

# Resveratrol Promotes Autophagic Cell Death in Chronic Myelogenous Leukemia Cells via JNK-Mediated p62/SQSTM1 Expression and AMPK Activation

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## Abstract

Autophagy that is induced by starvation or cellular stress can enable cancer cell survival by sustaining energy homeostasis and eliminating damaged organelles and proteins. In response to stress, cancer cells have been reported to accumulate the protein p62/SQSTM1 (p62), but its role in the regulation of autophagy is controversial. Here, we report that the plant phytoalexin resveratrol (RSV) triggers autophagy in imatinib-sensitive and imatinib-resistant chronic myelogenous leukemia (CML) cells via JNK-dependent accumulation of p62. JNK inhibition or p62 knockdown prevented RSV-mediated autophagy and antileukemic effects. RSV also stimulated AMPK, thereby inhibiting the mTOR pathway. AMPK knockdown or mTOR overexpression impaired RSV-induced autophagy but not JNK activation. Lastly, p62 expression and autophagy in CD34+ progenitors from patients with CML was induced by RSV, and disrupting autophagy protected CD34+ CML cells from RSV-mediated cell death. We concluded that RSV triggered autophagic cell death in CML cells via both JNK-mediated p62 overexpression and AMPK activation. Our findings show that the JNK and AMPK pathways can cooperate to eliminate CML cells via autophagy. *Cancer Res*; 70(3); 1042–52. ©2010 AACR.

## Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative syndrome linked to a hematopoietic stem cell disorder leading to the increased production of granulocytes at all stages of differentiation (1). Patients with CML carry the t(9;22) (q34;q11) translocation (2). This translocation is responsible for the expression of a 210 kDa chimeric fusion protein, p210 BCR-ABL, a constitutively active tyrosine kinase (3). The role of BCR-ABL in the pathogenesis of CML is linked to the activation of several downstream survival pathways including signal transducer and activator of transcription5/B cell lymphoma/leukemia x, Ras/Raf, MAP kinase-ERK kinase/Erk-1/2, Phosphatidylinositol 3 Kinase/Akt, and nuclear factor  $\kappa$ B (NF $\kappa$ B), which collectively provide proliferative advantages and resistance to apoptosis (4–6).

Resveratrol (*trans*-3,4',5-trihydroxystilbene; RSV), a naturally polyphenolic phytoalexin found in grapes, elicits several beneficial effects in human pathologies, noticeably in the pre-

vention of cardiovascular diseases (7), and is currently being used in phase I studies to treat obese and diabetic patients. In addition, RSV has also been shown to exhibit potent anticancer properties in some epithelial tumors and leukemia (8, 9). For instance, RSV has been reported to either protect CML cell lines from stress-induced apoptosis mainly through its antioxidant properties (10), or conversely, to induce cell death when used alone (11). However, the molecular mechanisms by which RSV exerts its antileukemic effects in CML cell lines remains incompletely understood (12). We have previously reported that RSV induces apoptosis in CML cell lines. More interestingly, we have shown that RSV also triggered cell death in imatinib-resistant (IM-R) cell lines and in Baf/3 cells carrying several mutated forms of BCR-ABL commonly found in resistant patients (13). However, the effects of RSV in CML cells are only partly inhibited by caspase inhibitors, strongly suggesting that RSV also exerts its potent antileukemic effect through caspase-independent cell death.

Autophagy is a catabolic process for the degradation and recycling of macromolecules and organelles, which is activated during stress conditions (12). Autophagy is initiated by the formation of double-membraned vesicles called autophagosomes, which fuse with lysosomes to form autophagolysosomes in which lysosomal hydrolases digest the vesicle contents for recycling (12, 14). The formation of autophagosomes is a three-step process characterized by nucleation, elongation, and completion of an isolation membrane or phagophore. The phagophore recruits different Atg proteins and its nucleation depends on PI3KCIII/Vps34 activity. Atg proteins, including Atg12, Atg5, and Atg16L are involved in the elongation of the isolation membrane, whereas LC3-II

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and P62 act as structural components of the autophagosomes (15). Autophagy is induced by various stimuli such as metabolic stress, energy need, and chemotherapy for instance (12, 16). Autophagy is considered as a survival mechanism induced in adverse conditions to maintain cell integrity, but paradoxically, it is also involved in a particular mode of death called autophagic cell death or type II cell death (12, 17, 18).

There is compelling evidence in the literature that RSV is capable of inducing autophagy in different cancer cell line models (19–23) but nothing is known regarding its effect on autophagy induction in leukemia. Moreover, the signaling pathways by which RSV mediates autophagy induction and exerts its antileukemic effects are far from being completely elucidated. Therefore, the purpose of this study was to decipher the mechanism of action of RSV in CML cells and to investigate whether autophagy contributes to the potent antileukemic effect of this compound. Here, we describe that RSV triggers autophagy in CML cell lines and CD34+ primary cells from CML patients through both AMPK-dependent production of phagophores and p62-dependent elongation and accumulation of autophagosomes. Therefore, the remarkable efficiency of RSV in IM-R cell lines is likely due to its capacity to trigger both apoptotic and autophagic cell death.

## Materials and Methods

**Reagents and antibodies.** Imatinib (Gleevec) was provided by Novartis Pharma. RPMI medium and fetal calf serum were from Life Technologies. Phenylmethylsulfonyl fluoride, aprotinin, leupeptin, resveratrol, and bafilomycin A1 were from Sigma. Ac-DEVD-AMC, Ac-DEVD-CHO, and zVAD-fmk were from Alexis Biochemicals. Rapamycin and SP600125 were from Calbiochem. Anti-p62, *c-Jun*, and HSP60 antibodies were purchased from Santa Cruz Biotechnology. Anti-LC3, ATG5, phosphorylated JNK (T183/Y185), phosphospecific antibodies directed against *c-Jun* (S63), AMPK $\alpha$  (T172), mTOR (S2448), P70/85-S6K (T389), S6 ribosomal (S235/236), 4EBP1 (T37/46) and AMPK $\alpha$ , mTOR, p70/85-S6K, S6 ribosomal, and 4EBP1 antibodies were from Cell Signaling. Other antibodies were from Dakopatts.

**Cell lines.** The human CML cell line K562 and their IM-R counterparts have been described elsewhere (24, 25). Ba/f3 p210BCR-ABL or p210BCR-ABL T315I were kindly provided by Pr. J.V. Melo (Department of Haematology, Imperial College of Hammersmith Hospital, London, UK) and Pr. F.X. Mahon (Laboratoire Hematopoïèse Leucémique et cible thérapeutique, INSERM U876, Université Victor Segalen, Bordeaux, France) (26).

**Primary cell isolation.** Blood samples were collected from patients newly diagnosed with CML as part of an institutionally approved protocol. Mononuclear cells were isolated by density centrifugation (Ficoll-Paque Plus), washed with PBS, 5% fetal calf serum, 2 mmol/L of EDTA, resuspended in cell culture medium (Iscove's modified Dulbecco's medium, 10% fetal bovine serum), and incubated overnight at 37°C before CD34+ cells' isolation by magnetic positive selection (StemSep Human CD34 Selection Kit; StemCell). Experiments were per-

formed using a StemSpan SFEM medium supplemented with 100 ng/mL of human recombinant SCF, FLT3-L, and 20 ng/mL of human recombinant interleukin-3, interleukin-6, and granulocyte colony-stimulating factor (PeproTech).

