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RESVERATROL INHIBITS PROLIFERATION AND SURVIVAL OF EPSTEIN BARR VIRUS (EBV)-INFECTED BURKITT’S LYMPHOMA CELLS DEPENDING ON VIRAL LATENCY PROGRAM

Running title: Apoptosis of EBV-infected BL cells by resveratrol

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Abstract

Resveratrol (3,4’, 5-trihydroxy-trans-stilbene; RV), a polyphenolic natural product, shows chemopreventive properties against several cancers, heart diseases, inflammation and viral infections. Epstein Barr Virus (EBV), a γ-herpesvirus, contributes to the development of several human cancers including Burkitt’s lymphoma (BL). In this study we asked whether treatment with RV would affect the viability of EBV-positive BL cells displaying different forms of latency. We report here that RV, regardless of EBV status, induces caspase-dependent apoptosis by arresting cell cycle progression in G1 phase. However, RV 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. RV-induced cell cycle arrest and apoptosis occurred in association with induction of p38 MAPK phosphorylation and suppression of ERK1/2 signaling pathway. Moreover, NFκB 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 in the higher resistance to the antiproliferative effect of RV since siRNA-mediated inhibition of LMP1 greatly increased the sensitivity of latency III BL as well as that of lymphoblastoid cell lines (LCLs) to the polyphenol. We propose that a combined RV/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.

Keywords: EBV, resveratrol, cell cycle, Burkitt’s lymphoma, latent infection, apoptosis, LMP1
Introduction

EBV, the causative agent of infectious mononucleosis, is associated with a variety of different human tumors including Burkitt’s lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin’s disease (HD) and post transplant lymphoproliferative disorders (PTLDs) occurring in immunocompromized 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 BL; the Latency III phenotype, defined by the expression of six nuclear (EBNA1, EBNA2, EBNALP, EBNA3A, EBNA3B, and EBNA3C) and three membrane antigens (LMP1, LMP2A and LMP2B) is present in most immunodeficiency-related B cell lymphoma and in the lymphoblastoid cell lines (LCLs) obtained by in vitro infection of B lymphocytes; latency II program, corresponding to the expression of EBNA1, LMP1 and LMP2, is mostly related to NPC and HD.

The hallmark of BL 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 BL cases in Africa and in 10-20% of all sporadic cases worldwide. Moreover, about 40-50% of AIDS-associated BL cases carry the viral DNA. Although in most BL 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 (3,4’, 5-trihidroxystilbene; RV), 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, anti-oxidant, anti-inflammatory, anti-bacterial and anti-viral activities (6). In addition, RV was found to inhibit proliferation and induce apoptosis in several human tumor cells (7, 8). It has been previously reported that RV is able to 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 RV on BLs 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 RV-induced susceptibility to apoptosis and studied the molecular mechanisms underneath.

We report here that regardless of EBV status, the treatment of BL cells with RV 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 RV-induced apoptosis as did in LCLs. These findings suggest that the combined use of RV 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 two human B lymphoblastoid cell lines (LCLs) were used. EBV-positive Raji and EBV negative Ramos cells were purchased from ATCC (LGC Promochem, UK). Akata with the EBV genome (11, 12), the isogenic EBV negative Akata subline 2A8 (13) and Jijoye M13 cells (4) were obtained from Dr. P.
Trivedi (Sapienza University, Rome, Italy). Lymphoblastoid cell lines 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% CO2 atmosphere and maintained at a cell density of 3.5 x 10^5/ml.

Cells, at a density of 3 x 10^5 cells/ml, were incubated in RPMI 1640/10% FCS with different concentrations of resveratrol (Sigma, St. Louis, MO, USA) 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 Burker chamber.

**Cytofluorimetric analysis**

Cell cycle studies were performed by FACS analysis as previously described (14). Apoptosis was evaluated by Annexin V-FITC apoptosis detection kit (Sigma), that measures Annexin V binding to phosphatidyserine in conjunction with propidium iodide staining, according to the accompanying procedure.

