

## Resveratrol Inhibits Proliferation and Survival of Epstein Barr Virus–Infected Burkitt's Lymphoma Cells Depending on Viral Latency Program

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### Abstract

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), a polyphenolic natural product, shows chemopreventive properties against several cancers, heart diseases, inflammation, and viral infections. Epstein Barr virus (EBV), a  $\gamma$ -herpesvirus, contributes to the development of several human cancers including Burkitt's lymphoma (BL). In this study, we asked whether treatment with resveratrol would affect the viability of EBV-positive BL cells displaying different forms of latency. We report here that resveratrol, regardless of EBV status, induces caspase-dependent apoptosis by arresting cell-cycle progression in G<sub>1</sub> phase. However, resveratrol strongly induced apoptosis in EBV(–) and latency I EBV(+) cells, whereas latency II and latency III EBV(+) BL cells showed a survival advantage that increased with the extent of the pattern of viral gene expression. Resveratrol-induced cell-cycle arrest and apoptosis occurred in association with induction of p38 MAPK phosphorylation and suppression of ERK1/2 signaling pathway. Moreover, NF- $\kappa$ B DNA-binding activity was inhibited in all BL lines except EBV (+) latency III cells.

*LMP1* oncogene, which is expressed in latency III phenotype, is involved with the higher resistance to the antiproliferative effect of resveratrol because siRNA-mediated inhibition of *LMP1* greatly increased the sensitivity of latency III BL cells as well as that of lymphoblastoid cell lines to the polyphenol. We propose that a combined resveratrol/siRNA strategy may be a novel approach for the treatment of EBV-associated B-cell malignancies in which the viral pattern of gene expression has been defined. *Mol Cancer Res*; 9(10); 1346–55. ©2011 AACR.

### Introduction

Epstein Barr virus (EBV), the causative agent of infectious mononucleosis, is associated with a variety of different human tumors including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease, and posttransplant lymphoproliferative disorders occurring in immunocompromised patients. In all these malignancies, the virus establishes a latent infection characterized by different EBV gene expression profiles. Three latency programs have been described. EBNA1 is the only viral antigen expressed in latency I phenotype mainly found in EBV-positive Burkitt's

lymphoma; the latency III phenotype, defined by the expression of 6 nuclear (EBNA1, EBNA2, EBNA3A, EBNA3B, and EBNA3C) and 3 membrane antigens (LMP1, LMP2A, and LMP2B), is present in most immunodeficiency-related B-cell lymphomas and in the lymphoblastoid cell lines (LCL) obtained by *in vitro* infection of B lymphocytes; latency II program, corresponding to the expression of EBNA1, LMP1, and LMP2, is mostly related to nasopharyngeal carcinoma and Hodgkin's disease.

The hallmark of Burkitt's lymphoma is the chromosomal translocation altering the proto-oncogene *c-myc* locus and the immunoglobulin (Ig) heavy or light chain genes, thereby causing deregulation of *c-myc*. The EBV genome is present in more than 95% of endemic Burkitt's lymphoma cases in Africa and in 10% to 20% of all sporadic cases worldwide. Moreover, about 40% to 50% of AIDS-associated Burkitt's lymphoma cases carry the viral DNA. Although in most Burkitt's lymphoma the virus displays a latency I program, unusual forms of latency have also been identified (1–3). Moreover, upon cultivation *in vitro*, BL cells tend to change their phenotype toward an LCL-like phenotype by broadening the pattern of EBV latent gene expression (4).

Antioxidant compounds present in dietary items have gained interest because of their beneficial effects on health as cancer chemopreventive agents. Resveratrol

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(3,4',5-trihydroxy-*trans*-stilbene), a polyphenolic phytoalexin found in the skin of red grapes and a variety of other fruits, functions in plants to protect against fungal infections (5). A variety of studies have reported that resveratrol has cardioprotective, antioxidant, anti-inflammatory, antibacterial, and antiviral activities (6). In addition, resveratrol was found to inhibit proliferation and induce apoptosis in several human tumor cells (7, 8). It has been previously reported that resveratrol can inhibit the growth of malignant B-cell lines in a dose- and time-dependent manner (9, 10). However, EBV infection might confer additional survival potential to malignant B cells, also depending on the different programs of latent gene expression adopted by the virus.

In this study, we have examined the antiproliferative activity of resveratrol on Burkitt's lymphomas with different forms of restricted virus latency. Thus, in the same cellular background, we have investigated the contribution that different patterns of EBV gene expression give to resveratrol-induced susceptibility to apoptosis and studied the molecular mechanisms underneath.

We report here that regardless of EBV status, the treatment of BL cells with resveratrol induces cell-cycle arrest and apoptosis; however, we also show that each form of EBV infection is associated with a specific degree of protection from apoptosis, more extended as a larger set of viral genes is expressed. Interestingly, silencing of the viral oncogene *LMP1* by siRNA decreased the protection of latency III BL cells to resveratrol-induced apoptosis, as it did in LCLs. These findings suggest that the combined use of resveratrol and siRNA directed to viral genes may configure a novel therapy for the treatment of EBV-positive B-cell malignancies with a known pattern of viral gene expression.

## Materials and Methods

### Cell culture and treatment with resveratrol

Human Burkitt's-derived cell lines, either positive or negative for EBV, and 2 human B lymphoblastoid cell lines were used. EBV-positive Raji and EBV(-) Ramos cells were purchased from American Type Culture Collection (LGC Promochem). Akata with the EBV genome (11, 12), the isogenic EBV(-) Akata subline 2A8 (13), and Jijoye M13 cells (4) were obtained from Dr. P. Trivedi (Sapienza University, Rome, Italy). Lymphoblastoids LCL1087 and LCL1260 were gifts from Dr. R. Dolcetti [Cancer Bioimmunotherapy Unit, Aviano (PN), Italy]. All cell lines were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and antibiotics in a 5% CO<sub>2</sub> atmosphere and maintained at a cell density of  $3.5 \times 10^5$ /mL.

Cells, at a density of  $3 \times 10^5$  cells/mL, were incubated in RPMI-1640/10% FCS with different concentrations of resveratrol (Sigma) from a stock solution of 50 mg/mL dissolved in ethanol and kept at -20°C protected from light. Control cells were treated with the diluent. At different times, viable cells excluding Trypan blue dye were counted in a Bürker chamber.

