Imatinib mesylate-resistant human chronic myelogenous leukemia cell lines exhibit high sensitivity to the phytoalexin resveratrol

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ABSTRACT **Imatinib is successfully used in the treatment of chronic myelogenous leukemia (CML), and the main mechanisms of resistance in refractory patients are now partially understood. In the present study, we investigated the mechanism of action of resveratrol in imatinib-sensitive (IM-S) and -resistant (IM-R) CML cell lines. Resveratrol induced loss of viability and apoptosis in IM-S and IM-R in a time- and dose-dependent fashion. Inhibition of cell viability was detected for concentrations of resveratrol as low as 5 μM, and the IC_{50} values for viability, clonogenic assays, apoptosis, and erythroid differentiation were in the 10–25 μM range. The effect of imatinib and resveratrol was additive in IM-S but not in IM-R clones in which the resveratrol effect was already maximal. The effect of resveratrol on apoptosis was partially rescued by zVAD-fmk, suggesting a caspase-independent contribution. Resveratrol action was independent of BCR-ABL expression and phosphorylation, and in agreement was additive to BCR-ABL silencing. Finally, phytoalexin inhibited the growth of BaF3 cells expressing mutant BCR-ABL proteins found in resistant patients, including the multiresistant T315I mutation. Our findings show that resveratrol induces apoptosis, caspase-independent death, and differentiation that collectively contribute to the specific elimination of CML cells. Resveratrol should provide therapeutic benefits in IM-R patients and in other hematopoietic malignancies.—Puissant, A., Grosso, S., Jacquel, A., Belhacene, N., Colosetti, P., Cassuto, J.-P., Auberger, P. Imatinib mesylate-resistant human chronic myelogenous leukemia cell lines exhibit high sensitivity to the phytoalexin resveratrol. *FASEB J.* 22, 1894–1904 (2008)**

Key Words: caspase-dependent cell death · caspase-independent cell death · anticancer therapy · BCR-ABL inhibitors

Chronic myelogenous leukemia (CML) is a myeloproliferative syndrome linked to a hematopoietic stem cell disorder leading to increased production of granulocytes at all stages of differentiation (1). Patients with CML consistently carry the t(9; 22) (q34; q11) translocation, commonly designated as the Philadelphia chromosome (2). This translocation is responsible for the expression of a 210 kDa chimeric fusion protein, p210 BCR-ABL, endowed with a constitutively active tyrosine kinase activity (3). The role of BCR-ABL in the pathogenesis of CML is currently well documented and has been confirmed in animal models (4, 5). BCR-ABL triggers several downstream survival pathways, including STAT5/Bcl-xL, Ras/Raf, MEK/Erk-1/2, PI3K/Akt, and NF-κB, that collectively provide proliferative advantages and resistance to apoptosis (6–8).

Imatinib mesylate (Gleevec), also known as STI571, targets the ATP-binding site of different tyrosine kinases, including BCR-ABL, the platelet-derived growth factor (PDGF) receptor (9), and c-Kit (10). Imatinib selectively induces the growth arrest and the apoptosis of BCR-ABL-positive leukemia cells with a minimal effect on normal hematopoietic progenitors (11–12). Of note, this agent has proven very effective with patients in the chronic phase of CML (13) and to a lesser extent in accelerated phase and blast crisis (14).

Although the treatment with imatinib achieves complete hematological remission in at least 95% of patients with CML, total cytogenetic and molecular responses are relatively rare events. Moreover, the resistance to imatinib is a relatively common feature for patients with accelerated phase or blast crisis (15).

To investigate the possible mechanisms of resistance to imatinib mesylate, several groups have recently reported the isolation and characterization of imatinib-resistant (IM-R) human CML cell clones isolated after prolonged culture in progressively increasing concentrations of the BCR-ABL inhibitor (16). Careful studies of these clones and of cells from IM-R patients have led

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to the conclusion that the resistance to imatinib can be accounted for by point mutations in the bcr-abl gene resulting in single amino acid substitutions that generally occurred in the ATP-binding pocket. Other mechanisms included bcr-abl gene amplification leading to the increased expression of p210 BCR-ABL (17, 18) or up-regulation of the multidrug resistance-1 (MDR-1) gene-encoded Pgp (19). More recently, other types of resistance independent of BCR-ABL and linked to an overexpression of the Src tyrosine kinases Lyn and Hck have been also evidenced (20).

Resveratrol (trans-3,4′,5-trihydroxystilbene) is a naturally polyphenolic phytoalexin found in grapes, peanuts, and a wide variety of plants that elicits several beneficial effects in human pathologies and noticeably in the prevention of cardiovascular diseases (21), and currently it is used in phase I studies to treat obese and diabetic patients. In addition to its well-documented cardioprotective action, resveratrol has also been shown to exhibit anticancer properties in some epithelial tumors and leukemia (22–23). For example, resveratrol has been shown either to protect CML cell lines from stress-induced apoptosis mainly through its antioxidant properties (24) or conversely to induce cell death when used alone (25). Nevertheless, the molecular mechanisms by which resveratrol exerts its effect on CML cell lines remain poorly defined, even though the inhibition of NF-κB has been proposed to mediate resveratrol antiproliferative and/or apoptotic effects (26).

Surprisingly, despite these promising *in vitro* and *in vivo* effects, resveratrol has not yet been analyzed as a potential therapeutic agent for the treatment of CML. Furthermore, no information exists concerning the effect of resveratrol on IM-R CML cell lines and whether resveratrol may exert a beneficial effect in IM-R patients remains an open question.

Thus, with the use of a panel of parental and BCR-ABL inhibitor-resistant K562 cell clones, the present study was conducted to investigate the effect of resveratrol on cell death, survival, and differentiation. We found that resveratrol induces a complex set of responses in sensitive and resistant CML cell lines, including increased caspase-dependent and -independent cell death and erythroid differentiation. These findings may have interesting implications for the future development of new therapeutic strategies to treat IM-R patients.

MATERIALS AND METHODS

Reagents and antibodies

Imatinib mesylate (STI571, Gleevec) was kindly provided by Novartis Pharma (Basel, Switzerland). Resveratrol was purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium and fetal calf serum (FCS) were purchased from Gibco-BRL (Paisley, UK). Sodium fluoride, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, and leupeptin were purchased from Sigma. Ac-DEVD-7-amino-4-methylcoumarin (AMC), Ac-LEHD-AMC, Ac-IETD-AMC, Ac-DEVD-CHO, Ac-LEHD-CHO, Ac-IETD-CHO, and zVAD-fmk were from Alexis Biochemicals (Lausan, Switzerland). Anti-ABL and anti-HSP60 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively.

HRP conjugated anti-mouse and anti-goat antibodies were from Dakopatts (Glostrup, Denmark). Anti-poly-(ADP)-ribose polymerase (PARP), anti-phospho-ABL, and peroxydase-conjugated anti-rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell lines

The human CML cell line K562 was grown at 37°C under 5% CO_2 in RPMI 1640 medium (Gibco-BRL) supplemented with 5% fetal calf serum (Gibco-BRL), 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mM sodium pyruvate (27). From the K562 cell line, we established resistant clones by adding increasing concentrations of imatinib (up to 10 μM) to the culture medium. Ten clones resistant to imatinib were selected by limiting dilutions. For convenience, only the results obtained with one of these clones (IIIF5 or IM-R) are presented. BaF3 cells transfected with wild-type BCR-ABL or mutated BCR-ABL (a kind gift of Francois X. Mahon, INSERM U876, Bordeaux, France) were grown in RPMI 1640 medium containing 10% FCS under 5% CO_2.