**Western Blot analysis**

Western blot analysis on whole cell lysates was performed 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 (1:1000), cyclin E (1:1000), cdk1 (1:1000), p27 (1:50), Mcl1 (1:200) (all by Santa Cruz Biotechnology, Santa Cruz CA, USA); cdk2 (1:1000, Biomol, Plymouth Meeting PA, USA); PARP (1:1000, Alexis Biochemicals, Enzo Life Sciences Plymouth Meeting, PA, USA); active caspase 3 (1:1000, AbCam, Cambridge, UK); XIAP and IAP1 (1:500, R&D System, Minneapolis, MN, USA); FLIP (1:500, Upstate Biotechnology, Waltham MA, USA);
RelA/p65 (1:100, Santa Cruz Biotechnology); phospho p38 (cat. n. 9211), total p38, phospho ERK1/2 (cat. n. 9101), total ERK1/2 (1:1000, Cell Signaling, Danvers, MA, USA); LMP1 (1:7500, Pharmingen, San Diego CA, USA); β-actin (1:1500, Sigma).

**Electrophoretic Mobility Shift Assay (EMSA)**

To determine NFkB and AP1 activation we performed EMSA as previously described (14, 16). Briefly, whole cell extracts were obtained from 2 x 10⁶ cells treated with RV or with the diluent for different lengths of time. 20 μg of proteins, as determined by a modified Lowry assay (RC DC protein assay, BioRad, Hercules CA, USA) were incubated with 30 fmol of a DIG-labeled kB DNA probe (17) for 20 min 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, Basel, Switzerland). The specificity of binding was also verified by competition with the unlabelled oligonucleotides. For supershift assay, cell extracts were incubated with antibodies against either p50 or p65 of NFkB (Santa Cruz Biotechnology) for 30 min at room temperature and then analyzed by EMSA. Quantitative evaluation of NFkB complex formation was performed by ImageJ freeshare software (http://rsbweb.nih.gov/ij).

**Fluorescence microscopy**

Cells incubated with RV or with diluent for 24 hours were washed with phosphate-buffered saline (PBS), spotted on slides and fixed with methanol for 5 min at -20°C. Fixed cells were incubated with p65 NFkB subunit antibodies (1:500, Santa Cruz Biotechnology) for 1 hour at 37°C, rinsed in PBS, and then incubated with FITC-conjugated secondary antibodies for 30 min at room temperature. Subsequently, cells were rinsed and nuclei stained with DAPI (0.2 μg/ml) for 15 min.
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 equipped with a FX 340 digital camera.

**LMP1 silencing and overexpression**

To obtain LMP1 down-regulation, we used a human H1 RNA pol III promoter-based shRNA vector, pSilencer 3.1-H1 hygro (Ambion, Austin TX, USA), to express siRNA that target the EBV LMP1 in Raji cells. The sense- and antisense-strand oligonucleotides for LMP1 shRNA were 5'-<br>gatccGGAATTTGCACGGACAGGCttcaagagaGCCTGTCCGTGCAAATTCCttttttttggaaa-3' and 5'-<br>agcttttccaaaaaaaaGGAATTTGCACGGACAGGCtctcttgaaGCCTGTCCGTGCAAATTCCg-3'.

A pSilencer hygro vector expressing an hairpin siRNA with limited homology to any known sequences in the human genome was used as negative control (control siRNA). The constructs were prepared by ligating the annealed oligonucleotides into the BamH1 and HindIII of pSilencer and the resulting vectors analyzed by DNA sequencing. 1.5 x 10^7 cells were electroporated with 30 μg of siRNA expression vectors at 975 μF, 260 V using a Gene Pulser II system (Bio-Rad). To select clones, electroporated cells were grown in medium containing 100 μg/ml hygromycin (Sigma). After three 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 x 10^7 EBV negative Akata 2A8 cells were electroporated (230 V, 960 μF) with 10 μg of LMP1 expression vector pSG5-LMP1 (a kind gift of Dr Martin Rowe, Birmingham University Medical School, UK) or of the corresponding control vector pSG5.