### Cytofluorimetric analysis

Cell-cycle studies were conducted by fluorescence-activated cell-sorting (FACS) analysis as previously described (14). Apoptosis was evaluated by Annexin V-FITC apoptosis detection kit (Sigma), which measures Annexin V binding to phosphatidylserine in conjunction with propidium iodide (PI) staining, according to the accompanying procedure.

### Western blot analysis

Western blot analysis on whole-cell lysates was conducted as previously described (14). Where needed, nuclei were isolated (15) and lysed in Laemmli buffer. The blots were probed with the following primary antibodies: cyclin A (1:1,000), cyclin E (1:1,000), cdk1 (1:1,000), p27 (1:50), Mcl-1 (1:200; all by Santa Cruz Biotechnology); cdk2 (1:1,000; Biomol); PARP (1:1,000; Alexis Biochemicals, Enzo Life Sciences); active caspase 3 (1:1,000; AbCam); XIAP and IAP1 (1:500; R&D System); FLIP (1:500; Upstate Biotechnology); RelA/p65 (1:100; Santa Cruz Biotechnology); phospho-p38 (catalogue no. 9211), total p38, phospho-ERK1/2 (catalogue no. 9101), total ERK1/2 (1:1,000; Cell Signaling); LMP1 (1:7,500; Pharmingen); and  $\beta$ -actin (1:1,500; Sigma).

### Electrophoretic mobility shift assay

To determine NF- $\kappa$ B and AP1 activation, we carried out electrophoretic mobility shift assay (EMSA) as previously described (14, 16). Briefly, whole-cell extracts were obtained from  $2 \times 10^6$  cells treated with resveratrol or with the diluent for different lengths of time. Twenty micrograms of proteins, as determined by a modified Lowry assay (RC DC Protein Assay; BioRad), were incubated with 30 fmol of a DIG-labeled  $\kappa$ B DNA probe (17) for 20 minutes at room temperature. DNA-protein complexes formed were separated from free oligonucleotides on a 4% native polyacrylamide gel, transferred to a nylon membrane, and detected by chemiluminescence (DIG Luminescent Detection Kit; Roche Applied Science). The specificity of binding was also verified by competition with the unlabeled oligonucleotides. For supershift assay, cell extracts were incubated with antibodies against either p50 or p65 of NF- $\kappa$ B (Santa Cruz Biotechnology) for 30 minutes at room temperature and then analyzed by EMSA. Quantitative evaluation of NF- $\kappa$ B complex formation was carried out by ImageJ Freeshare Software (<http://rsbweb.nih.gov/ij>).

### Fluorescence microscopy

Cells incubated with resveratrol or with diluent for 24 hours were washed with PBS, spotted on slides, and fixed with methanol for 5 minutes at -20°C. Fixed cells were incubated with p65 NF- $\kappa$ B subunit antibodies (1:500; Santa Cruz Biotechnology) for 1 hour at 37°C, rinsed in PBS, and then incubated with fluorescein isothiocyanate-conjugated secondary antibodies for 30 minutes at room temperature. Subsequently, cells were rinsed and nuclei stained with 4',6-diamidino-2-phenylindole (DAPI; 0.2  $\mu$ g/mL) for 15 minutes. The coverslips were mounted

with 0.1% (w/v) *p*-phenylenediamine in 10% (v/v) PBS, 90% (v/v) glycerol, pH 8 (18), and the specimens observed by immunofluorescence microscopy using a Leica DM4000 fluorescence microscope equipped with an FX 340 digital camera.

### LMP1 silencing and overexpression

To obtain LMP1 downregulation, we used a human H1 RNA polymerase III promoter-based short hairpin RNA (shRNA) vector, pSilencer 3.1-H1 hygro (Ambion), to express siRNA that targets the EBV LMP1 in Raji cells. The sense- and antisense-strand oligonucleotides for LMP1 shRNA were 5'-gatccGGAATTTGCACGGACAGGCTTcaagagaGCCTGTCCGTGCAAATTCCTTTTTTggaaa-3' and 5'-agcttttccaaaaaaGGAATTTGCACGGACAGGCTctcttgaaGCCTGTCCGTGCAAATTCcg-3'. A pSilencer hygro vector expressing a hairpin siRNA with limited homology to any known sequences in the human genome was used as a negative control (control siRNA). The constructs were prepared by ligating the annealed oligonucleotides into the *Bam*H1 and *Hind*III of pSilencer and the resulting vectors analyzed by DNA sequencing. A total of  $1.5 \times 10^7$  cells were electroporated with 30  $\mu$ g of siRNA expression vectors at 975  $\mu$ F, 260 V, using a Gene Pulser II system (Bio-Rad). To select clones, electroporated cells were grown in medium containing 100  $\mu$ g/mL hygromycin (Sigma). After 3 weeks, resistant clones were grown up and tested for LMP1 protein expression.

Transient expression of siLMP1 in LCLs was obtained by electroporation with the specific siRNA and the control plasmid according to the conditions used for Raji cells.

To enforce transient LMP1 expression,  $1.5 \times 10^7$  EBV (-) Akata 2A8 cells were electroporated (960  $\mu$ F, 230 V) with 10  $\mu$ g of LMP1 expression vector pSG5-LMP1 (a kind gift of Dr Martin Rowe, Birmingham University Medical School, United Kingdom) or of the corresponding control vector pSG5.