**Western blot.** Western blot experiments have been described in detail previously (5).

**p62 immunoprecipitation.** K562 cells were suspended in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 20 mmol/L EDTA, 1 mmol/L DTT, 50 mmol/L NaF, 0.1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L phenylmethylsulfonyl fluoride, 2  $\mu$ g/mL aprotinin, and 0.5% NP40]. Lysates (500  $\mu$ L) were then incubated with 1.5  $\mu$ g of monoclonal mouse anti-p62 antibody (Santa Cruz Biotechnology) and 30  $\mu$ L of protein G-Sepharose (Zymed Laboratories) at 4°C overnight. Beads were washed five times with 1 mL of lysis buffer before boiling in Laemmli sample buffer and performing SDS-PAGE, transfer to polyvinylidene difluoride membranes, and immunoblotting.

**Confocal microscopy.** GFP-LC3 construct (a gift from Pr. Maria I. Colombo, Laboratorio de Biología Celular y Molecular, Universidad Nacional de Cuyo, Mendoza, Argentina) was stably transfected in K562 cells. After treatment, cells were washed with ice-cold PBS and were fixed and permeabilized with Cytofix/Cytoperm solution (BD Bioscience). After two washes with Perm/Wash solution at room temperature, cells were incubated for 30 min with polyclonal rabbit anti-p62 antibody and with anti-rabbit Alexa594 coupled antibody. Cells were finally incubated with 1 mg/mL of 4',6-diamidino-2-phenylindole, mounted on glass slides in Fluoromount-G (Southern Biotechnology Associates), and photographed with a confocal laser microscope (Carl Zeiss).

**Transmission electron microscopy.** Electron microscopy experiment have been described previously (27).

**Knockdown by small interfering RNA.** The protocol for knockdown experiments has been described earlier (24). Stealth small interfering RNAs (siRNA) targeting ATG5, p62, AMPK $\alpha$ 1, and AMPK $\alpha$ 2 were purchased from Invitrogen, whereas LC3 siRNA were from Dharmacon.

**Cell viability (XTT).** Cell viability assays have been described earlier (28).

**Colony formation assay.** Colony formation assays have been reported elsewhere (13).

**Reverse transcription-PCR analysis.** Total RNA was prepared from K562 cells exposed or not to RSV and SP for 24 h using Trizol (Invitrogen) and used as a template for reverse transcription. For p62 amplification, the following specific primers were used (GTGGTAGGAACCCGCTACAA and GCGATCTTCCTCATCTGCTC).

**Overexpression of mTOR.** K562 cells were transiently transfected with pcDNA3-CT or pcDNA3-mTOR wild-type (a gift from Dr. Sophie Giorgetti-Peraldi, INSERM U895 Team 6, Université de Nice Sophia-Antipolis, Nice, France) using the jetPEI protocol (PolyPlus transfection, Inc.). One day after transfection with plasmid CT, or mTOR wild-type at a final concentration of 2.5  $\mu$ g, cells were treated or not with RSV for an additional 2 d.

**Flow cytometry.** After stimulation, CD34+ CML primary cells were washed with ice-cold PBS and were fixed and permeabilized with Cytofix/Cytoperm solution (BD Bioscience). After two washes with Perm/Wash solution at room

temperature, cells were incubated for 30 min with anti-phosphorylated S6 ribosomal (S235/236)-Alexa488 or anti-phosphorylated 4EBP1 (T37/46)-Alexa488 antibodies (Cell Signaling). Finally, cells were resuspended in Perm/Wash solution and analyzed with a FACScan (Becton Dickinson).

**Statistical analysis.** Results are expressed as the mean  $\pm$  SD. Statistical analysis was performed using Student's *t* test with  $P < 0.05$  deemed as statistically significant. All experiments were repeated at least thrice unless otherwise stated.

## Results

**RSV induces autophagy in imatinib-sensitive and -resistant CML cells.** We previously reported that treatment of imatinib-sensitive (IM-S) or IM-R K562 cells with RSV for 24 to 48 hours resulted in loss of cell viability and induction of apoptosis (13). However, the effect of RSV was only partly inhibited by the pan-caspase inhibitor zVAD-fmk, suggesting that other modes of cell death were also induced (13). Accordingly, electronic microscopy images of IM-S and IM-R cells treated for 48 hours with RSV showed typical images of autophagy including accumulation of numerous vesicles with distinct double membranes (Fig. 1A). RSV also induced LC3-II accumulation, a hallmark of autophagy (Fig. 1B). In the meantime, imatinib failed to induce LC3-II accumulation in contrast with rapamycin, used as a positive control for autophagy induction. RSV-mediated induction of autophagy was accompanied by increased expression of ATG3 and p62 (Fig. 1B). The increased accumulation of p62 is consistent with RSV triggering stress-mediated autophagy. Thus, autophagy may function to prevent the accumulation of damaged organelles during RSV-induced metabolic stress. Coimmunoprecipitation analysis established that endogenous LC3 and p62 interacted together (Fig. 1C). Importantly, both LC3-I and LC3-II were able to interact with p62 in IM-S and IM-R cells and this interaction was increased in RSV-treated cells. Colocalization of p62 with GFP-LC3 was confirmed further by confocal microscopy (Fig. 1D). This is the first evidence that LC3 and p62 could bind together in intact cells.

**Pharmacologic inhibition of caspases and vacuolar ATPase prevents RSV-mediated loss of cell viability.** We have reported that RSV induced caspase activation in both IM-S and IM-R K562 cells (13). We thus investigated whether inhibition of caspases or targeting lysosomes altered the antileukemic effect of RSV. Inhibition of vacuolar ATPase by bafilomycin A1 (Baf A1) or caspases by zVAD-fmk prevented RSV-mediated loss of cell viability by 35% and 45% in IM-S cells, respectively (Fig. 2A).

The combination of both inhibitors was most potent that each one separately, inducing an overall 60% increase in cell viability in RSV-treated cells. Although zVAD-fmk clearly protects K562 cells from imatinib-induced loss of cell viability, Baf A1 had no effect. These results strongly suggest that the intrinsic mechanisms underlying the effect of RSV and imatinib are different. In addition, Baf A1 and zVAD-fmk also protected IM-R cells from RSV-mediated loss of viability (Fig. 2A, left).

In long-term clonogenic assays, the percentage of colony-forming IM-S cells was drastically reduced upon imatinib and RSV treatment but only the RSV effect was prevented by Baf A1 (Fig. 2A, right). As expected, the clonogenic potential of IM-R cells was not altered by imatinib treatment.

