Cells were stained with PE-conjugated antibodies against activated caspase-3 and the frequency of cells with activated caspase-3 was measured by flow cytometer. Data represent the average of triplicate samples, and error bars represent 1 SD from the mean for triplicate wells. Results are representative of 2 independent experiments. \* $P$ <0.01 (S3I-201- versus S3I-201+).