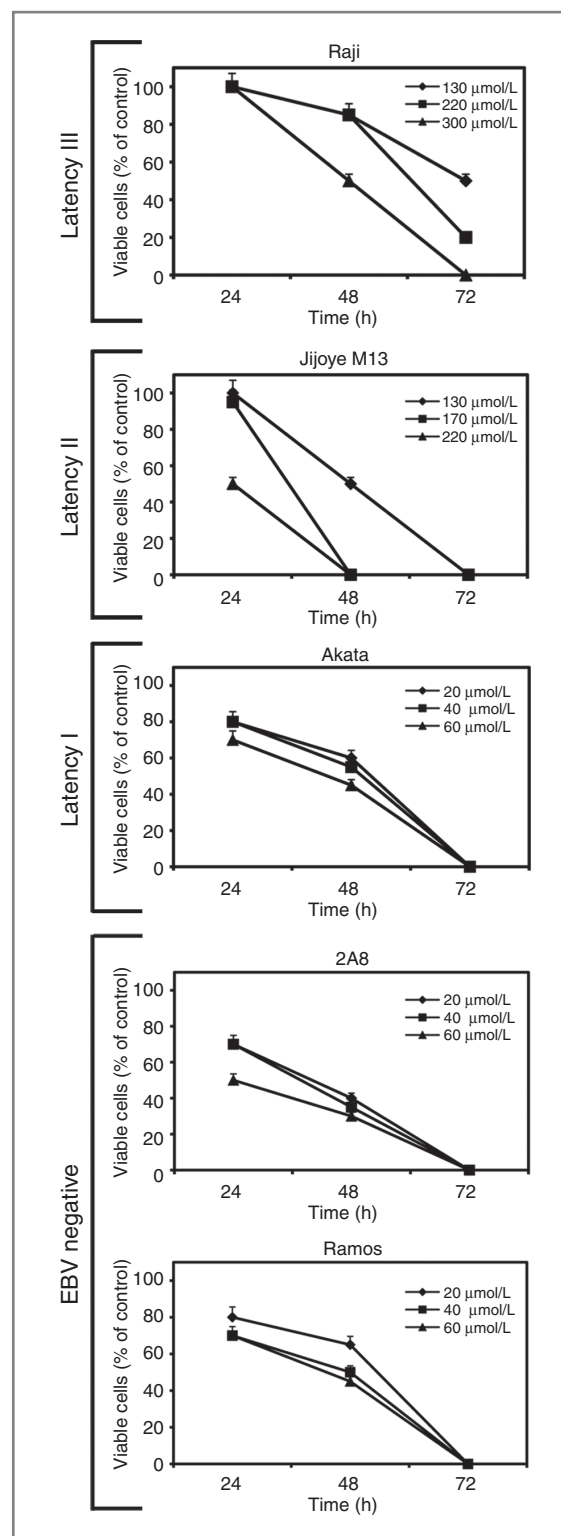
### Statistical analysis

Statistical analysis was carried out using ANOVA followed by post hoc tests (Bonferroni-Dunn). The level of significance was set at  $P < 0.05$ .

## Results

### Resveratrol inhibits proliferation of EBV-positive BL cells

As we aimed to determine whether EBV gene expression played a role on the susceptibility of BL cells to resveratrol, we tested 2 EBV(-) BL cell lines (2A8 and Ramos) and 3 EBV(+) BL cell lines, the latter expressing different patterns of EBV latency genes. In particular, Raji cells show a latency III phenotype; Jijoye M13, a subline of Jijoye cells classified as group II like (4), express EBNA1 and LMP2A, whereas Akata cells display a latency I program (Supplementary Fig. S1). The cell lines, exposed to resveratrol concentrations increasing from 20 to 300  $\mu$ mol/L, for various periods of time, showed different degrees of sensitivity to the polyphenol. Figure 1 shows that the growth of each cell



**Figure 1.** Resveratrol-induced inhibition of BL cell proliferation. Cells were incubated with resveratrol at the concentrations indicated. After 24, 48, and 72 hours, cell counts were assessed by Trypan blue staining. Percentage of proliferation was determined as proliferation of treated cells  $\times$  100/proliferation of control cells treated with the diluent. Each point represents the mean  $\pm$  SD of 3 similar experiments.

line was inhibited by resveratrol in a concentration- and time-dependent manner. The concentrations reducing cell proliferation by 50% at 48 hours were of about 300  $\mu\text{mol/L}$  for Raji, 130  $\mu\text{mol/L}$  for Jijoye M13 cells, and 40  $\mu\text{mol/L}$  for Akata and EBV(-) BL cells. The values determined for latency III and latency II cells were significantly different between each other and with respect to latency I and EBV(-) cells ( $P < 0.05$ ). Altogether, resveratrol cytotoxicity for EBV(-) and Akata cells was approximately 3- and 7-fold greater than that observed for Jijoye and Raji cells, respectively.

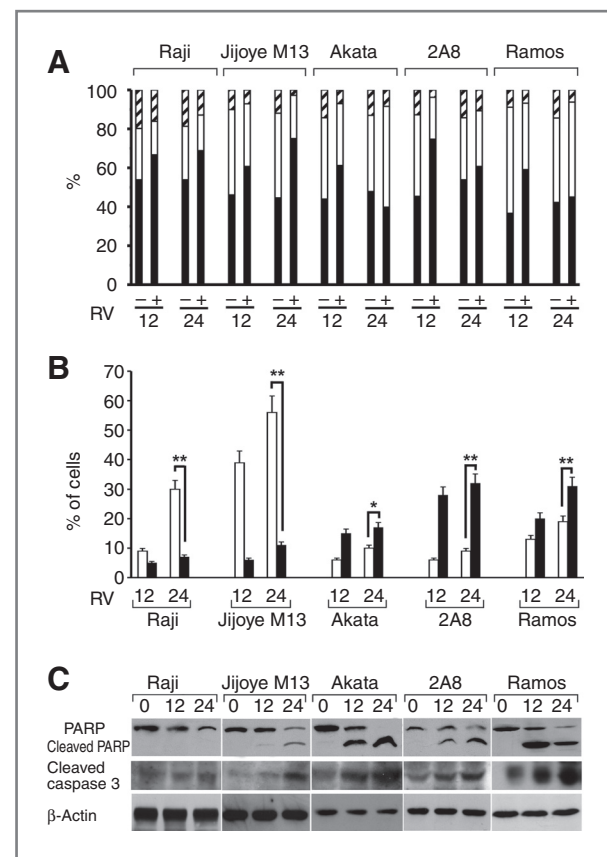
### Resveratrol induces cell-cycle arrest and apoptosis of EBV-infected BL cells

To investigate the antiproliferative effect of resveratrol on BL cells, we analyzed the cell-cycle distribution after incubation with the polyphenol. To compare the different cell lines, all the following experiments were carried out by exposing each cell line to the resveratrol concentration reducing cell proliferation by 50% at 48 hours, as previously determined. Cells were harvested at 12 and 24 hours, and the DNA content was determined by PI staining and flow cytometric analysis. The bar graph in Figure 2A shows that 12-hour treatment with resveratrol increased significantly ( $P < 0.01$ ) the percentage of cells in the  $G_1$  phase with a reduction of cells in S and  $G_2$ -M phases. After 24-hour incubation, the  $G_1$  population further increased in Raji and Jijoye M13 cells whereas decreased in Akata cells, 2A8, and Ramos cells. In the latter (latency I and EBV(-) Burkitt's lymphoma), the proportion of cells in the sub  $G_0$ - $G_1$  phase, representing apoptotic events, augmented after 24-hour incubation (data not shown), suggesting a relation between cell-cycle arrest and apoptosis.