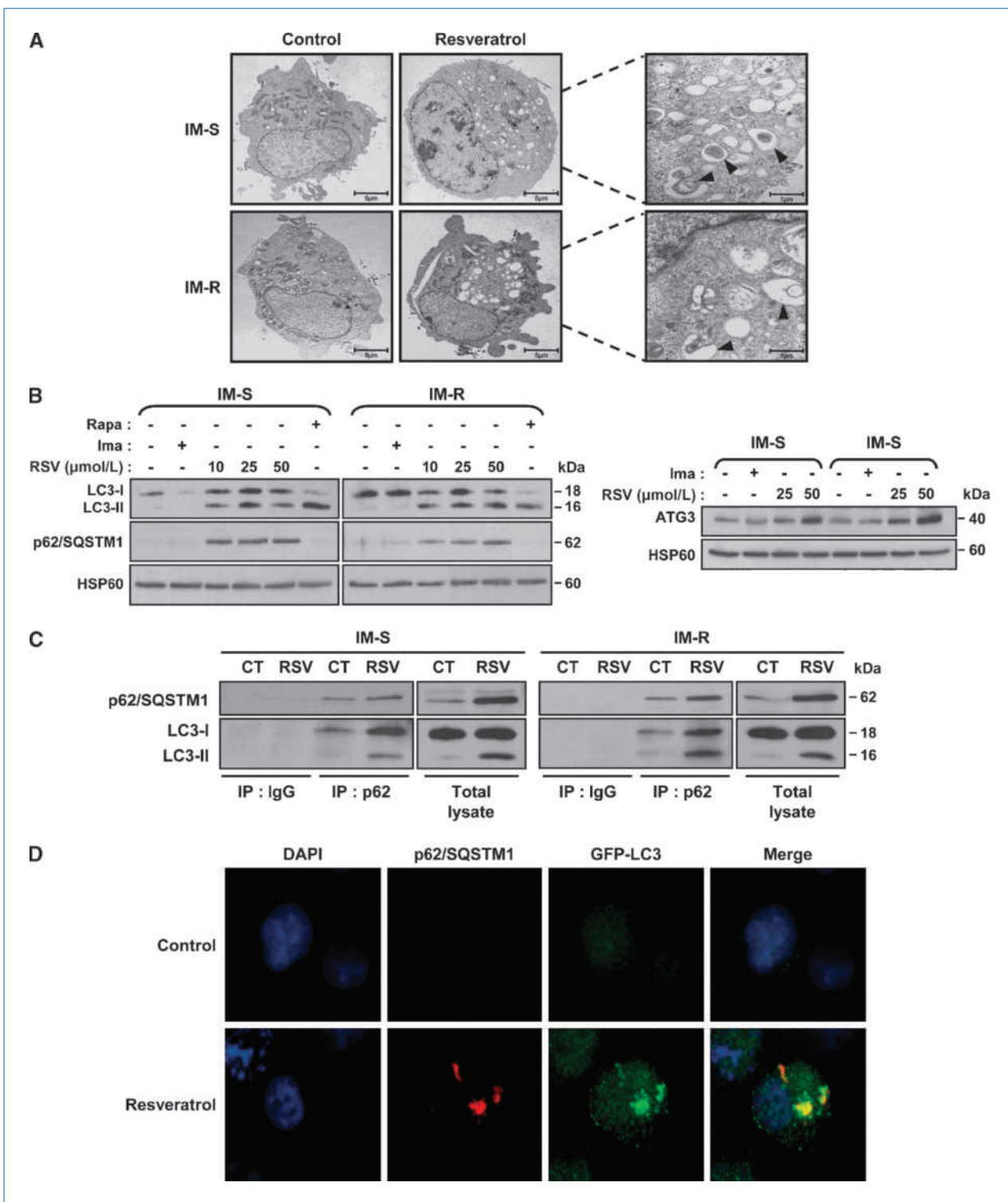
To investigate whether autophagy was involved in the effect of RSV, we inhibited it using LC3 or ATG5 siRNAs. None of these siRNAs, which drastically reduced the expression of both proteins (Fig. 2B), were capable of promoting increased cell viability in the presence of imatinib (Fig. 2C). In contrast, knockdown of ATG5 (Fig. 2C, left) or LC3 promoted protection against RSV-mediated cell death in IM-S and IM-R cells (Fig. 2C, right). As RSV triggers both apoptosis and autophagy in K562 cells, we next analyzed whether these two events could depend on each other. Caspase 3 activation and LC3-II accumulation were detected for low doses of RSV (Supplementary Fig. S1A). Moreover, both accounted for RSV-mediated loss of cell metabolism because the combination of zVAD-fmk and Baf A1 was more potent in preventing RSV-induced cell death compared with each drug separately (Supplementary Fig. S1B).

**RSV activates the JNK pathway to induce p62.** The molecular mechanisms underlying the antileukemic effect of RSV are ill-defined. To further decipher the mode of action of this compound, we analyzed the effect of RSV on the main cellular signaling pathways that are constitutively modulated in CML cells, including p38MAPK, JNK, ERK1/2, PI3K, mTOR, PKA, and PKC. Pharmacologic inhibition suggested that JNK was important for the RSV effect (data not shown). Accordingly, RSV triggered JNK activation, as soon as 2 to 4 hours after its addition in IM-S and IM-R cells, a response maintained for at least 48 hours (Fig. 3A).

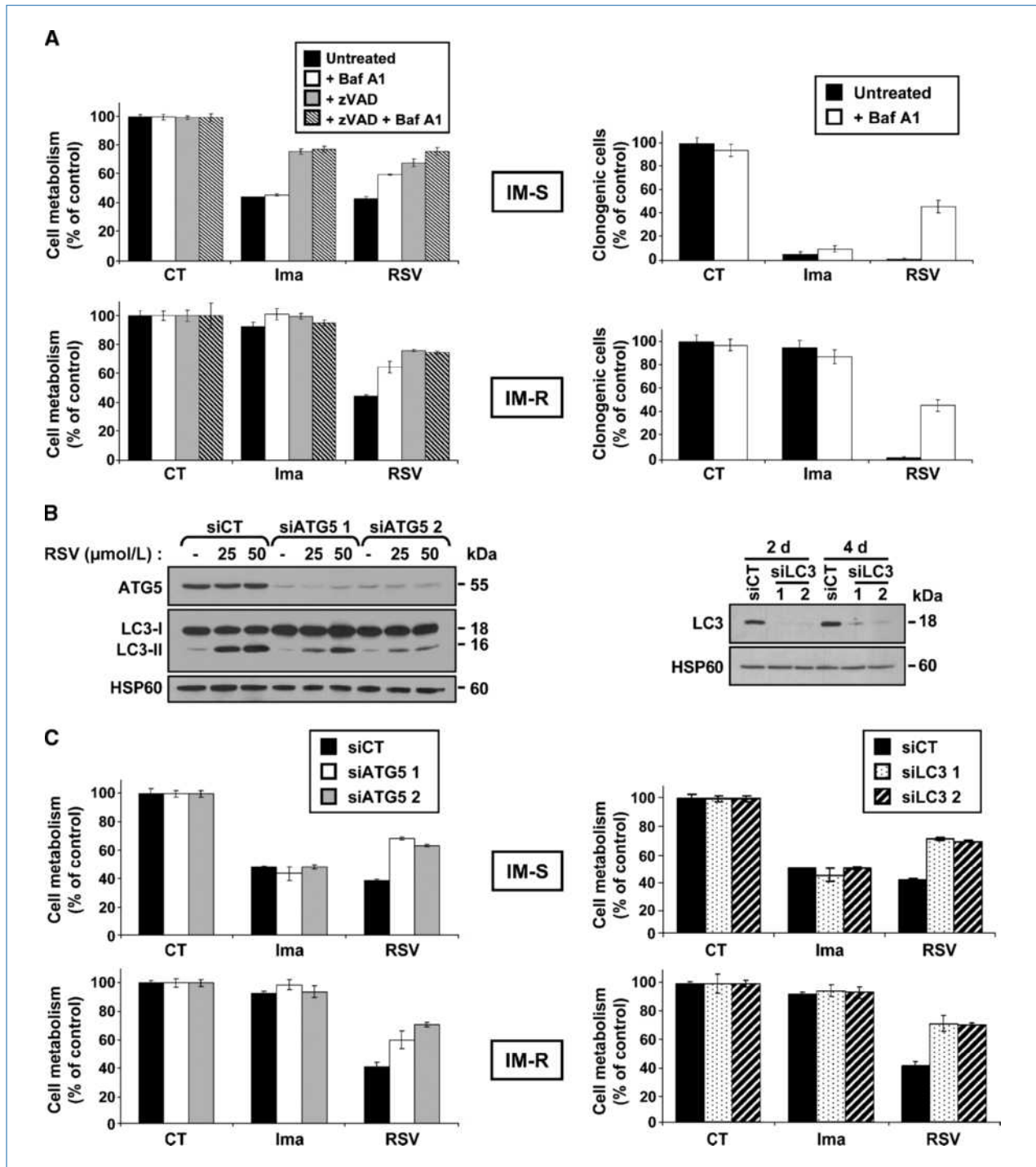
Of note, JNK activation correlated with an increased *c-Jun* phosphorylation (Fig. 3A). As expected, the effect of RSV on JNK activation and *c-Jun* phosphorylation were abrogated by SP600125 (SP), a specific JNK inhibitor. Because RSV induced p62 expression in K562 cells (Fig. 1), we investigated whether JNK was implicated. In support of this, an RSV-mediated increase in p62 mRNA level was prevented by SP in IM-S and IM-R clones as well (Fig. 3B). In addition, electron microscopy images revealed that induction of autophagy by RSV was also abrogated by SP (Fig. 3C). Accordingly, SP drastically reduced both p62 expression and LC3-II accumulation (Fig. 3D). These findings show that RSV triggered autophagy and p62 accumulation through the activation of JNK in CML cell lines.