Caspase activities measurements

After stimulation, cells were lysed for 30 min at 4°C in lysis buffer (50 mM HEPES, pH 8; 150 mM NaCl; 20 mM EDTA; 1 mM PMSF; 10 μg/ml leupeptin; 10 μg/ml aprotinin; and 0.2% Triton X-100), and lysates were cleared at 10,000 *g* for 15 min at 4°C. Each assay (in quaduplicate) was performed with 50 μg of protein prepared from control or stimulated cells. Briefly, cellular extracts were then incubated in a 96-well plate, with 0.2 mM of Ac-DEVD-AMC, Ac-IETD-AMC, or Ac-LEHD-AMC as substrates for various times at 37°C as described previously (28).

Caspase activities were measured by after emission at 460 nm (excitation at 390 nm) in the presence or not of 1 μM of Ac-DEVD-CHO, Ac-IETD-CHO, or Ac-LEHD-CHO. Enzyme activities were expressed in arbitrary units per milligrams of protein.

Cell viability (XTT)

Cells (15×10^3 cells/100 μl) were incubated in a 96-well plate with different effectors for the times indicated in the figure legends. Fifty microliters of sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) reagent was added to each well. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolically active cells. The absorbance of the formazan product, reflecting cell viability, was measured at 490 nm. Each assay was performed in quadruplicate.

Colony formation assay

Resveratrol in the 1–100 μM range or 1 μM imatinib was added to imatinib-sensitive (IM-S) or IM-R CML cell lines growing in semisolid methyl cellulose medium (0.5×10^3 cells/ml; MethoCult H4236; StemCell Technologies Inc., Vancouver, BC, Canada). Colonies were detected after 10 days of culture by adding 1 mg/ml of 3-(4,5-dimethylthiazol-

2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent and were scored by Image J quantification software (U.S. National Institutes of Health, Bethesda, MD, USA).

Benzidine staining and phase contrast microscopy

Cell hemoglobinization was analyzed by benzidine staining. One thousand microliters of (0.5×10^6 cells/ml) was mixed with 200 μl of benzidine dihydrochloride reagent (Sigma). Morphological changes characteristic of erythroid differentiation were visualized using standard phase optics (Zeiss, Oberkochen, Germany).

Flow cytometry

After stimulation, cells were washed with ice-cold PBS and were fixed and permeabilized with Cytofix/Cytoperm solution. After two washes with Perm/Wash solution at room temperature, cells were incubated for 30 min with anti-active-caspase-3-FITC monoclonal or anti-α-globin antibodies. Finally, cells were washed and resuspended in Perm/Wash solution. All reagents were from BD Bioscience (San Diego, CA, USA). Anti-α-globin antibody was purchased from Santa Cruz Biotechnology.

In some experiments, control cells or BCR-ABL inhibitor-treated cells were incubated for 48 h in the presence or the absence of zVAD-fmk and were stained with the annexin-V-fluos staining kit (Roche, Meylan, France) according to the manufacturer's procedure. Fluorescence was measured by using the FL1 and FL2 channels of a fluorescence-activated cell sorter apparatus (FACScan, Becton-Dickinson, Cowley, UK).

BCR-ABL silencing by short hairpin RNA

Small interference RNA (siRNA)-expressing plasmids were constructed targeting a specific region of human bcr-abl (5′-AGCAGAGTTCAAAAGCCCT-3′; ref. 29). The oligonucleotides purchased from Eurogentec (Seraing, Belgium) were annealed and cloned into the pTER vector (a generous gift from Hans Clevers, Hubrecht Laboratory, Utrecht, The Netherlands) using the *Bgl*II and *Hind*III restriction sites (30). This results in a tet-inducible small hairpin RNA (shRNA) expression system when stably cotransfected into K562 (tet-on) cells with the pcDNA6/TR vector (Invitrogen, Paisley, UK).

Western blot

After stimulation, cells were lysed for 30 min at 4°C in lysis buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 20 mM EDTA; 100 μM NaF; 10 mM Na_3VO_4; 1 mM PMSF; 10 μg/ml leupeptin; 10 μg/ml aprotinin; and 1% Triton X-100). Lysates were centrifuged at 10,000 *g* for 15 min at 4°C, and the supernatants were supplemented with concentrated SDS sample buffer. A total of 100 μg of protein was separated on 8% polyacrylamide gel and transferred onto polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA) in a 20 mM Tris, 150 mM glycine, and 20% ethanol buffer at 500 mA during 4 h at 4°C. After blocking nonspecific binding sites in saturation buffer (50 mM Tris, pH 7.5; 50 mM NaCl; 0.15% Tween; 3% BSA; and 0.5% gelatin), the membranes were incubated with specific antibodies. The membranes were washed 3× using TNA-1% Nonidet P-40 (50 mM Tris, pH 7.5, and 150 mM NaCl) and incubated further with HRP conjugated antibody for 1 h at room temperature. Immunoblots were revealed using the enhanced chemiluminescence detection kit (Amersham Biosciences, Uppsala, Sweden).

Assessement of the cell cycle by flow cytometry

IM-S and IM-R cells were exposed to either 1 μM imatinib or different concentrations of resveratrol for 24 or 48 h at 37°C. Thereafter, cells were washed, fixed in citrate buffer, and finally left 1 h at −20°C. Cells were next incubated in a glycine/NaCl buffer containing 0.1% Nonidet P-40, 10 μg/ml RNase A, and 40 μg/ml of propidium iodide (PI) for 1 h at 4°C. Cell distribution across the different phases of the cell cycle was analyzed with a FACScan.

RESULTS

Resveratrol decreased cell viability in both IM-S and IM-R K562 cell lines

The effect of imatinib mesylate on the viability of the different K562 cell clones used in the present study has been recently published, and it was shown that both resistant clones exhibit reduced sensitivity to imatinib (30; Fig. 2*B*). Moreover, apoptosis, assessed by caspase activity measurement or annexin-V binding, accounted for approximatively one-half of the BCR-ABL inhibitor total effect (31). To investigate the effect of resveratrol on the metabolic status of the IM-S K562 cell line *vs.* its IM-R counterpart, both cell lines were treated with increasing concentrations of this polyphenolic compound for 48 and 72 h. As shown in **Fig. 1*A*, *B***, the two kinds of cells showed equivalent sensitivity to resveratrol measured by the XTT test until 25 μM, even though a slightly more important inhibition was achieved in the IM-R clone for concentrations in the 50–100 μM range. Inhibition was detected for concentrations of resveratrol as low as 5 μM, and the IC_{50} values were ~25 μM for both clones. The inhibitory effect of resveratrol on IM-S and IM-R cell proliferation was further confirmed in colony formation assays in methyl cellulose that more specifically measured a proliferation index. Indeed, as depicted in Fig. 1*C*, *D*, resveratrol dose dependently reduced the proliferation potential of IM-S and IM-R clones. Inhibition was detected for concentrations of resveratrol as low as 1 μM. As expected, imatinib was found to abrogate proliferation of the IM-S but not that of the IM-R clone (Fig. 1*C*, *D*).