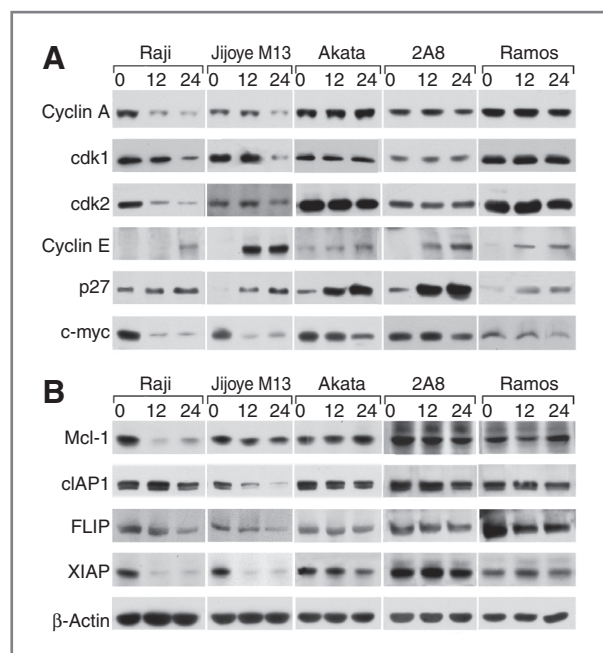
Therefore, we evaluated apoptosis in cells incubated with resveratrol for 12 and 24 hours, after staining with Annexin V and PI. Annexin V binding to phosphatidylserine, on the outer side of the cell membrane, characterizes the cells entering apoptosis, whereas cells double stained by Annexin V and PI represent the fraction of late apoptotic/necrotic cells. As it appears from Figure 2B, the ratio between Annexin V and Annexin V/PI-positive cells appeared to be dramatically different among the cell lines. In fact, after 24-hour exposure to resveratrol, the percentages of early apoptotic cells in Raji and Jijoye were about 30% and 55% whereas the fractions of late apoptotic/necrotic cells were about 8% and 12%, respectively. In addition, the percentage of Annexin V-positive cells in Jijoye M13 was significantly higher than that measured in Raji ( $P < 0.0001$ ). Conversely, in Akata cells as well as in 2A8 and Ramos cells, at both time points, the percentages of cells double stained by Annexin V and PI were larger than those of cells stained only by Annexin V. In particular, in EBV(-) 2A8 and Ramos, Annexin V/PI-positive cells reached about 30% whereas single stained cells at 24 hours were about 12% and 22%, respectively. In Akata, the fraction of double-positive cells was lower than those measured for EBV(-) cell lines but still higher than that of cells stained only by Annexin V. Taken together, these

data seemed to indicate that EBV(-) BL cells and latency I Akata were more prone to resveratrol-induced apoptosis than latency II and latency III cell lines.

Western blot analysis of PARP processing confirmed that resveratrol differentially induced apoptosis in BL cells. As illustrated in Figure 2C, after 12-hour incubation, the 85-kDa cleavage product of PARP appeared in latency I Akata and EBV(-) cell lines and further increased after 24 hours. In contrast, PARP apoptotic form was detectable in Jijoye M13 cells after 24-hour exposure to resveratrol and in Raji cells only after 48 hours (data not shown), indicating that latency III BL cells were more resistant to resveratrol-induced apoptosis than the other cell lines. To investigate the apoptotic pathway, we analyzed the activation of effector caspase 3 by measuring the levels of the 17-kDa cleaved form.



**Figure 2.** Resveratrol (RV) induces cell-cycle arrest in  $G_1$  phase and apoptosis of BL cells. Each cell line was cultured with the resveratrol concentration reducing proliferation by 50% at 48 hours or with the diluent for 12 and 24 hours. A, fixed cells were stained with PI and analyzed by flow cytometry. The percentages of cells in  $G_1$  (black bars), S (white bars) or  $G_2$ -M phase (hatched bars) are shown. B, evaluation of apoptosis by FACS analysis after Annexin V and PI double staining. Annexin V-positive cells (empty columns) and Annexin V/PI-stained cells (black columns), mean values  $\pm$  SD. \*,  $P < 0.01$  or \*\*,  $P < 0.001$  vs. Annexin V/PI. C, detection of PARP cleavage and active caspase 3. At the indicated times, cell lysates were prepared and analyzed by Western blots.  $\beta$ -Actin was used as the internal marker. The data are representative of those obtained in at least 3 independent experiments with similar results.



**Figure 3.** Western blot analysis of cell cycle (A) and apoptosis (B) regulatory proteins following resveratrol treatment of BL cells. At the indicated times (hours), cell lysates were analyzed by 12% SDS-PAGE followed by immunoblot analysis. Specific signals were visualized and quantified as described in Materials and Methods. A representative result of 3 independent experiments is shown.  $\beta$ -Actin was used as the internal marker.

Data reported in Figure 2C showed higher levels of the biologically active caspase 3 along with increased amounts of the processed PARP, thus indicating that resveratrol induces apoptosis by the caspase-dependent pathway.

#### Modulation of cell-cycle and apoptosis-associated proteins by resveratrol

To clarify the molecular mechanism of resveratrol-induced cell-cycle arrest in  $G_1$  phase and the subsequent apoptosis, we examined by Western blot analysis the expression of several cell-cycle and apoptosis-related proteins (Fig. 3). Exposure to resveratrol increased the expression of the cdk inhibitor *p27* and that of *cyclin E* in all cell lines; in contrast, *cyclin A*, *cdk1*, and *cdk2* were downregulated in Raji and Jijoye M13 cells but did not vary significantly in Akata and in the EBV(-) cell lines (Fig. 3A). Because S-phase *cdk1/2* and *cyclin A* are essential for DNA synthesis, their decrement along with the increment of cdk inhibitor *p27* indicate that resveratrol blocks specific cell-cycle-associated activity involved in the progression from  $G_1$  to S-phase. Moreover, the levels of the *c-myc* oncogene were strongly downregulated in Raji and Jijoye M13 cells and a significant decrement was detected in Akata as well as in 2A8 and Ramos cells. The levels of expression of the antiapoptotic proteins IAP1, FLIP, and XIAP appeared to diminish in all cell lines, whereas Mcl-1 levels were reduced by 80% in Raji and 40% in Jijoye M13 but only slightly in the other cell lines (Fig. 3B). Conversely, the

Bcl-2 protein, whose gene is deleted in Ramos, was overexpressed in the other cell lines; furthermore, the tumor suppressor *p53* gene, deleted in Akata, was upregulated in Raji, Jijoye M13, and Ramos cells (data not shown).