**p62 is required for RSV-mediated autophagy.** To investigate whether p62 accumulation was responsible for RSV-mediated autophagy and CML cell death, we knocked down p62 using two different siRNAs. We verified that both siRNAs abrogated p62 expression 4 days after their addition in the culture medium (Fig. 4A). p62 knockdown resulted in a total blockage of LC3-II accumulation, and importantly, promoted increased cell viability in the presence of RSV in IM-S (Fig. 4B, top) and IM-R cells (Fig. 4B, bottom). By contrast, p62 inhibition failed to restore cell viability in the presence of imatinib (data not shown).

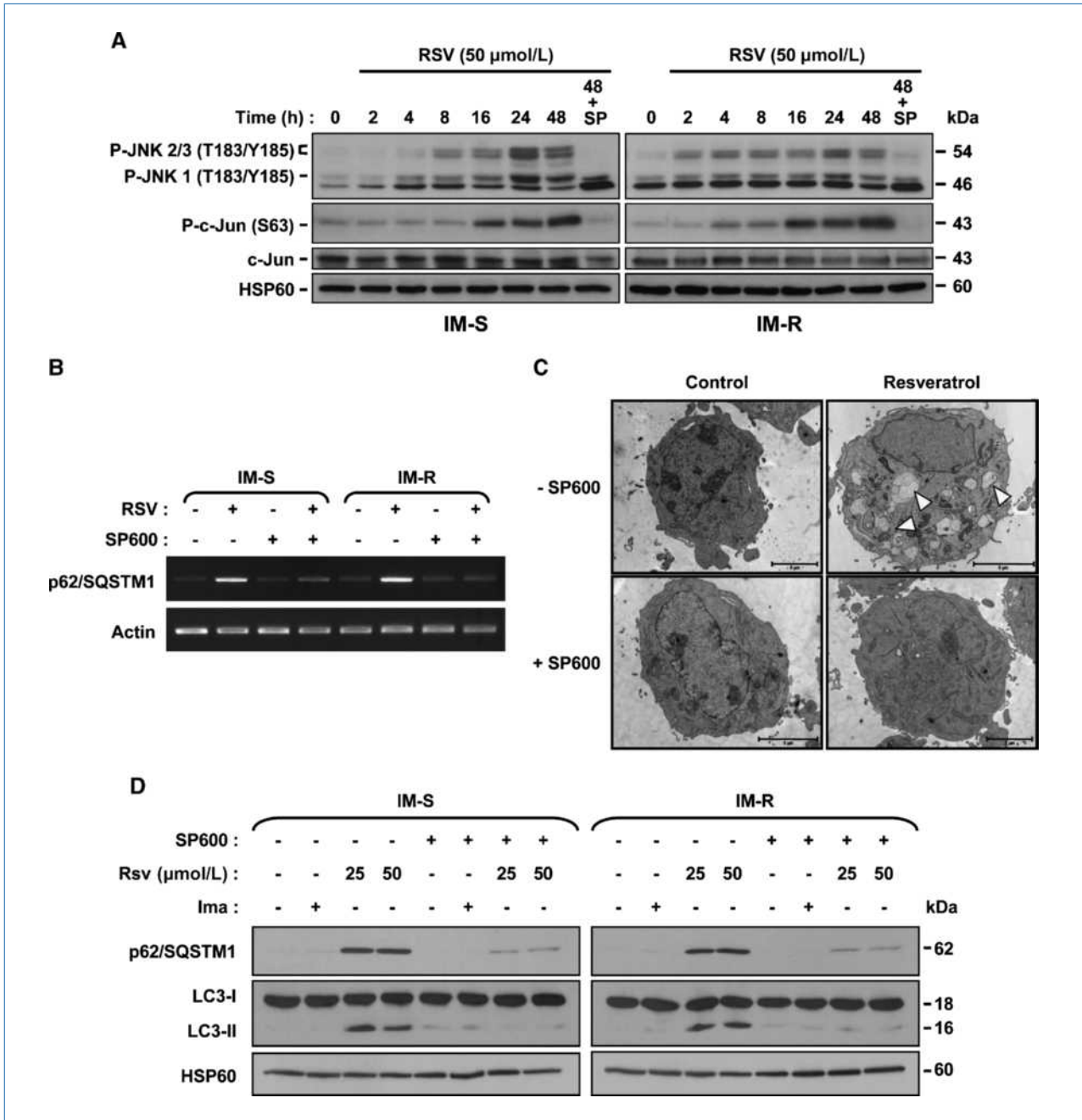
**RSV also regulates autophagy through AMPK-dependent inhibition of the mTOR pathway.** Because recent results in



**Figure 1.** RSV induces autophagy in IM-S and IM-R K562 cells. A, IM-S and IM-R cells ( $10^5$ /mL) were incubated for 48 h at 37°C with 50 μmol/L of RSV. After inclusion, preparations were observed with an electron microscope mounted with a CCD camera. B, cells ( $10^5$ /mL) were incubated for 48 h with 3 μmol/L imatinib or various concentrations of RSV. Cell lysates were analyzed by SDS-PAGE. Proteins were blotted with anti-LC3, p62, ATG3, or HSP60 antibodies. Rapamycin (100 nmol/L) was used as a control of autophagy induction. C, cells were stimulated for 72 h with RSV (50 μmol/L). Lysates were incubated with control IgG or a mouse anti-p62 antibody and immunoprecipitates were analyzed by immunoblotting using rabbit anti-p62 or anti-LC3 antibodies. D, K562 cells stably expressing GFP-LC3 (green) were incubated for 48 h with 50 μmol/L of RSV, fixed, and then permeabilized. Cells were then incubated with an anti-p62 (red) antibody and 4',6-diamidino-2-phenylindole (DAPI; blue). Immunofluorescent staining was viewed by confocal microscopy.