In a second set of experiments, the effects of various doses of resveratrol were tested in combination with different doses of imatinib. Imatinib induced a dose-dependent decrease of cell metabolism in IM-S cells but not in its IM-R counterpart as expected (**Fig. 2*A*, *B***). According to the results of Fig. 1, resveratrol drastically decreased cell metabolism in both IM-S and IM-R cells; however, there was a more pronounced effect in the latter cell line. Interestingly, the combination of resveratrol and imatinib was found to exert additive effects on cell metabolism.

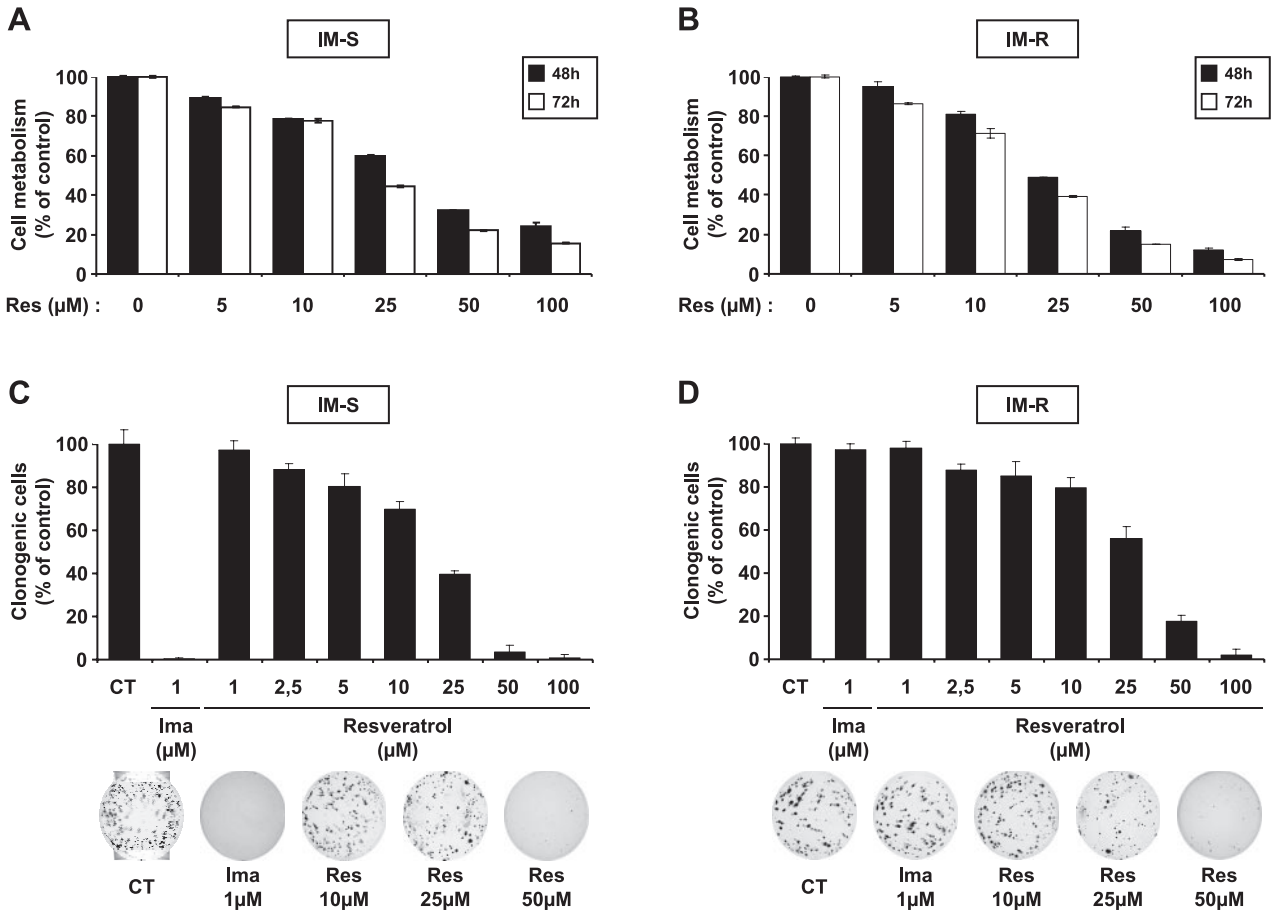

Figure 1. Resveratrol (Res) induces loss of cell viability in IM-S and IM-R cells. IM-S (*A*) and IM-R (*B*) K562 cells (10^5/ml) were incubated for 48 h (filled bars) or 72 h (open bars) at 37°C with increasing concentrations of resveratrol in 100 μl of RPMI 1640 medium containing 5% FCS in 96-well plates. Cell viability was measured by the XTT assay as described in Materials and Methods. Results are mean ± SD of 4 different determinations. Error bars = 95% confidence intervals. Resveratrol in the 1–100 μM range or 1 μM imatinib (Ima) was added to IM-S (*C*) or IM-R (*D*) CML cell lines growing in semisolid methyl cellulose medium (0.5×10^3 cells/ml). Colonies were detected after 10 days of culture by adding 1 mg/ml of MTT reagent and were scored by Image J quantification software. Results are expressed as the percentage of colony forming cells after drug treatment in comparison with the untreated control cells (CT). Results are means ± SD of 3 different determinations. Error bars = 95% confidence intervals. Photographs of IM-S and IM-R cultures treated for 10 days with various concentrations of resveratrol or 1 μM imatinib are also shown.

Resveratrol blocks IM-S and IM-R cells in the G_0/G_1 phase of the cell cycle

The percentage of cells in individual cell-cycle phases was assessed during incubation with imatinib or different concentrations of resveratrol. Representative histograms are shown in **Fig. 3**. A marked decrease of the cell fraction with fully replicated DNA (G_2/M) occurs within 24 h after imatinib addition in IM-S K562 cells (Fig. 3*A*). This correlated with an increased proportion of cells in the G_0/G_1 phase of the cell cycle. Later on, an increasing number of cells accumulated in the subG_1 phase of the cell cycle in the presence of imatinib (Fig. 3*C*). Neither a decrease in G_2/M nor a G_0/G_1 or subG_1 accumulation was detected in IM-R cells on imatinib treatment (Fig. 3*B*, *D*).

Resveratrol increased the percentage of cells in the G_1 phase of the cell cycle and reduced the fraction of cells in G_2/M in both IM-S and IM-R cells after 24 h (Fig. 3*A*, *B*). After 48 h of resveratrol treatment, a significant fraction of IM-S or IM-R cells accumulated in the subG_1 phase together with a drastic reduction of the percentage of cells in the G_2/M phase of the cell cycle (Fig. 3*C*, *D*).