**Statistical analysis**

Statistical analysis was carried out using the ANOVA followed by post hoc tests...
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 RV, we tested two EBV (-) (2A8 and Ramos) and three 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, while Akata cells display a latency I program (Fig. S1). The cell lines, exposed to RV concentrations increasing from 20 to 300 μM, for various periods of time, showed different degrees of sensitivity to the polyphenol. Fig 1 shows that the growth of each cell line was inhibited by RV in a concentrations and time-dependent manner. The concentrations reducing cell proliferation by 50% at 48 hours were of about 300 μM for Raji, 130 μM for Jijoye M13 cells and 40 μM 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 negative cells (P<0.05).

Altogether, RV cytotoxicity for EBV (-) and Akata cells was approximately 3-fold 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 RV on BL cells, we analyzed the cell cycle distribution after incubation with the polyphenol. In order to compare the different cell lines, all the following experiments were carried out by exposing each cell line to the RV concentration reducing cell proliferation by 50% at 48 hours, as previously determined. Cells were harvested at 12 and 24 hours, and the DNA content determined by PI staining and flow cytometric analysis. The bar graph in Fig. 2a shows that a 12 hours treatment with RV increased significantly (P< 0.01) the percentage
of cells in the G1 phase with a reduction of cells in S and G2/M phases. After 24 incubation, the G1 population further increased in Raji and Jijoye M13 cells while decreased in Akata cells, in the 2A8 and in Ramos cells. In the latter (Latency I and EBV (-) BL) the proportion of cells in the sub G0/G1 phase, representing apoptotic events, augmented after 24 hours incubation (data not shown), suggesting a relation between cell cycle arrest and apoptosis.

Therefore, we evaluated apoptosis in cells incubated with RV for 12 and 24 hour, 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, while cells double stained by Annexin V and PI, represent the fraction of late apoptotic/necrotic cells. As it appears from Fig 2b, the ratio between Annexin V and Annexin V/PI positive cells appeared to be dramatically different among the cell lines. In fact, after a 24 hours exposure to RV, the percentages of early apoptotic cells in Raji and Jijoye were about 30% and 55% while the fractions of late apoptotic/necrotic cells were about 8% and 12 %, respectively. In addition, the percentage of AnnexinV-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, AnnexinV/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 by Annexin V only. Taken together, these data appeared to indicate that EBV (-) BL cells and latency I Akata were more prone to RV-induced apoptosis than Latency II and Latency III cell lines.

Western blot analysis of poly (ADP-ribose) polymerase (PARP) processing confirmed that RV differentially induced apoptosis in BL cells. As illustrated in Fig 2c, after 12 hours 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 hours exposure to RV and in Raji cells only after 48 hours (data not shown), indicating that
Latency III BL cells were more resistant than the other cell lines to RV-induced apoptosis. To investigate the apoptotic pathway, we analyzed the activation of effector caspase 3 by measuring the levels of the 17 KDa cleaved form. Data reported in Fig 2c showed higher levels of the biologically active caspase 3 along with increased amounts of the processed PARP, thus indicating that RV 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 G1 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 RV increased the expression of the cyclin dependent kinase (cdk) inhibitor p27 and that of cyclin E in all cell lines; in contrast, cyclin A, cdk1 and cdk2 were down-regulated 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 RV blocks specific cell cycle associated activity involved in the progression from G1 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 while Mcl1 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 up regulated 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 MAP kinase and ERK signaling pathways may be important in determining cell proliferation or apoptosis.
To investigate whether RV treatment of BL cell lines affected MAPKs pathways, we incubated (EBV+) and (EBV-) BL cells with the polyphenol for different periods of time and analyzed the cell lysates by Western blot with phospho-specific antibodies (Fig. 4). For each cell line a positive control for MAPK activation pathway was obtained by exposing the cells to TPA for one hour. Activation of p38 in Akata and Ramos cells was detected three hours after addition of RV, as the signal identifying phospho-p38 raised about four and five fold, respectively, with respect to that of time 0. A similar strong activation of p38 MAPK was observed for Raji and Jijoye M13 after six hours of incubation with RV. In contrast, the signals corresponding to phospho-ERK1/2, highly represented in untreated cells, rapidly disappeared following addition of RV, 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 RV in 2A8 cells although they were both activated by TPA treatment. In addition, the analysis of the phosphorylation pathways of JNK and PKC did not reveal significant variations in all BL cell lines exposed to RV (data not shown).