**Figure 2.** RSV-mediated autophagy decreases cell viability and clonogenicity. **A**, left, cells ( $10^5/\text{mL}$ ) were preincubated for 1 h with or without zVAD-fmk ( $50 \mu\text{mol/L}$ ), bafilomycin A1 ( $5 \text{ nmol/L}$ ), or the combination of both effectors and exposed to imatinib ( $1 \mu\text{mol/L}$ ) or RSV ( $50 \mu\text{mol/L}$ ) for the next 48 h. Cell viability was assessed using the XTT assay. **A**, right, K562 cells were incubated for 8 d in semisolid methylcellulose medium ( $0.5 \times 10^3$  cells/mL) with imatinib ( $1 \mu\text{mol/L}$ ) or RSV ( $50 \mu\text{mol/L}$ ) in the presence or the absence of bafilomycin A1 ( $5 \text{ nmol/L}$ ). Colonies were detected by adding 1 mg/mL of MTT reagent and scored by Image J quantification software. Columns, mean of four different determinations; bars, SD (95% confidence intervals). **B**, left, K562 cells ( $10^5/\text{mL}$ ) were transfected for 4 d with a control siRNA or two specific ATG5 siRNAs. RSV ( $25$  or  $50 \mu\text{mol/L}$ ) was added for the last 2 d. ATG5 silencing and RSV-mediated LC3 cleavage were analyzed by immunoblotting using specific antibodies. HSP60 was used as a loading control. **B**, right, LC3 knockdown was analyzed 2 and 4 d after transfection with two LC3 siRNAs using specific antibody. **C**, cells were transfected for 4 d with a control siRNA or two ATG5 siRNAs (left) or LC3 (right). Imatinib ( $1 \mu\text{mol/L}$ ) or RSV ( $50 \mu\text{mol/L}$ ) were added for the last 2 d. Cell viability was assessed using the XTT assay. Columns, mean of four different determinations; bars, SD (95% confidence intervals).



**Figure 3.** RSV activates the JNK pathway to induce p62. A, cells ( $10^5$ /mL) were incubated for various times at  $37^\circ\text{C}$  with  $50\ \mu\text{mol/L}$  of RSV in the presence or absence of  $10\ \mu\text{mol/L}$  of SP600125 (SP). Phosphorylation of JNK and *c-Jun* was detected by immunoblotting. B and C, cells ( $10^5$ /mL) were stimulated with RSV ( $50\ \mu\text{mol/L}$ ), SP ( $10\ \mu\text{mol/L}$ ), or the combination of both effectors. B, RT-PCR analysis of p62 was performed after 24 h of treatment. C, after 48 h, IM-S cells were fixed and observed with an electron microscope mounted with a CCD camera. D, cells were incubated for 48 h with imatinib ( $1\ \mu\text{mol/L}$ ) or RSV with or without SP ( $10\ \mu\text{mol/L}$ ). p62 and LC3 expression were visualized by immunoblotting using specific antibodies. HSP60 or actin were used as a loading control.

the literature indicate that RSV may activate AMPK (29, 30), we verified whether this was also the case in CML cell lines. RSV increased AMPK phosphorylation on threonine 172 in both IM-S and IM-R cells with a maximum at 4 hours (Fig. 5A). This was accompanied by a net decrease in the

status of phosphorylation of mTOR, p70-S6 kinase, S6 ribosomal protein, and 4-EBP1 suggesting a blockade of this pathway at the TSC1/TSC2 level. To address the role of AMPK on RSV-mediated increase in autophagy, we knocked down both the  $\alpha 1$  and  $\alpha 2$  AMPK subunits using RNA interference

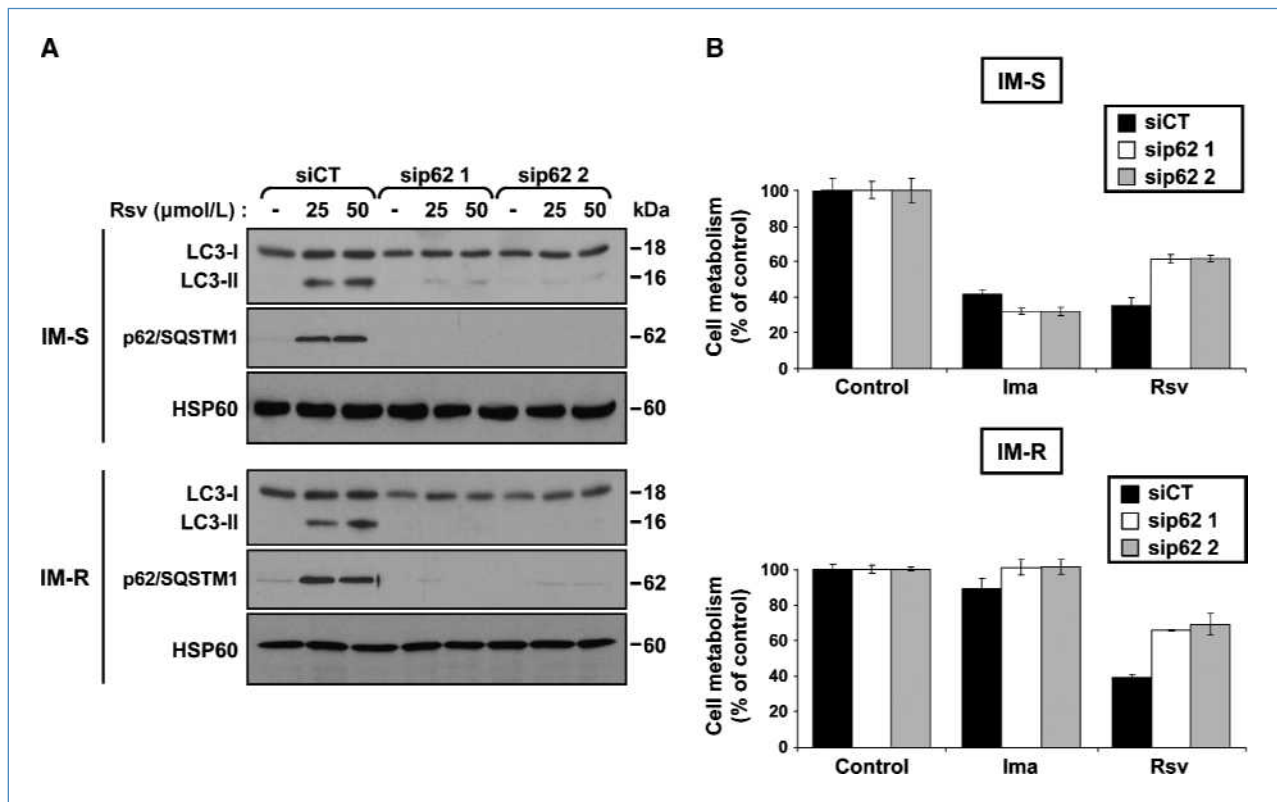
because redundancy had been previously reported for these isoforms (31).