Resveratrol-induced cell death proceeds by both caspase-dependent and -independent pathways

As shown in **Fig. 4*A*, *B*** (filled bars), optimal concentrations of imatinib and resveratrol induced an equivalent increase in caspase-3 and -9 activities in IM-S cells. Caspase-8 activity was not significantly affected by imatinib but was slightly increased by resveratrol (Fig. 4*C*). The effect of imatinib and resveratrol on caspase-3 and -9 activities was fully additive in the IM-S cell line. While drastically resistant to imatinib in terms of caspase-3 and -9 activities, the IM-R clone was sensitized to the

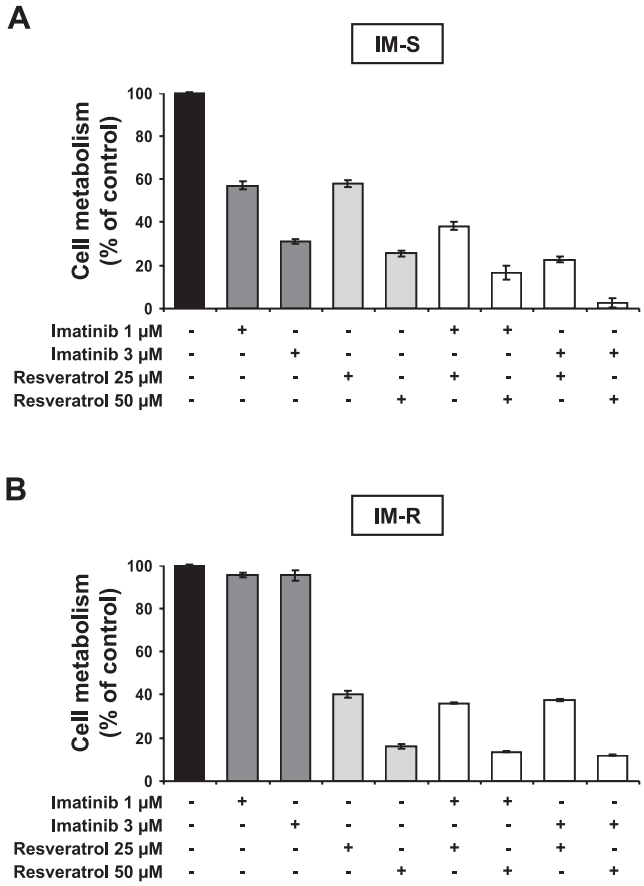

Figure 2. Resveratrol sensitizes IM-S and IM-R cells to the effect of BCR-ABL inhibitor. IM-S (*A*) and IM-R (*B*) K562 cells (10^5/ml) were incubated for 48 h at 37°C in 100 μl of RPMI 1640 medium containing 5% FCS in 96-well plates with either imatinib (1 or 3 μM), resveratrol (25 or 50 μM), or the combination of these effectors. Cell viability was measured by the XTT assay as described in Materials and Methods. Results are mean ± SD of 4 different determinations. Error bars = 95% confidence intervals.

effect of resveratrol and the level of caspase activity achieved by the polyphenolic compound was twice that found in IM-S cells (Fig. 4, open bars). Finally, as shown earlier for the cell metabolism assay, the level of caspase-3 and -9 activities induced by the combination of both effectors was found to be identical in IM-S cells as compared with its IM-R counterpart. Interestingly, the level of caspase activation achieved with resveratrol in the IM-R clone was almost equivalent to that reached with the combination of resveratrol and imatinib.

Figure 4*D* shows that an exposure of IM-S cells to 50 or 100 μM resveratrol induced cell death (annexin V^+/PI^+) in ~45 and 65% of cells, respectively, as compared with 11% of untreated cells. At the same time, 50 and 100 μM of resveratrol also induced death (annexin V^+/PI^+) in 50 and 62% of IM-R cells, respectively, *vs.* 7% of untreated cells. As expected, imatinib induced apoptotic cell death (annexinV^+/PI^- cells) in 18% of IM-S cells compared with only 3% in of IM-R cells.

A nonnegligible part of the resveratrol effect (25%) can be accounted for by apoptosis in IM-S and IM-R cells since ~15% of cells were annexinV^+/PI^- (Fig. 4*D*). The effect of the combination of both effectors was additive on apoptotic death (annexinV^+/PI^- cells) but not on nonapoptotic death (annexinV^+/PI^+ cells). IM-R cells, although totally resistant to imatinib-mediated apoptosis, remained sensitive to apoptosis triggered by resveratrol. Interestingly, they also exhibited a hypersensitivity to cell death induced by the combination of both effectors (not shown).

As also shown in Fig. 4*D*, the effect of imatinib on apoptosis was abrogated by the pan-caspase inhibitor zVAD-fmk in IM-S cells. By contrast, a significant part of the effect of resveratrol on cell death (40–50%) was not affected by zVAD-fmk, suggesting that this phytoalexin also induced caspase-independent death (CID). Inter-

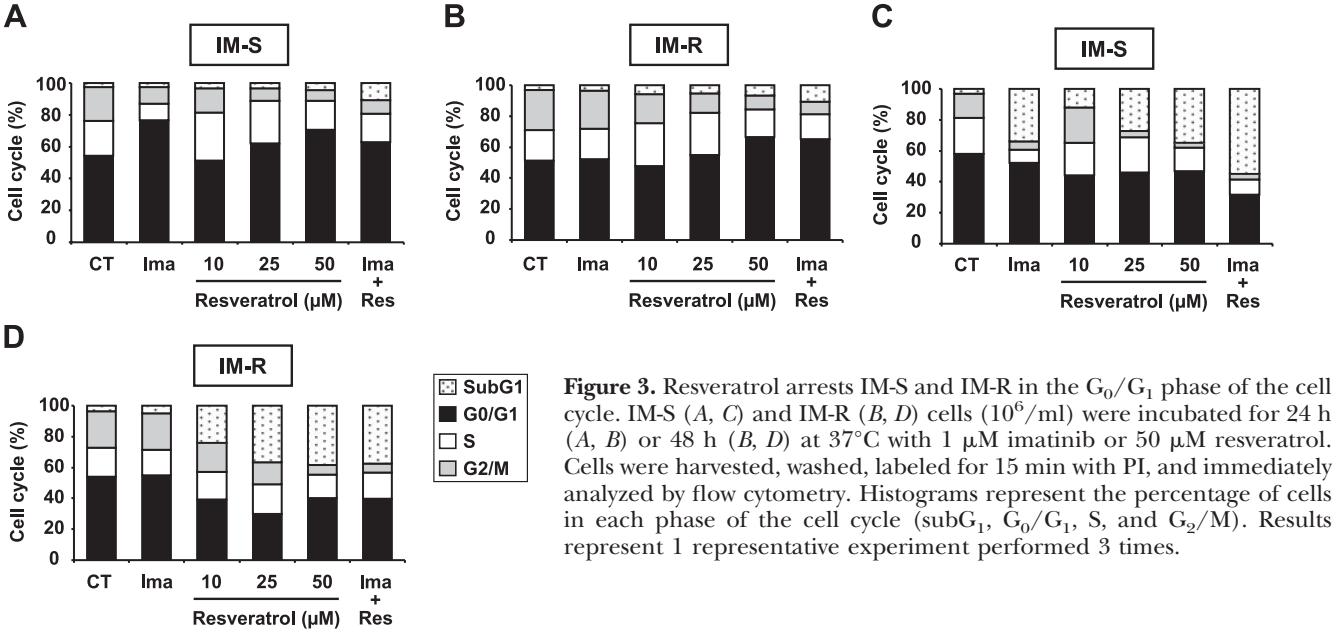

Figure 3. Resveratrol arrests IM-S and IM-R in the G_0/G_1 phase of the cell cycle. IM-S (*A*, *C*) and IM-R (*B*, *D*) cells (10^6/ml) were incubated for 24 h (*A*, *B*) or 48 h (*B*, *D*) at 37°C with 1 μM imatinib or 50 μM resveratrol. Cells were harvested, washed, labeled for 15 min with PI, and immediately analyzed by flow cytometry. Histograms represent the percentage of cells in each phase of the cell cycle (subG_1, G_0/G_1, S, and G_2/M). Results represent 1 representative experiment performed 3 times.