Resveratrol inhibition of NFkB activity in BL cells

In BL tumor cells, c-myc overexpression is linked to chromosomal translocation in one of the immunoglobulin gene loci; it has been shown that NFkB activity is required for c-myc expression and constitutive NFkB DNA-binding activity in different types of B cell malignancies, including Burkitt’s lymphoma, has been reported. To investigate the effect of RV on NFkB activity in BL cells, EBV (+) and EBV (-) cell lines were treated with RV or control diluent for up to 9 hours. After different periods of incubation, whole cell extracts were used to measure NFkB activity by EMSA. Fig. 5a shows that Raji and Akata cells displayed high constitutive NFkB DNA-binding activity. RV caused a marked inhibition of NFkB activity in Akata cells already after one hour of treatment. In Jijoye M13, 2A8 and Ramos cells, after a rapid and transient increment, NFkB activity decreased under control levels. In contrast, exposure of Raji cells to RV did not significantly alter NFkB DNA-binding activity during the first 9 hours of treatment. To confirm that the retarded band
visualized by EMSA was indeed NFκB, cell extracts were incubated with antibodies to either p50 or p65 and then analyzed by EMSA. Antibodies to either subunit of NFκB shifted the band to higher molecular weight suggesting that the active complex consisted of p50 and p65 subunits (Supplementary Fig S2). The effect of RV on NFκB activity appeared to be specific for this transcription factor since 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-negative cells (Supplementary Fig S3).

NFκB binding activity decreased, however, in all cell lines treated for prolonged time with RV (data not shown). Immunofluorescence studies and Western blot analysis performed on Raji cells exposed to the polyphenol for 24 hours showed a dramatic decrement of the p65 subunit (Fig. 5 b 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 RV-treated cells.

**LMP1 expression modulates the sensitivity of BL cells to RV**

Since Raji cells appeared to be more protected from RV-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 RV 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 RV or to the diluent for 24 hours and viable cells determined by Trypan blue staining. Fig. 6a shows that in Raji 5E cell proliferation was reduced by about 40% as compared to 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 RV. Analysis of NFκB DNA binding activity by EMSA in Raji control and in 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 RV, we transfected EBV (-) 2A8 cells with the LMP1 plasmid or the control vector; 24 hours later cells were exposed to either RV or the diluent and viable cells determined by Trypan blue staining after a further 24 hours period. Fig. 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 RV as compared to control cells treated with the diluent. In contrast, proliferation of 2A8 cells expressing the viral oncogene and treated with RV was similar to that observed for the control cells (Fig 6e). These results clearly indicate that LMP1 expression increases the resistance of BL cells to the polyphenol.

LMP1 downregulation increases the antiproliferative effect of RV on LCLs

In order to validate the cooperative effects of LMP1 suppression and RV treatment in inhibiting cell proliferation of EBV-infected latency III cells, we exposed to the polyphenol two LCLs silenced for LMP1. To this end, cells were first electroporated with LMP1 siRNA or control plasmid and 24 hours later treated or not for the next 24 hours with RV, at concentration previously found to effectively inhibit LCLs proliferation (Supplementary Fig. S4). Cell counts were determined by trypan blue exclusion assay and apoptosis levels assessed by detection of PARP cleavage on Western blots. Fig. 7a shows that electroporation of LCL1260 with control plasmid (siRNA C) did not affect LMP1 expression; in contrast, transfection of the cells with LMP1siRNA, decreased LMP1 levels by about 30-40 % at 24 hours and by about 80% at 48 hours. Following a 24 hours exposure of the cells to 130 μM or 220 μM RV, proliferation of LCLs with control siRNA was inhibited by 45% and 55%, respectively (Fig. 7b). However, LMP1 down-regulation 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 RV-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, RV is a dietary chemopreventive phytochemical that has gained considerable attention for its remarkable inhibitory effects on different stages of carcinogenesis (23). Several evidences for the contributions EBV can make to transformed cells have been provided (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 three EBV positive BL lines that express respectively, just EBNA1 as in Akata, a Latency II-like pattern with the exception of LMP1( EBNA1+/LMP2A+) as in Jijoye M13, and the Latency III phenotype as in Raji, have allowed us to evaluate the antiproliferative effect of RV on cells in which EBV infection was associated with a specific degree of protection from apoptosis. We report here for the first time that RV 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 appears 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 in Jijoye M13 and Latency III in Raji cells, further broadening EBV latent genes expression pattern, progressively diminish the sensitivity of BL cells to RV-induced apoptosis. The different susceptibility of BL cells to RV is reflected in the variable concentrations of the polyphenol necessary to reduce proliferation by 50%. Our results show that the growth
inhibitory potential of RV is mainly due to the induction of cell cycle arrest and apoptosis since a significant fraction of the cell population accumulated in the G1 phase of the cell cycle after exposure to the polyphenol. Cytofluorimetric evaluation of Annexin V and AnnexinV/PI positive cells revealed that G1 block triggered a high percentage of EBV (-) and Latency I BL cells to rapidly proceed towards late apoptosis/necrosis, whereas the proportion of early apoptotic cells further increased in Latency II-like and Latency III BL cells during the 24 hours treatment with the polyphenol.