The combination of both siRNAs led to a nearly complete abrogation of AMPK $\alpha$  and LC3-II accumulation (Fig. 5B), indicating that AMPK also mediated the effect of RSV on autophagy. In agreement with these findings, the expression of a constitutively activated mTOR plasmid in K562 cells abrogated RSV-mediated LC3-II accumulation but increased S6 ribosomal and 4-EBP1 phosphorylation, underlying the role of AMPK in RSV-mediated autophagy (Fig. 5C). The next question was to determine if the effect of RSV on AMPK and JNK were interrelated. In a first approach, we established that SP failed to inhibit the effect of RSV on P70/85-S6K, S6 ribosomal and 4-EBP1 phosphorylations suggesting that RSV activated AMPK and JNK independently or that AMPK acted upstream of JNK. Knockdown of AMPK failed to inhibit RSV-mediated activation of JNK (Fig. 5B) and p62 expression (data not shown), definitely demonstrating the nonredundant role of both pathways in the RSV effect. Interestingly, all the signal transduction pathways mediated by RSV occurred in Baf/3-p210BCR-ABL wild-type and Baf/3-p210BCR-ABL T315I cells as well (Supplementary Fig. S2). Next, we analyzed the possible relationship between apoptosis and autophagy in the RSV ef-

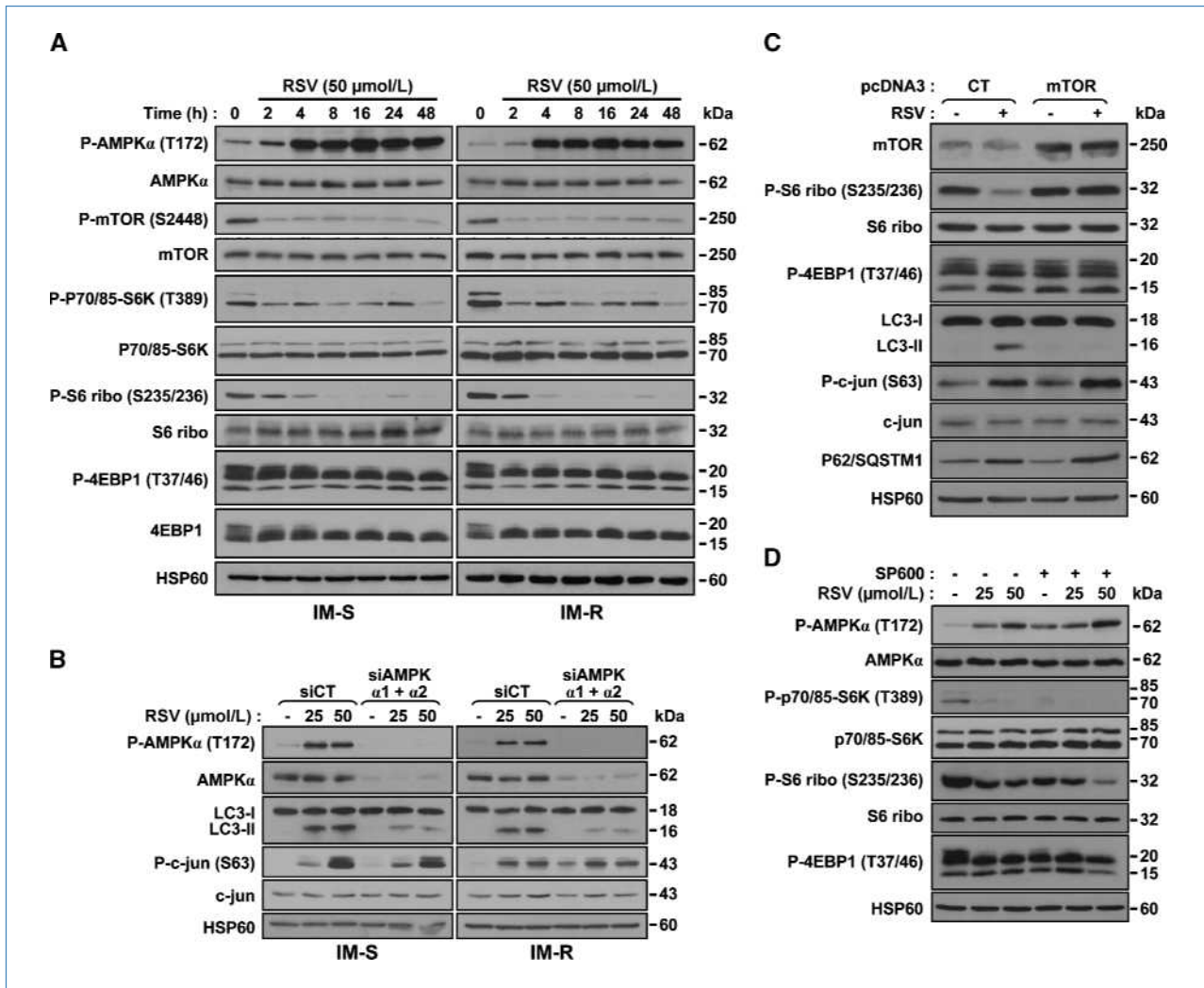
fect. Inhibition of RSV-mediated caspase activation by zVAD-fmk neither modulated LC3-II accumulation nor p62 induction (Supplementary Fig. S3A), indicating that caspases did not participate in autophagy. By contrast, knockdown of either LC3, Atg5, or AMPK (Supplementary Fig. S3B–D) inhibited RSV-mediated caspase 3 activation as well as the JNK inhibitor SP600125 (Supplementary Fig. S3D). Altogether, these findings indicate that impairing caspase activation has no effect on RSV-mediated autophagy, whereas inhibition of autophagy inhibits RSV-induced caspase activation and apoptosis.

**RSV-mediated autophagic cell death in CD34+ cells from patients with CML.** Finally, we investigated the effect of RSV on CD34+ stem cells from patients with CML. CD34+ cells were purified on magnetic beads coated with anti-CD34 monoclonal antibodies. For each patient, the purity of CD34+ estimated by fluorescence-activated cell sorting was found to be at least 90%. RSV induced LC3-II accumulation in CD34+ cells are shown for a representative patient (Fig. 6A). As in CML cell lines, this was accompanied by a net increase in p62 expression and *c-Jun* phosphorylation.

In addition, RSV significantly reduced S6 and 4-EBP1 phosphorylation in CD34+ cell samples from patients with CML as judged by phosphoflow experiments (Fig. 6B).