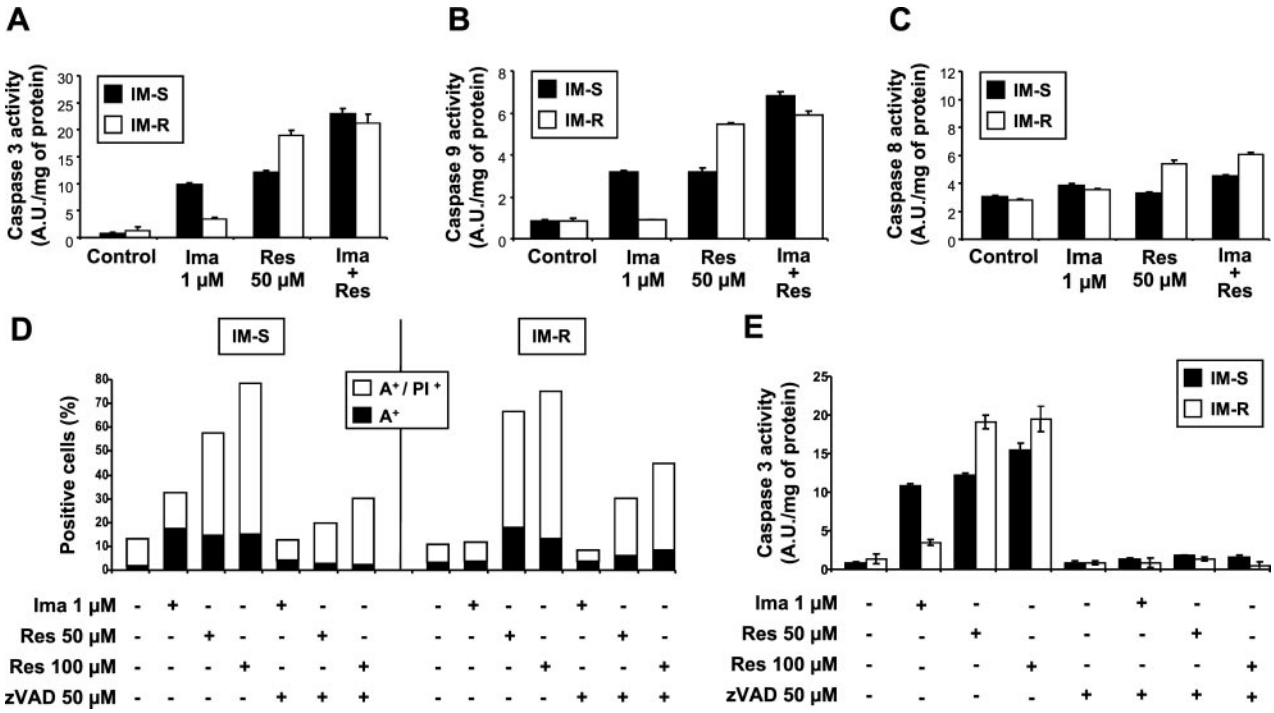

Figure 4. Resveratrol induced both caspase-dependent and -independent cell death. *A–D*) Resveratrol increased caspase activities in IM-S and IM-R cell lines: IM-S (filled bars) and IM-R (open bars) K562 cells (10^6/ml) were incubated for 48 h in the presence of 1 μM imatinib, 50 μM resveratrol, or the combination of both effectors. Cells were harvested, washed, and lysed in caspase buffer. Caspase-3 (*A*), -9 (*B*), and -8 (*C*) activities were evaluated in quadruplicate using 0.2 mM Ac-DEVD-AMC, Ac-LEHD-AMC, or Ac-IETD-AMC, respectively, as substrates. Hydrolysis of each substrate was determined after emission at 460 nm and excitation at 390 nm. To allow specific assessment of caspase activity, hydrolysis was followed as a function of time in the presence or the absence of either Ac-DEVD-CHO, Ac-LEHD-CHO, or Ac-IETD-CHO, respectively. Results are expressed as arbitrary units (A.U.) and are means ± SD of 4 independent experiments made in quadruplicate. Error bars = 95% confidence intervals. *D*) Resveratrol induced loss of cell viability by both caspase-dependent and -independent mechanisms: IM-S and IM-R cells (10^6/ml) were preincubated for 1 h in the presence or the absence of the pan-caspase inhibitor zVAD-fmk (50 μM) and exposed to imatinib (1 μM) or resveratrol (50 or 100 μM) for the next 48 h. Cells were stained with PI and annexin-V-fluos staining kit according to the manufacturer's indications. Histograms show both annexin-V^+/PI^+ cells (open bars) and annexin-V^+/PI^- cells (filled bars). A representative experiment is shown. *E*) zVAD-fmk abrogates caspase-3 activity in imatinib or resveratrol IM-S and IM-R treated cells. Caspase-3 activity was measured as described in *A*.

estingly, IM-R cells exhibited a diminished sensitivity to zVAD-fmk compared with IM-S cells together with a higher proportion of caspase-independent cell death. Finally, we checked that in these experiments zVAD-fmk abrogated caspase activation (Fig. 4*E*).

Imatinib but not resveratrol-mediated erythroid differentiation is altered in IM-R K562 cells

We (8, 31) and others (32–33, 34) have previously shown that besides inducing cell death, imatinib or BCR-ABL silencing by RNA interference triggered differentiation of K562 cells toward the erythroid lineage. Moreover, resveratrol has been previously reported to induce the erythroid differentiation of this cell line (35).

We thus investigated whether erythroid differentiation mediated by imatinib, resveratrol, or the combination of both effectors was altered in IM-R cells compared with their IM-S counterpart. **Figure 5*A*** shows that imatinib (1 μM) is far more effective than resveratrol in inducing hemoglobinization, a hallmark of erythroid differentiation in IM-S cells. At 25 μM of resveratrol, there was a 20-fold increase in the percentage of cells expressing hemoglobin as compared with untreated cells, which represents approximatively one-half of the effect triggered by 1 μM imatinib. The highest concentrations of resveratrol progressively decreased the percentage of benzidine-positive cells, which reaches the control level at 100 μM of the polyphenolic compound. Interestingly, in the presence of the combination of both effectors the index of erythroid differentiation dropped to low levels, confirming the conflict between erythroid differentiation and cell death in this cellular model (31). Of note, resveratrol significantly reduced the imatinib-induced erythroid differentiation in IM-S cells.