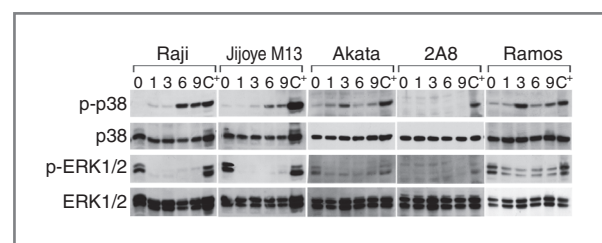
#### Resveratrol activates p38 and inhibits ERK1/2 MAPK pathways

It has been reported that p38 MAPK and ERK signaling pathways may be important in determining cell proliferation or apoptosis.

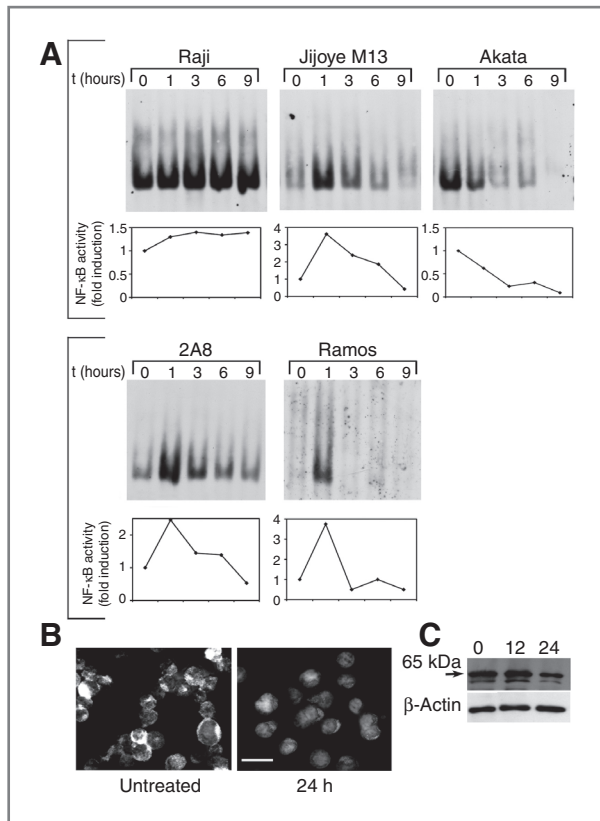
To investigate whether resveratrol treatment of BL cell lines affected MAPK pathways, we incubated EBV(+) and EBV(-) BL cells with the polyphenol for different periods of time and analyzed the cell lysates by Western blotting with phospho-specific antibodies (Fig. 4). For each cell line, a positive control for MAPK activation pathway was obtained by exposing the cells to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 1 hour. Activation of p38 in Akata and Ramos cells was detected 3 hours after the addition of resveratrol, as the signal identifying phospho-p38 elevated to about 4- and 5-fold, respectively, with respect to that at time 0. A similar strong activation of p38 MAPK was observed for Raji and Jijoye M13 after 6 hours of incubation with resveratrol. In contrast, the signals corresponding to phospho-ERK1/2, highly represented in untreated cells, rapidly disappeared following the addition of resveratrol, indicating that this pathway was dramatically inhibited by exposure of the cells to the polyphenol. Unexpectedly, both MAPK signaling pathways did not appear to be modulated by resveratrol in 2A8 cells, although they were both activated by TPA treatment. In addition, the analysis of the phosphorylation pathways of *c-jun*-NH<sub>2</sub>-kinase and protein kinase C did not reveal significant variations in all BL cell lines exposed to resveratrol (data not shown).

#### Resveratrol inhibition of NF- $\kappa$ B activity in BL cells

In BL tumor cells, *c-myc* overexpression is linked to chromosomal translocation in one of the Ig gene loci; it has been shown that NF- $\kappa$ B activity is required for *c-myc* expression and constitutive NF- $\kappa$ B DNA-binding activity in different types of B-cell malignancies, including Burkitt's lymphoma, has been reported. To investigate the effect of



**Figure 4.** Western blot analysis of phosphorylation of p38 and ERK1/2 MAPK in resveratrol-treated BL cells. At the indicated times (hours), cell lysates were prepared and the phosphorylation pattern of p38 and ERK was evaluated by Western blot analysis with the specific antibodies for each protein and its phosphorylated (p-) form; C<sup>+</sup>, TPA-treated cells as the positive control. Specific signals were visualized and quantified as described in Materials and Methods.



**Figure 5.** NF- $\kappa$ B activity in resveratrol-treated BL cells. A, at the indicated times (hours), whole-cell extracts were incubated with a consensus NF- $\kappa$ B-binding site oligonucleotide to evaluate NF- $\kappa$ B activity by EMSA (see Materials and Methods). The signals, quantified by densitometry, are expressed as fold induction of time 0. The data shown are representative of 3 independent experiments with similar results. B, Raji cells untreated or treated with resveratrol for the times indicated were analyzed with RelA/p65 antibodies by indirect immunofluorescence; DNA was visualized with DAPI, and the merge images are displayed; scale bar, 20  $\mu$ m. C, 50  $\mu$ g of nuclear proteins from Raji cells untreated (0) or treated with resveratrol for 12 and 24 hours were subjected to SDS-PAGE and Western blot analysis with RelA/p65 antibodies as described (see Materials and Methods).

resveratrol on NF- $\kappa$ B activity in BL cells, EBV(+) and EBV(-) cell lines were treated with resveratrol or control diluent for up to 9 hours. After different periods of incubation, whole-cell extracts were used to measure NF- $\kappa$ B activity by EMSA. Figure 5A shows that Raji and Akata cells displayed high constitutive NF- $\kappa$ B DNA-binding activity. Resveratrol caused a marked inhibition of NF- $\kappa$ B activity in Akata cells already after 1 hour of treatment. In Jijoye M13, 2A8, and Ramos cells, after a rapid and transient increment, NF- $\kappa$ B activity decreased under control levels. In contrast, exposure of Raji cells to resveratrol did not significantly alter NF- $\kappa$ B DNA-binding activity during the first 9 hours of treatment. To confirm that the retarded band visualized by EMSA was indeed NF- $\kappa$ B, cell extracts were incubated with antibodies to either p50 or p65 and then analyzed by EMSA. Antibodies to either subunit of NF- $\kappa$ B shifted the band to higher molecular weight, suggesting that the

active complex consisted of p50 and p65 subunits (Supplementary Fig. S2). The effect of resveratrol on NF- $\kappa$ B activity seemed to be specific for this transcription factor because EMSA carried out to measure AP1 activity in parallel samples did not reveal a significant decrement in EBV-positive cell lines and variable levels were detected in EBV(-) cells (Supplementary Fig. S3).