RV-induced cell cycle arrest in G1 appeared to be associated with the modulation of cell cycle regulatory proteins involved in the G1/S transition, since 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 down-regulation is a critical molecular event of RV-mediated antiproliferative activity, closely associated with growth suppression, cell cycle arrest and apoptosis (27, 28). Our data indicate that RV 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 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, appears 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 RV may induce cell death, at least in part, through caspase-dependent pathway. We have found that RV-induced apoptosis occurs in conjunction with downregulation of the antiapoptotic proteins cIAP1, FLIP and XIAP. Since these proteins inhibit caspase activity (31-33) it is conceivable that RV might induce apoptosis by downregulating their expression. Our data concerning the analysis of cell cycle and apoptosis related factors in RV-treated BL cells also showed that overall, the variations of protein
levels were much more pronounced in Raji and in Jijoye M13 cells as compared to 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 RV-induced block of cell cycle progression.

Several experimental evidences have been provided of p38 MAP kinase involvement in apoptosis triggered by a variety of agents, as well as in the regulation of cell cycle G1/S and G2/M checkpoints in response to cellular stress and DNA damage (34, 35). While p38 MAPK is generally believed to be a kinase that mediates cell death, ERK1/2 mitogen activated protein kinases pathway 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 RV on BL cells. It has been proposed that ERK1/2 kinases regulate G1 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 RV-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 RV-induced cell cycle arrest.

NFkB 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, NFkB exerts an anti-apoptotic activity by switching on genes that hamper the effects of pro-apoptotic stimuli thereby suppressing cell death pathways. Genes whose activity is positively regulated by NFkB include members of the Bcl-2 family, TRAF1/2, cIAP1/2, cFLIP, and XIAP (39). BL cells often present aberrant NFkB regulation and express constitutive high levels of NFkB activity (40, 41). Our data, indicating that RV markedly inhibits NFkB 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 Lat III Raji cells expressing the EBV oncogene LMP1, suppression of
NFkB activity was observed only after prolonged treatment with the polyphenol.

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

LMP1, as the critical activator of NFkB (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 down-regulation decreases the protection of Raji cells to RV-induced apoptosis and conversely that LMP1 overexpression protects EBV (-) 2A8 cells from RV treatment. Because of the LMP1 stimulatory effect on NFkB, it is conceivable that LMP1 increases the resistance of latency III BL cells to RV by activating the transcription factor activity.

Several EBV latency-associated gene products linked to enhanced cell survival, are capable of re-setting the apoptosis threshold and so might contribute to the resistance of EBV-positive BL cells (45). We report that induction of apoptosis mediated by RV is clearly different between EBV (-) and EBV (+) Latency I, II or Latency III BL cells. Almost all endemic BLs carry EBV as an EBNA1-only (Latency I) infection. Nevertheless, one endemic BL was found unusually heterogeneous and in early passage cultures yielded clones that, besides c-myc translocation, differed in EBV status showing three forms of restricted latency correlated with specific degree of protection from apoptosis (2, 25, 26). The observation that EBV-associated BL is a heterogeneous disease reflected by the expression of different viral latency programs may have great impact for the treatment and sub-classification of BL. Therefore, it is extremely important to correlate prognosis and response to chemotherapy with the type of viral latency involved. In this respect, EBV-associated post-transplant 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 demonstrated that silencing of the LMP1 viral oncogene dramatically increases the antitumor potential of RV in Lat III BL and in LCLs.