However, as previously demonstrated, IM-R cells exhibited reduced erythroid differentiation in the presence of imatinib, as compared with IM-S cells, while resveratrol-induced erythroid differentiation was maintained to approximatively the IM-S level in IM-R cells. These results were also confirmed by FACS analysis of α-globin levels (Fig. 5*B*).

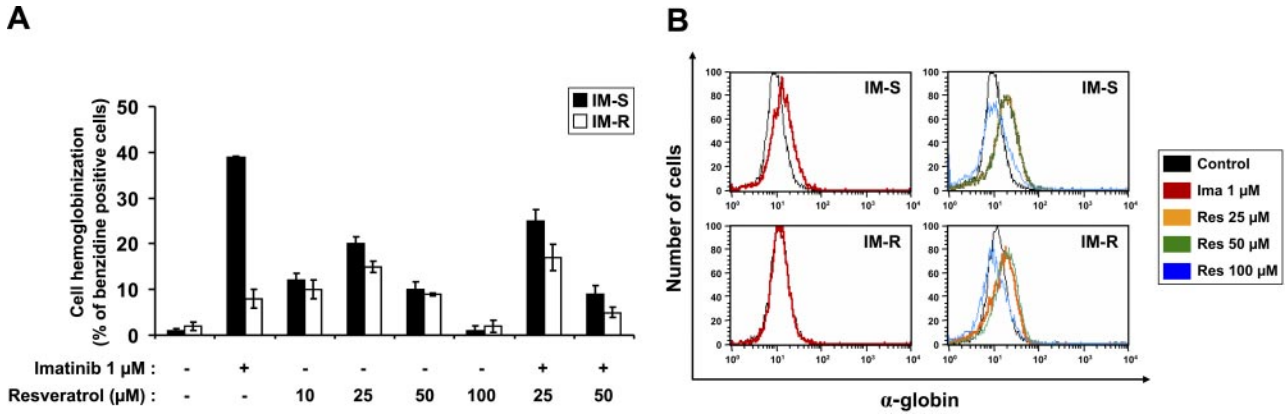

Figure 5. Resveratrol triggers erythroid differentiation but blocks imatinib-mediated erythroid differentiation. IM-S (filled bars) and IM-R (open bars) K562 cells (10^6/ml) were grown for 48 h with either 1 μM imatinib or increasing concentrations of resveratrol (in the 10–100 μM range). *A*) After 48 h, cells were stained with benzidine stain to estimate erythroid differentiation as described in Materials and Methods. Results are the means ± SD of 4 different determinations. Error bars = 95% confidence intervals. *B*) Erythroid differentiation was also assessed by flow cytometry analysis of α-globin expression. Fluorescence was analyzed by using the FL1 channel of a FACScan.

Effect of resveratrol is independent of BCR-ABL activity

We thought to analyze whether the effect of resveratrol on IM-S and IM-R cell death relied on BCR-ABL activity. For that purpose, the expression and the phosphorylation status of BCR-ABL were analyzed in parallel by Western blotting in IM-S and IM-R cells exposed to either imatinib or various concentrations of resveratrol (**Fig. 6*A***).

As expected, imatinib (1 μM) drastically decreased the phosphorylation status of BCR-ABL, while resveratrol (25–100 μM) failed to affect the phosphorylation of the chimeric protein (Fig. 6*A*). In IM-R cells, imatinib also elicited the complete dephosphorylation of BCR-ABL strongly suggesting that resistance to imatinib occurred downstream of BCR-ABL in this cell clone. BCR-ABL expression was identical whatever the cell lines and the treatment used (Fig. 6*A*). Finally, the proapoptotic effect of resveratrol was confirmed by a significant and dose-dependent cleavage of PARP regardless of its effect on BCR-ABL expression and phosphorylation (Fig. 6*B*). As expected, imatinib induced a complete cleavage of PARP in IM-S but had no effect in IM-R cells.

Effect of resveratrol is additive to that of BCR-ABL silencing

Besides their well-documented effects as BCR-ABL inhibitors, imatinib may also affect other kinases such as c-Kit, the PDGF receptor, and to a lesser extent Src kinases (10, 36). To specifically assess the role of BCR-ABL inhibition in the cell death program of K562 cells, we generated several clones inducible for the expression of a shRNA directed against BCR-ABL. Stimulation of one of these clones with tetracycline for 3, 5, or 7 days diminished BCR-ABL protein expression by 50, 70, and 90%, respectively, with no evident effect on ABL expression (**Fig. 7*A***).

Apoptosis was next assessed by FACS using an FITC-coupled anti-active caspase-3 monoclonal antibody. BCR-ABL silencing induced by a 5 day treatment with tetracycline followed by a 48 h incubation in the

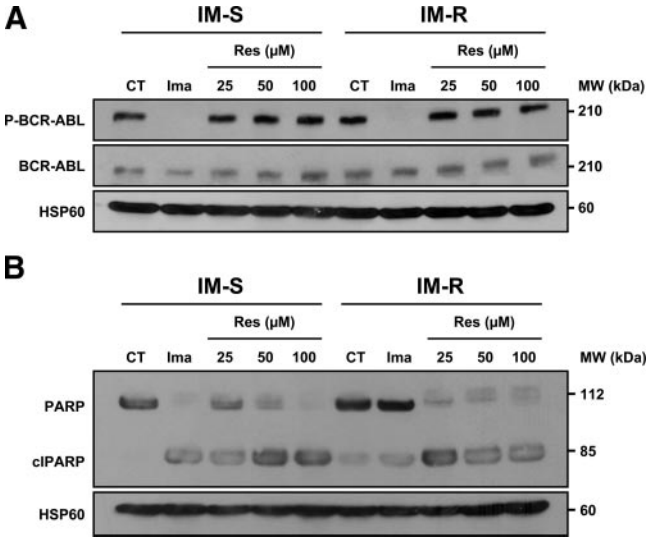

Figure 6. Resveratrol alters neither the status of BCR-ABL phosphorylation nor BCR-ABL expression. IM-S or IM-R K562 cells (10^6/ml) were incubated for either 15 min (optimal phosphorylation conditions; *A*) or 48 h (optimal PARP cleavage; *B*) in the presence of 1 μM imatinib or 50 μM resveratrol. Cells were harvested, washed, and finally lysed. Protein extracts were prepared, and 100 μg was subjected to sodium dodecyl-sulfate polyacrylamide gels (8%) followed by immunoblot analysis. BCR-ABL phosphorylation and expression were visualized using antiphospho-ABL and anti-ABL antibodies, respectively. PARP cleavage was used as a positive control of apoptosis, and HSP60 was used as a loading control. Both experiments were performed 3 times with similar results. A representative experiment is shown.