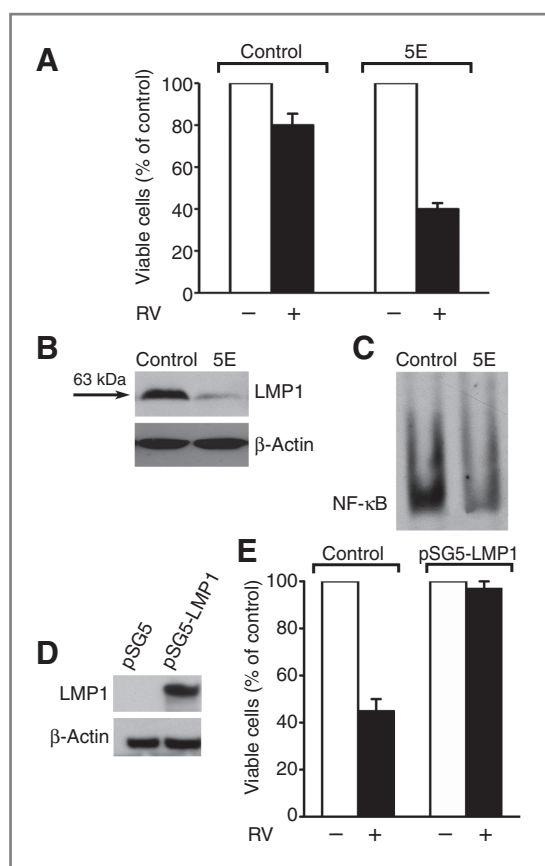
NF- $\kappa$ B-binding activity decreased, however, in all cell lines treated for prolonged time with resveratrol (data not shown). Immunofluorescence studies and Western blot analysis conducted on Raji cells exposed to the polyphenol for 24 hours showed a dramatic decrement of the p65 subunit (Fig. 5B and C). In particular, the fluorescent signal decorating the nucleus and the cytoplasm of untreated cells in bright patches appeared weak and mainly localized to the cytoplasm in resveratrol-treated cells.

### LMP1 expression modulates the sensitivity of BL cells to resveratrol

Because Raji cells seemed to be more protected from resveratrol-mediated apoptosis, we looked for EBV genes expressed in the latency III program that would confer additional survival advantage against the effects of the polyphenol. *LMP1* is the principal EBV oncogene with the potential to antagonize apoptosis as well as to promote cellular survival and proliferation (19–21). We therefore set to investigate the effects of resveratrol after silencing *LMP1* expression in Raji cells.

Raji 5E, a clone selected after transfection of Raji cells with a construct expressing *LMP1* siRNA, and Raji control transfected with a control plasmid were exposed to resveratrol or to the diluent for 24 hours and viable cells determined by Trypan blue staining. Figure 6A shows that in Raji 5E, cell proliferation was reduced by about 40% as compared with that measured in the control. This result clearly indicated that inhibition of *LMP1* expression in clone 5E, as confirmed by Western blot analysis (Fig. 6B), dramatically increased the sensitivity of latency III Raji cells to resveratrol. The analysis of NF- $\kappa$ B DNA-binding activity by EMSA in Raji control and Raji 5E revealed in the latter a strong reduction of the band corresponding to the DNA-protein complex, indicating that inhibition of *LMP1* expression in Raji 5E resulted in a lower stimulation of the transcription factor (Fig. 6C).

To further investigate links between *LMP1* expression and sensitivity of BL cells to resveratrol, we transfected EBV(-) 2A8 cells with the *LMP1* plasmid or the control vector; 24 hours later, cells were exposed to either resveratrol or the diluent and viable cells determined by Trypan blue staining after a further 24-hour period. Figure 6D shows that 24 hours after transfection, *LMP1* plasmid had efficiently promoted the expression of the protein. As expected, cell proliferation of EBV(-) 2A8 cells transfected with the control plasmid was strongly reduced by exposure to resveratrol as compared with control cells treated with the diluent. In contrast, proliferation of 2A8 cells expressing the viral oncogene and treated with resveratrol was similar to that observed for the control cells (Fig. 6E). These results



**Figure 6.** Influence of LMP1 expression on Burkitt's lymphoma sensitivity to resveratrol (RV). A, Raji cells expressing control siRNA (control) or siRNAs targeting LMP1 (5E) were treated with resveratrol (black columns) or with diluent (empty columns) for 24 hours and cell viability was measured by Trypan blue staining; values are means  $\pm$  SD of 3 different experiments and are presented as the percentage of control; B, LMP1 protein levels in Raji cells expressing control siRNA (control) or siRNAs targeting LMP1 (5E) were assessed by Western blot analysis;  $\beta$ -actin was used as a loading control. C, NF- $\kappa$ B DNA-binding activity in Raji control and Raji 5E was detected by EMSA (see Materials and Methods). D, 2A8 cells were electroporated with either pSG5 or pSG5-LMP1 plasmid and 24 hours later analyzed by Western blotting for LMP1 expression. E, 2A8 cells treated as in (D) were exposed to resveratrol (black columns) or the diluent (empty columns) for 24 hours and cell viability was measured by Trypan blue staining; values are means  $\pm$  SD of 3 different experiments and are presented as the percentage of control.

clearly indicate that LMP1 expression increases the resistance of BL cells to the polyphenol.

#### LMP1 downregulation increases the antiproliferative effect of resveratrol on LCLs

To validate the cooperative effects of LMP1 suppression and resveratrol treatment in inhibiting cell proliferation of EBV-infected latency III cells, we exposed to the polyphenol 2 LCLs silenced for LMP1. To this end, cells were first electroporated with LMP1 siRNA or control plasmid and 24 hours later treated with or without resveratrol for the next 24 hours at concentration previously found to effectively inhibit LCLs proliferation (Supplementary Fig. S4).