**Figure 4.** p62 is required for RSV-mediated autophagy. A and B, K562 cells ( $10^5$ /mL) were transfected for 4 d with a control siRNA or two p62 siRNAs. Imatinib (1  $\mu$ mol/L) or RSV were added for the last 2 d. A, LC3-I cleavage and p62 knockdown were visualized by immunoblotting using specific antibodies. HSP60 was used as a loading control. B, cell viability was assessed using the XTT assay. Columns, mean of four different determinations; bars, SD (95% confidence intervals).



**Figure 5.** RSV activates AMPK to initiate an autophagic process. A, lysates from cells treated for various times with 50  $\mu\text{mol/L}$  of RSV were analyzed by SDS-PAGE. Phosphorylation and expression level of AMPK $\alpha$ , mTOR, p70/85-S6K, S6 ribosomal, and 4EBP1 were determined using specific antibodies. B, cells ( $10^5/\text{mL}$ ) were transfected for 4 d with a control siRNA or the combination of two siRNA directed against AMPK $\alpha 1$  and AMPK $\alpha 2$ . RSV was added for the last 2 d. LC3-I cleavage, AMPK $\alpha$  knockdown, and c-*Jun* phosphorylation status were visualized by immunoblotting using specific antibodies. C, IM-S cells ( $10^5/\text{mL}$ ) were transfected with pcDNA3 or pcDNA3-mTOR. Two days later RSV (50  $\mu\text{mol/L}$ ) was added 2 more days. Protein level and phosphorylation status of mTOR, S6 ribosomal, 4EBP1, p62 and c-*Jun* and LC3 cleavage were visualized by immunoblotting with specific antibodies. D, IM-S cells ( $10^5/\text{mL}$ ) were stimulated for 48 h with RSV (25 and 50  $\mu\text{mol/L}$ ), SP (10  $\mu\text{mol/L}$ ), or the combination of both effectors. Cell lysates were analyzed by SDS-PAGE and the phosphorylation status of AMPK $\alpha$ , p70/85-S6K, S6 ribosomal, and 4EBP1 proteins was analyzed with specific antibodies. HSP60 was used as a loading control.

Next, we investigated the effect of RSV on CD34+ cell viability and metabolism. RSV induced the death of purified CD34+ cells from CML patients with a maximal effect at 50  $\mu\text{mol/L}$  as shown by phase contrast microscopy (Fig. 6C). Finally, cell viability was determined on CD34+ cells from five different patients. RSV was found to be more effective than imatinib in inhibiting cell viability (Fig. 6D). Baf A1 inhibited RSV but not imatinib-mediated loss of cell metabolism, in agreement with the result of Fig. 2, confirming the importance of lysosomes and autophagy in the RSV effect. In contrast, Baf A1 failed to prevent imatinib-mediated cell death in CD34+ cells.

### Discussion

The antiproliferative and anticarcinogenic effects of RSV involved various signaling mechanisms including increased antioxidant capacity through the induction of phase II enzymes, cell cycle arrest, induction of apoptosis of transformed cells, suppression of invasion and metastasis, and sensitization to chemotherapy-triggered apoptosis (32). However, the signaling pathways mediated by RSV to induce its complex antileukemic effects in cancer cells remain incompletely elucidated. We have previously reported that RSV induces caspase activation leading to the apoptosis of IM-S and



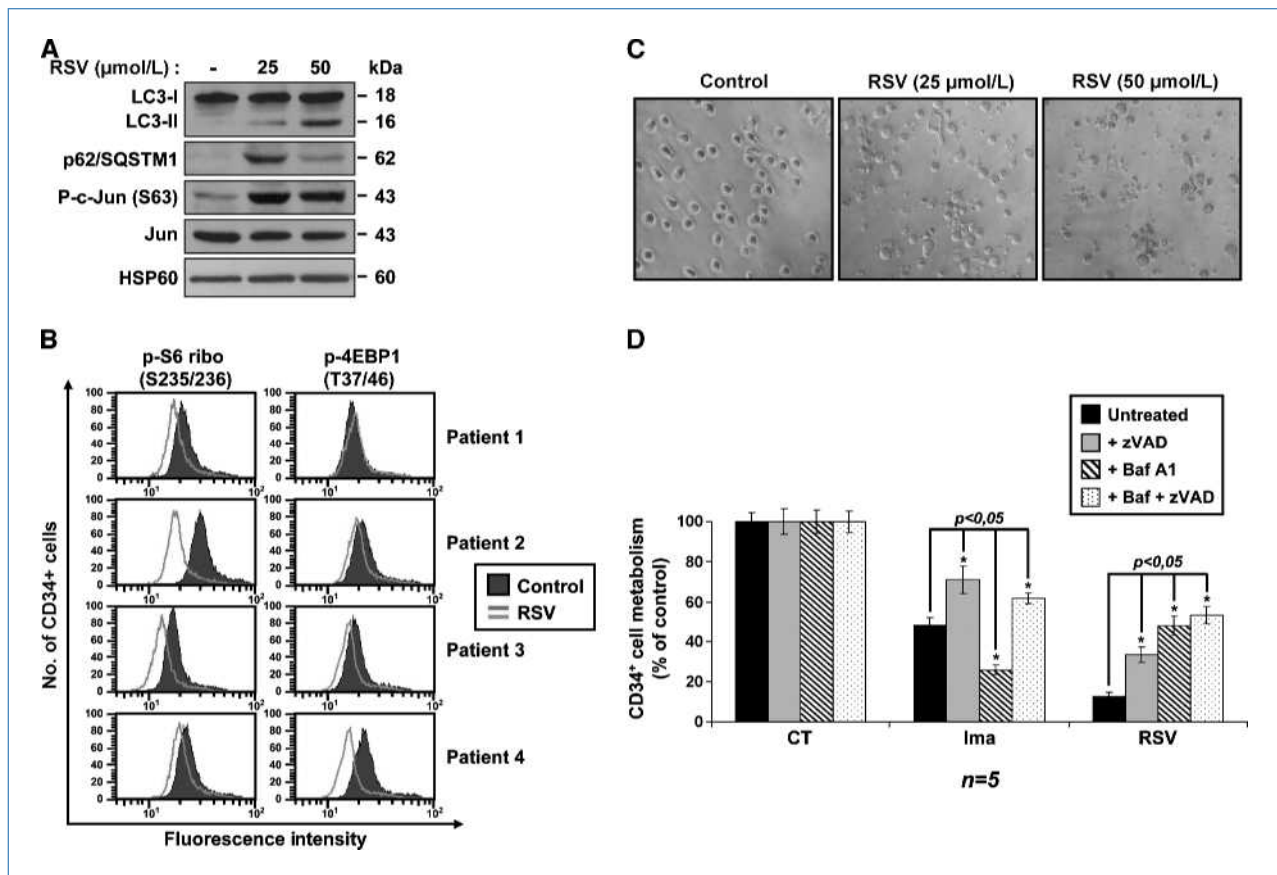
IM-R CML cell lines, a mechanism that explains, only in part, its potent antileukemic effect (13).