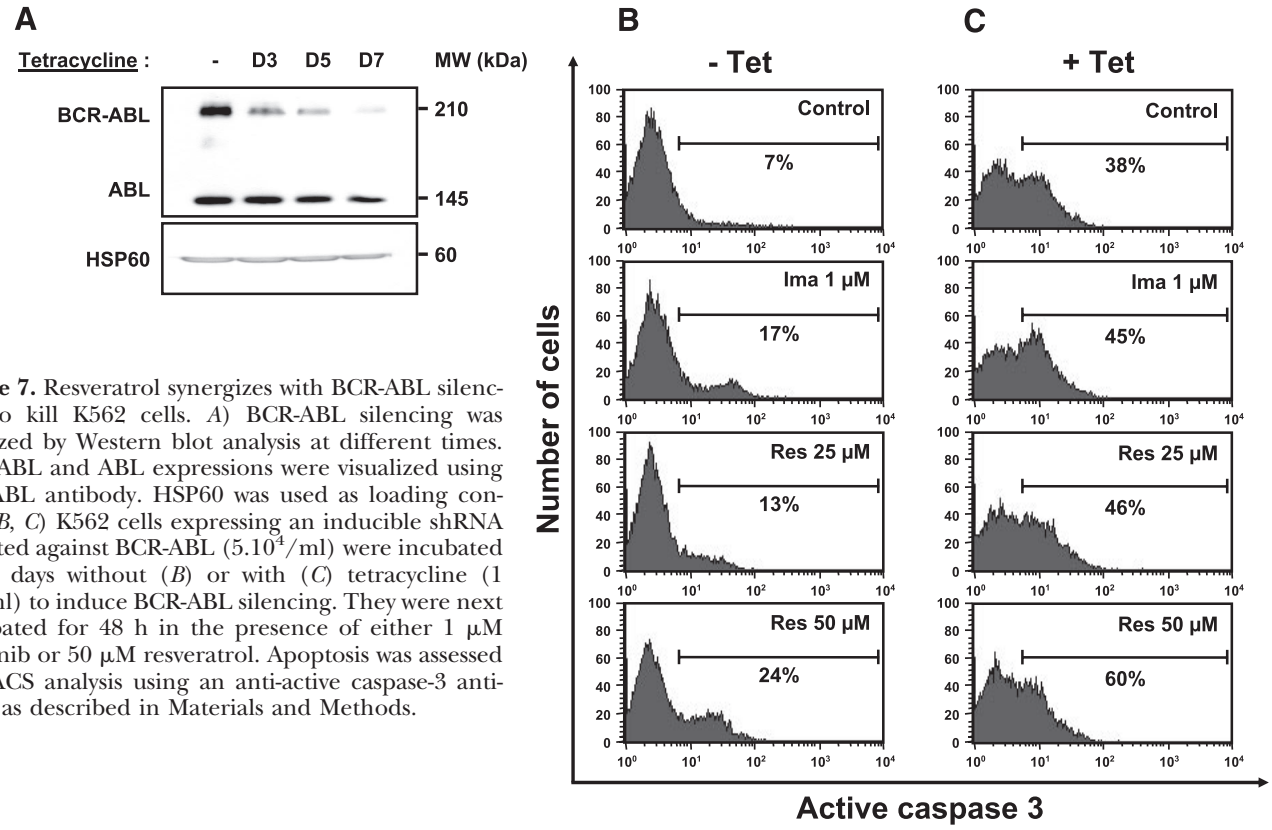

Figure 7. Resveratrol synergizes with BCR-ABL silencing to kill K562 cells. *A*) BCR-ABL silencing was analyzed by Western blot analysis at different times. BCR-ABL and ABL expressions were visualized using anti-ABL antibody. HSP60 was used as loading control. *B*, *C*) K562 cells expressing an inducible shRNA directed against BCR-ABL (5.10^4/ml) were incubated for 5 days without (*B*) or with (*C*) tetracycline (1 μg/ml) to induce BCR-ABL silencing. They were next incubated for 48 h in the presence of either 1 μM imatinib or 50 μM resveratrol. Apoptosis was assessed by FACS analysis using an anti-active caspase-3 antibody as described in Materials and Methods.

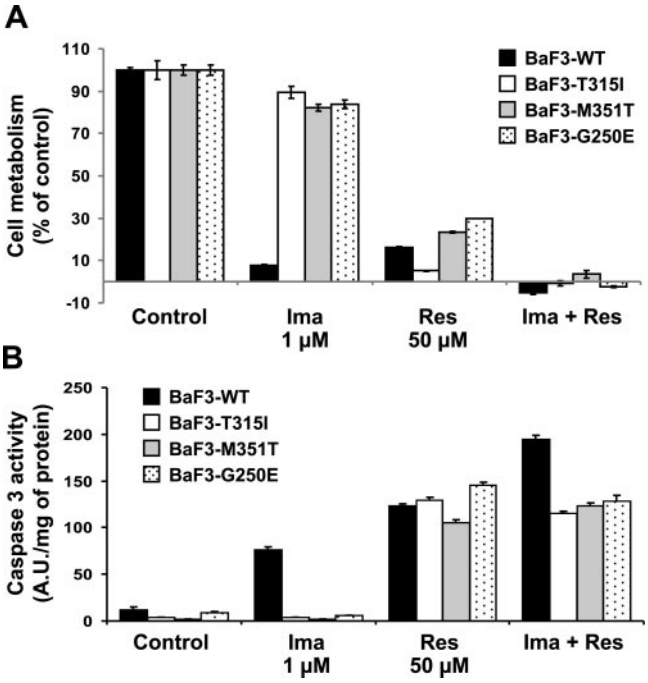

Figure 8. Resveratrol is highly effective in killing BaF3 cells exhibiting different BCR-ABL mutations. Wild-type or mutated-BCR-ABL-expressing BaF3 cells were incubated for 48 h at 37°C with either 1 μM imatinib or 50 μM resveratrol in 96-well plates. *A*) Cell viability was measured by the XTT assay as described in Materials and Methods. Results are means ± SD of 4 different determinations. Error bars = 95% confidence intervals. *B*) Caspase-3 activity was measured in quadruplicate as described above using Ac-DEVD-AMC as substrate. Results are means ± SD of 3 independent determinations. Error bars = 95% confidence intervals.

presence or the absence of different concentrations of resveratrol resulted in the induction of apoptosis.

BCR-ABL silencing by itself increased apoptosis assessed by FACS analysis of active caspase-3 from 7% in untreated cells to 38% in tetracycline-stimulated K562 cells (Fig. 7*B*). When used alone, resveratrol (25 or 50 μM) increased caspase activation in 13 and 24% of K562 cells, respectively, and the effect of the phytoalexin was found to be additive to that of BCR-ABL silencing. Indeed, the combination of resveratrol (25 or 50 μM) and BCR-ABL silencing increased the proportion of cells with active caspase-3 to 46 and 60%, respectively. These findings confirmed that the effect of resveratrol is independent of BCR-ABL and that combination of the phytoalexin and BCR-ABL silencing is highly effective in killing CML cell lines.

Resveratrol inhibits the growth of BaF3 cells carrying BCR-ABL mutations

We took advantage of the existing BaF3 cell lines expressing either the wild-type form of BCR-ABL (BaF3-WT) or different mutated forms of BCR-ABL commonly found in IM-R patients, namely the BaF3-T315I, BaF3-M351T, and BaF3-G250E. The four cell lines were exposed to either 1 μM imatinib, 50 μM resveratrol, or the combination of both effectors for 48 h. Then, cell viability and caspase assays were performed. Imatinib induced a drastic loss of cell viability concomitant with caspase-3 activation in BaF3-WT cells (**Fig. 8*A*, *B***).