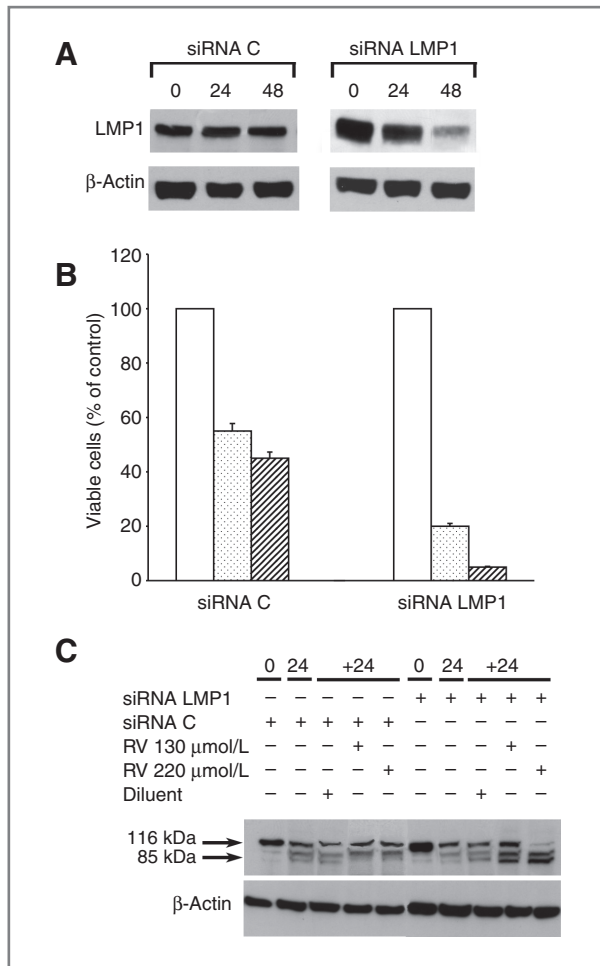
Cell counts were determined by Trypan blue exclusion assay, and apoptosis levels were assessed by detection of PARP cleavage on Western blots. Figure 7A shows that electroporation of LCL1260 with control plasmid (siRNA C) did not affect LMP1 expression; in contrast, transfection of the cells with LMP1 siRNA decreased LMP1 levels by about 30% to 40% at 24 hours and by about 80% at 48 hours. Following 24-hour exposure of the cells to 130 or 220  $\mu$ mol/L resveratrol, proliferation of LCLs with control siRNA was inhibited by 45% and 55%, respectively (Fig. 7B). However, LMP1 downregulation by siRNA dramatically reduced the viability of the cells treated with the polyphenol and strongly induced apoptosis as revealed by the high levels of PARP cleavage product (Fig. 7C). Similar results were obtained with LCL1087 (data not shown). Altogether, our data indicated that LCLs expressing lower levels of LMP1 were more sensitive to resveratrol-induced apoptosis.

#### Discussion

A great variety of naturally occurring compounds have been shown to inhibit, delay, or reverse cellular events associated with carcinogenesis, representing a promising alternative to conventional therapies for the management of cancer (22). Among them, resveratrol is a dietary chemopreventive phytochemical that has gained considerable attention for its remarkable inhibitory effects on different stages of carcinogenesis (23). A great deal of evidence has been provided for the contributions EBV can make to transformed cells (24). Multiple viral genes can block apoptosis and different Burkitt's lymphomas express different combinations of these antiapoptotic genes, indicating that EBV contributes survival functions of these tumors in multiple ways (2, 3, 25, 26).

The experiments carried out in 3 EBV-positive BL lines that express just EBNA1 as in Akata, a latency II-like pattern with the exception of LMP1 (EBNA1<sup>+</sup>/LMP2A<sup>+</sup>) as in Jijoye M13, and the latency III phenotype as in Raji, respectively, have allowed us to evaluate the antiproliferative effect of resveratrol on cells in which EBV infection was associated with a specific degree of protection from apoptosis.

We report here for the first time that resveratrol causes cell-cycle arrest and apoptosis of EBV-infected BL cells independently of the latency program the virus established in the host cells. However, the efficacy of the polyphenol seems to be inversely related to the restriction pattern of viral gene expression. Thus, latency I infection in Akata cells confers a small but significant protective effect whereas latency II-like infection in Jijoye M13 and latency III in Raji cells, further broadening EBV latent genes expression pattern, progressively diminish the sensitivity of BL cells to resveratrol-induced apoptosis. The different susceptibility of BL cells to resveratrol is reflected in the variable concentrations of the polyphenol necessary to reduce proliferation by 50%. Our results show that the growth inhibitory potential of resveratrol is mainly due to



**Figure 7.** Downregulation of LMP1 expression increases resveratrol (RV)-induced cell growth inhibition and apoptosis of LCLs. A, cells were electroporated with control siRNA (siRNA C) or siRNAs targeting LMP1 (siRNA LMP1) and, at the time indicated, LMP1 protein levels analyzed by Western blotting; B, LCLs treated as in (A) and incubated for 24 hours were exposed to 130  $\mu\text{mol/L}$  (pointed columns), 220  $\mu\text{mol/L}$  (dashed columns) resveratrol, or to diluent (empty columns) for 24 hours. Cell counts were determined by Trypan blue staining, and the values expressed as the percentage of control cells treated with the diluent. Each value represents the mean  $\pm$  SD of 3 similar experiments. C, samples of cells treated as in (B) were collected after electroporation (0), 24 hours later (24), and following exposure for 24 hours to either resveratrol or the diluent (+24). A total of 20  $\mu\text{g}$  of cell lysates was resolved on 8% to 16% acrylamide gel and analyzed by Western blotting with PARP antibodies. Data shown are representative of 3 independent experiments.

the induction of cell-cycle arrest and apoptosis because a significant fraction of the cell population accumulated in the  $G_1$  phase of the cell cycle after exposure to the polyphenol. Cytofluorimetric evaluation of Annexin V and Annexin V/PI-positive cells revealed that  $G_1$  block triggered a high percentage of EBV(-) and latency I BL cells to rapidly proceed toward late apoptosis/necrosis, whereas the proportion of early apoptotic cells further increased in latency II-like and latency III BL cells during 24-hour treatment with the polyphenol.

Resveratrol-induced cell-cycle arrest in  $G_1$  seemed to be associated with the modulation of cell-cycle regulatory proteins involved in the  $G_1$ -S transition because a decreased expression of *cyclin A*, *cdk1*, and *cdk2* as well as an upregulation of *cyclin E* and the cdk inhibitor *p27* were detected.