In this study, we show that RSV induces autophagy in IM-S and IM-R cells as well. This effect is mediated through both the activation of AMPK and JNK that participate independently with the initiation and elongation steps of autophagy, respectively. Indeed, RSV through AMPK-dependent mTOR inhibition promotes the formation of phagophores, whereas activation of JNK favors elongation via induction of p62 expression and its binding to LC3. In agreement with this model (Supplementary Fig. S4), blockage of autophagy by siRNAs directed against LC3, p62, or AMPK promotes CML cell survival, suggesting that RSV induces autophagic cell death.

Scarletti and colleagues reported recently that RSV induces caspase-dependent and -independent cell death in MCF7 cells expressing caspase 3 but only caspase-independent cell death in MCF7 cells lacking this caspase (21). Indeed, RSV activates noncanonical Beclin-1-independent autophagy in both cell lines that, however, acts as a caspase-independent cell death

mechanism only in MCF7 cells deficient for caspase 3. Accordingly, we reported earlier that in CML cells, RSV induces both caspase-dependent and -independent cell death (13). However, inhibition of caspase activation by zVAD-fmk neither affected RSV-induced LC3-II accumulation nor p62 expression, suggesting that RSV-mediated apoptosis did not interfere with induction of autophagy (Supplementary Fig. S3). By contrast, inhibition of RSV-mediated autophagy partly prevented caspase 3 activation, suggesting that RSV-mediated autophagy contributes to RSV-induced apoptosis (Supplementary Fig. S3C). Therefore, in CML cells, RSV mediated autophagic cell death by caspase-independent mechanisms. However, RSV-mediated autophagy is involved at least partly in the induction of apoptosis (Supplementary Fig. S4).

We also show that RSV triggered JNK activation and that inhibition of JNK by SP prevented RSV-mediated induction of autophagy and CML cell death. In contrast, JNK inhibition fails to alter RSV-mediated apoptosis (Fig. 5D). Therefore, JNK plays a crucial role in RSV-mediated autophagic but not apoptotic cell



**Figure 6.** RSV increases autophagic cell death in CD34+ CML primary cells. A, CD34+ primary cells from patients were stimulated for 48 h with RSV. LC3-I cleavage, p62 expression, and *c-Jun* phosphorylation and expression were analyzed by immunoblotting using specific antibodies. HSP60 was used as a loading control. B, CD34+ cells from four patients were stimulated for 48 h with 50  $\mu\text{mol/L}$  of RSV. Cells were fixed, permeabilized, and incubated with anti-phosphorylated S6 or anti-phosphorylated 4EBP1 antibodies and analyzed by flow cytometry. C, CD34+ primary cells were left untreated or stimulated with RSV (25 and 50  $\mu\text{mol/L}$ ) and photographed 48 h later (magnification,  $\times 200$ ). D, CD34+ primary cells were preincubated for 1 h with or without zVAD-fmk (50  $\mu\text{mol/L}$ ), bafilomycin A1 (5 nmol/L), or the combination of both effectors and next exposed to imatinib (1  $\mu\text{mol/L}$ ) or RSV (50  $\mu\text{mol/L}$ ) for the next 48 h. Cell viability was assessed using the XTT assay. Columns, mean of four different determinations; bars, SD ( $P < 0.05$  was considered statistically significant).

death. The function of JNK in autophagy regulation is emerging. It has been reported that JNK activation elicited by IRE1 in the early phase of endoplasmic reticulum stress is crucial for autophagosome formation and accumulation (33). In the same line, Li and colleagues described previously that SP could inhibit autophagy induction by ceramide (34). They proposed that activation of the JNK pathway mediates Beclin-1 expression, which in turn, plays a key role in autophagic cell death in cancer cells. Clearly, this is not the case in our study because RSV fails to induce the expression of Beclin-1 (data not shown). With regards to RSV, there are conflicting results in the literature regarding its effect on JNK modulation. Indeed, some groups reported activation of JNK by this phytoalexin (35, 36), whereas others described inhibition (37, 38). Nevertheless, our findings clearly show that in CML cells, RSV acts as a potent inducer of JNK and *c-Jun* phosphorylation.

An interesting finding of our study is that RSV-mediated JNK activation leads to increased expression of p62 mRNA and protein levels. Although JNK has been associated with an increase in autophagy gene expression (34, 39, 40), this is to the best of our knowledge, the first evidence that p62 expression is regulated by JNK. Of note, we (27) and others (41) have previously reported an increased expression of p62 with phorbol esters, whereas pangenomic profiles have identified p62 as a gene upregulated by RSV in both IM-S and IM-R cells.<sup>7</sup> However, whether or not this effect relies on JNK activation by phorbol 12-myristate 13-acetate remains to be determined. p62 plays an important role in the control of bone homeostasis because p62 knockout mice display defects in osteoclastogenesis as a consequence of inefficient activation of NF $\kappa$ B in osteoclasts (42). p62 also has a role in the control and the clearance of polyubiquitinated proteins, thereby lessening toxicity and tissue damage (43). In addition, recent studies in the literature have highlighted a new and important function of p62 in promoting tumorigenesis. Indeed, Duran and colleagues showed that p62 is an important NF $\kappa$ B mediator in tumorigenesis in a Ras-induced model of lung carcinoma in mice (44). In addition, Mathew and colleagues reported that autophagy induction triggered p62 elimination, thereby suppressing tumorigenesis (45). Although this suggested the potential function of p62 as a tumor suppressor, the role of NF $\kappa$ B in both studies seems contradictory. RSV has been shown to inhibit tumor necrosis factor- $\alpha$ -mediated NF $\kappa$ B activation in the K562 cell line (46). Therefore, it is very unlikely that NF $\kappa$ B could be involved in p62 expression in CML cells treated with RSV.

<sup>7</sup> Puissant and colleagues, unpublished data.

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RSV also induced phase II enzyme expression such as NQO1 and HMOX-1 by activation of NRF2, a transcription factor that binds to antioxidant response elements located in the promoter region of phase II enzymes (47). Thus, an interesting possibility would be that RSV triggered p62 expression through NRF2 activation. In addition, p62 expression is stimulated by Ras and during metabolic stress in apoptosis and autophagy-deficient tumor cells (44).

Importantly, p62 mRNA expression is induced during Ras-induced transformation through both PI3K and Erk1/2. In this study, it was shown that the p62 promoter could bind activator protein-1 and that deletion or point mutation in this site was sufficient to abolish p62 transcription in response to Ras. We (27) and others (41) have shown that phorbol esters drastically increased p62 expression in CML cells. As JNK is known to induce activator protein-1 expression, one interesting possibility would be that RSV increased p62 expression through activator protein-1.

It has been proposed that RSV might transduce part of its effect through AMPK activation (29, 30). In agreement with these findings, we report that RSV does activate AMPK in CML cells. In addition, we showed that AMPK activation by RSV is required for the formation of phagophores, but not for JNK and p62-dependent elongation of phagophores. Finally, these two pathways seem to be both necessary to jointly induce autophagic cell death in CML cells.

In conclusion, we report here that RSV promotes autophagic cell death in CML cells through both JNK-mediated p62 expression and AMPK activation, an effect which participates significantly to its antileukemic effects. To the best of our knowledge, this is the first demonstration that the JNK and AMPK pathways cooperate to induce autophagic elimination of CML cells. Therefore, induction of autophagic cell death could be beneficial especially in IM-R CD34+ cells from CML patients that displayed impaired-drug induced apoptotic cell death.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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