Resveratrol was as potent as imatinib to decrease cell viability and increase caspase-3 activation in this cell line. BaF3-T315I, M351T, and G250E cell lines were resistant to the effect of the BCR-ABL inhibitor as expected but highly sensitive to the effect of resveratrol (Fig. 8*A*, *B*).

Interestingly, the T315I mutation, which confers resistance to most known tyrosine kinase inhibitors, was drastically sensitive to the action of resveratrol. Finally, the combination of imatinib and resveratrol induced a complete loss of viability in all the tested cell lines (Fig. 8*A*).

DISCUSSION

The aim of the present study was to analyze whether resveratrol could affect cell death and survival of CML cell lines either sensitive or resistant to imatinib mesylate and to decipher the mechanism of action of this phytoalexin. The precise mechanisms of resistance to imatinib in the IM-R clones are currently unknown, but recent data from our group indicate that they are due neither to MDR overexpression nor mutations in the kinase domain of BCR-ABL. Moreover pangenomic profiling of IM-S and IM-R clones strongly suggest that resistance could rely on BCR-ABL downstream targets, including some tyrosine and serine/threonine kinases and/or important surface molecules (unpublished results).

An interesting finding of the present study was the observation that resveratrol inhibited the proliferation of CML cell lines regardless of whether they were sensitive or resistant to imatinib.

Resveratrol effect was found to be additive to that of imatinib or to BCR-ABL silencing and to rely on both caspase-dependent and -independent mechanisms. Indeed, resveratrol increased caspase-9 and -3 activities as efficiently as imatinib but moderately affected caspase-8 activity. Interestingly, resveratrol-mediated caspase activation was even more pronounced in IM-R cells. As previously mentioned, apoptosis was only one of the possible mechanisms used by resveratrol to induce cell death, since a significant part (40–50%) of the resveratrol inhibitory effect was not impaired by zVAD-fmk, a pan-caspase inhibitor (Fig. 2), or Ac-DEVD-CHO (not shown).

We and others have previously reported that besides inducing apoptosis in CML cell lines imatinib also triggered their erythroid differentiation. Resveratrol also induced erythroid differentiation in both IM-S and IM-R cell clones, although to a lesser extent than imatinib.

Thus, the exact contribution of cell death and differentiation in the resveratrol effect remains to be established, but nevertheless induction of erythroid differentiation does not seem to be a major process by which this phytoalexin mediated loss of cell viability. The exact nature of the CID counterpart is currently under investigation, but preliminary data strongly suggest that type II autophagic cell death may be involved.

We next investigated whether the effect of resveratrol was dependent on BCR-ABL activity. Conversely to imatinib, resveratrol neither inhibited BCR-ABL phosphorylation nor affected its expression in the 25–100 μM range. Thus, the resveratrol effect did not seem to rely on BCR-ABL kinase inhibition, confirming a BCR-ABL-independent mechanism.

Several recent studies (37) in leukemic and nonleukemic cells have shown that the resveratrol effect on cell death and survival could be mediated by NF-κB inhibition leading to the modulation of the transcription of genes mainly involved in cell death and survival or in cell-cycle control. The antiproliferative action of resveratrol in a variety of cell models has also been attributed to a reversible delay or an irreversible arrest of the cell cycle, presumably *via* the inhibition of DNA polymerase and ribonucleotide reductase activity (38). In the present study, we failed to find evidence of any modulation of the S phase of the cell cycle in the presence of resveratrol, while a clear decrease in the proportion of cells exhibiting fully replicated DNA (G_2/M) concomitant with an increase in the proportion of cells that accumulate in the G_0/G_1 phase was detected after a 24 h treatment with the phytoalexin.

We also analyzed the pangenomic expression profile of IM-S and IM-R CML cell lines treated with resveratrol and found that this phytoalexin modulates the expression of several sets of genes involved in cell cycle, regulation of apoptosis, and autophagy (unpublished results). Interestingly, among the regulated genes, we were able to show a decrease in DNA polymerase 3 expression, but, unexpectedly, we also detected an up-regulation of ribonucleotide reductase mRNA expression in K562 cells stimulated for 24 h with resveratrol. Taken together, our results indicate that an irreversible S-phase arrest due to ribonucleotide reductase inhibition is unlikely to explain the antiproliferative action of resveratrol on IM-R and IM-R CML cell lines.

More precisely, it appears that the major effect of the phytoalexin is to induce accumulation of CML cells lines in the G_0/G_1 phase of the cell cycle. In line with this, the detailed analysis of the specific resveratrol transcriptome in both cell lines should help bring about new and important information concerning the mode of action of this phytoalexin (unpublished results). Interestingly, we found that the effect of resveratrol is reminiscent of that of 5-aminoimidazole-4-carboxamide riboside (AICAR), an AMP-activated kinase (AMPK) activator, suggesting that resveratrol may activate the AMPK pathway. These findings are consistent with several recent data from the literature showing that resveratrol does activate the AMPK pathway (39–41). Nevertheless, further studies are needed to decipher the exact mechanisms of action of resveratrol in IM-S and IM-R CML cell clones.

To the best of our knowledge, this is the first report on the ability of resveratrol to overcome imatinib resistance in CML cell lines. Similar results have been

recently reported by Bhardwaj *et al.* (42) in multiple myeloma (MM) where resveratrol was shown to sensitize MM-resistant cell lines to melphalan. Moreover, in this study the effect of resveratrol was additive to that of bortezomib, a drug currently used in patients resistant to the combination of prednisone and melphalan.

Finally, resveratrol has been shown to be well tolerated in animal studies, with very little toxicity, and is currently used in phase I clinical assays more particularly for the treatment of obese and diabetic patients. In this context, the present study establishes the early beginnings of future therapy in CML patients. This study, however, has some potential limitations. For example, the remarkable efficiency of resveratrol to induce cell death in IM-R CML cell lines should first be confirmed in preclinical studies aimed at determining its efficiency in xenografts of IM-S and IM-R K562 cells in nude mice.

Along the same line, we have also shown that in primary leukemic cells from CML patients, resveratrol induced a significant increase in erythroid colony formation in methyl cellulose to the detriment of myeloid colony formation. This was correlated with a strong reduction of cell viability after 4 days of incubation with resveratrol (not shown), confirming our *in vitro* studies with the K562 CML cell line. Finally, an important point of interest of the present study is the observation that resveratrol is capable of inducing cell death of BaF3, which carries several BCR-ABL mutations and particularly the T315I mutation, which is resistant to most known drugs currently used for the treatment of CML.

To conclude, we have demonstrated that resveratrol treatment of various CML cells induces a complex set of responses, including cell-cycle arrest, caspase-dependent and independent cell death, and erythroid differentiation. Interestingly, the resveratrol effect was even more pronounced in IM-R CML cell lines. The remarkable efficiency of resveratrol to induce cell death in IM-R CML cell lines may find therapeutic application for the treatment of imatinib refractory patients. FJ

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