It has been shown that *c-myc* downregulation is a critical molecular event of resveratrol-mediated antiproliferative activity, closely associated with growth suppression, cell-cycle arrest, and apoptosis (27, 28). Our data indicate that resveratrol suppresses *c-myc* expression in all BL cell lines, regardless of EBV status. Because *c-myc* is the master transcription factor responsible for proliferation of BL cells, EBV (-) and latency I BL cell lines might be very sensitive to *c-myc* repression and cell-cycle arrest and readily die by apoptosis or necrosis rather than sustaining a prolonged arrest of proliferation. Moreover, the higher protection of Raji and Jijoye M13 cells from apoptosis, despite the striking and rapid decrement of *c-myc* levels, seems most likely due to the expression profile of EBV genes in these cell lines. With respect to Jijoye M13, protection from apoptosis has been previously reported in LMP2A-expressing cell lines (29, 30).

PARP cleavage and caspase 3 activation detected in all BL cells suggest that resveratrol may induce cell death, at least in part, through caspase-dependent pathway. We have found that resveratrol-induced apoptosis occurs in conjunction with downregulation of the antiapoptotic proteins cIAP1, FLIP, and XIAP. Because these proteins inhibit caspase activity (31-33), it is conceivable that resveratrol might induce apoptosis by downregulating their expression. Our data concerning the analysis of cell-cycle- and apoptosis-related factors in resveratrol-treated BL cells also showed that, overall, the variations of protein levels were much more pronounced in Raji and Jijoye M13 cells than in Akata or EBV(-) cell lines. A likely explanation for the lower reactivity of the latter cell lines can be found in the rapid and massive cell death that follows resveratrol-induced block of cell-cycle progression.

A great deal of experimental evidence has been provided for p38 MAPK involvement in apoptosis triggered by a variety of agents, as well as in the regulation of cell cycle  $G_1$ -S and  $G_2$ -M checkpoints in response to cellular stress and DNA damage (34, 35). Although p38 MAPK is generally believed to be a kinase that mediates cell death, ERK1/2 MAPK pathways have been shown to promote survival and cell growth (36). Our findings indicating inhibition of ERK1/2 activity and the concomitant activation of p38 signaling cascade confirm the opposite effects these kinases play in determining cell survival/death and may account for the observed antiproliferative activity of resveratrol on BL cells. It has been proposed that ERK1/2 kinases regulate  $G_1$  phase progression through various mechanisms including *cyclin D1* induction, enhanced *c-myc* protein stability, *p27* downregulation, and decreased expression of antiproliferative genes (36, 37). Although cyclin D1 protein levels were barely detectable in resveratrol-treated BL cells, our results showing *c-myc* suppression and the increment of *p27* protein levels suggest the involvement of ERK1/2 pathway in mediating resveratrol-induced cell-cycle arrest.



NF- $\kappa$ B activation has been linked to several aspects of oncogenesis, including the control of cell migration, cell-cycle progression, and differentiation, as well as apoptosis (38). In general, NF- $\kappa$ B exerts an antiapoptotic activity by switching on genes that hamper the effects of proapoptotic stimuli, thereby suppressing cell death pathways. Genes whose activity is positively regulated by NF- $\kappa$ B include members of the Bcl-2 family, *TRAF1/2*, *cIAP1/2*, *cFLIP*, and *XIAP* (39).

BL cells often present aberrant NF- $\kappa$ B regulation and express constitutive high levels of NF- $\kappa$ B activity (40, 41). Our data, indicating that resveratrol markedly inhibits NF- $\kappa$ B activity in EBV(-) 2A8 and Ramos, latency I (Akata), and latency II-like (Jijoye M13) BL cells, are in agreement with what previously reported on the inhibitory effect of the polyphenol on the activity of the transcription factor (42). However, in latency III Raji cells expressing the EBV oncogene *LMP1*, suppression of NF- $\kappa$ B activity was observed only after prolonged treatment with the polyphenol.

Among the BL cell lines examined in this study, only Raji expresses the EBV oncogene *LMP1* because the Jijoye subline M13, deleted for EBNA2, lacks the viral latent membrane protein.

*LMP1*, as the critical activator of NF- $\kappa$ B (43, 44), is responsible for the dramatic differences in the growth pattern and phenotype of cells driven into proliferation by either *c-myc* or the EBV latency III program (20). We report here that *LMP1* downregulation decreases the protection of Raji cells to resveratrol-induced apoptosis and conversely that *LMP1* overexpression protects EBV(-) 2A8 cells from resveratrol treatment. Because of the *LMP1* stimulatory effect on NF- $\kappa$ B, it is conceivable that *LMP1* increases the resistance of latency III BL cells to resveratrol by transcription factor activation.

Several EBV latency-associated gene products linked to enhanced cell survival are capable of resetting the apoptosis threshold and thus might contribute to the resistance of EBV-positive BL cells (45). We report that induction of apoptosis mediated by resveratrol is clearly different between EBV(-) and EBV(+) latency I, II, or III BL cells. Almost all endemic Burkitt's lymphomas carry EBV as an EBNA1-only (latency I) infection. Nevertheless, one endemic Burkitt's lymphoma case was found to be unusually heterogeneous and in early-passage cultures yielded clones that, besides *c-myc* translocation, differed in EBV status

showing 3 forms of restricted latency correlated with specific degree of protection from apoptosis (2, 25, 26). The observation that EBV-associated Burkitt's lymphoma is a heterogeneous disease reflected by the expression of different viral latency programs may have great impact for the treatment and subclassification of Burkitt's lymphoma. Therefore, it is extremely important to correlate prognosis and response to chemotherapy with the type of viral latency involved. In this respect, EBV-associated posttransplant lymphoproliferative diseases and EBV-positive diffuse large B-cell lymphomas that express *in vivo* the full pattern of EBV latent genes might be the least responsive to chemotherapy. We showed that silencing of the *LMP1* viral oncogene dramatically increases the antitumor potential of resveratrol in latency III BL cells and LCLs.

Our findings, therefore, enlighten a previously unknown aspect of the chemotherapeutic potential of resveratrol, that is, its use in combination with the delivery of siRNA for posttranscriptional silencing of latent EBV genes. We envisage the possibility to develop novel therapeutic strategies for the treatment of EBV-associated malignancies in which resveratrol, because of its safety and lack of known toxicity, might be used by itself or in combination with siRNA specific for EBV genes whose expression characterize the latency pattern of the tumor.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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