Our findings therefore, enlight a previously unknown aspect of the chemotherapeutic potential of RV: i.e. its use in combination with the delivery of siRNA for post-transcriptional silencing of
latent EBV genes. We envisage the possibility to develop novel therapeutic strategies for the
treatment of EBV-associated malignancies in which RV, 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.

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Legend to the figures

Fig. 1 Resveratrol-induced inhibition of BL cell proliferation. Cells were incubated with RV at the concentrations indicated. After 24h, 48h and 72h cell counts were assessed by trypan blue staining. Percentage of proliferation was determined as proliferation of treated cells x 100/proliferation of control cells, treated with the diluent. Each point represents the mean ± SD of three similar experiments.

Fig. 2 Resveratrol induces cell cycle arrest in G1 phase and apoptosis of BL cells. Each cell line was cultured with the RV concentration reducing proliferation by 50% at 48 hours or with the diluent, for 12h and 24h. (a) Fixed cells were stained with propidium iodide and analyzed by flow citometry. The percentages of cells in G1 (black bars), S (white bars) or G2/M phase (hatched bars) are shown. (b) Evaluation of apoptosis by FACS analysis after Annexin V and propidium iodide staining. AnnexinV positive cells (empty columns) and AnnexinV/PI stained cells (black columns), mean values ± 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. β-actin was used as the internal marker. The data are representative of those obtained in at least three independent experiments with similar results.

Fig. 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 Methods. A representative result of three independent experiments is shown. β-actin was used as the internal marker.

Fig. 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 evaluated by Western blot analysis with the specific antibodies for each protein and its phosphorylated (p-) form; C+, TPA-treated cells, as positive control. Specific signals were visualized and quantified as described in Methods.

**Fig. 5 NFkB activity in resveratrol-treated BL cells.** (a) At the indicated times (hours), whole-cell extracts were incubated with a consensus NFkB binding site oligonucleotide to evaluate NFkB activity by EMSA (see Methods). The signals, quantified by densitometry, are expressed as fold induction of time zero. The data shown are representative of three independent experiments with similar results. (b) Raji cells untreated or treated with RV for the times indicated were analyzed with RelA/p65 antibodies by indirect immunofluorescence; the DNA was visualized with DAPI and the merge images are displayed; scale bar, 20 μm (c) 50 μg of nuclear proteins from Raji cells untreated (0) or treated with RV for 12 and 24 hrs were subjected to SDS-PAGE and Western blot analysis with RelA/p65 antibodies as described (see Methods).

**Fig. 6 Influence of LMP1 expression on BL sensitivity to RV.** (a) Raji cells expressing control siRNA (control) or siRNA targeting LMP1 (5E) were treated with RV (black columns) or with diluent (empty columns) for 24h and cell viability measured by trypan blue staining; values are means ± SD of three different experiments and are presented as % of control; (b) LMP1 protein levels in Raji cells expressing control siRNA (control) or siRNA targeting LMP1 (5E) were assessed by Western blot analysis; β-actin was used as a loading control. (c) NFkB DNA binding activity in Raji control and Raji 5E was detected by EMSA (see Methods). (d) 2A8 cells were electroporated with either pSG5 or pSG5-LMP1 plasmid and 24 hours later analyzed by Western blot for LMP1 expression. (e) 2A8 cells treated as in (d) were exposed to RV (black columns) or the diluent (empty columns) for 24 hours and cell viability measured by trypan blue staining; values are means ± SD of three different experiments and are presented as % of control.
Fig. 7 Downregulation of LMP1 expression increases resveratrol-induced cell growth inhibition and apoptosis of LCLs. (a) Cells were electroporated with control siRNA (siRNA C) or siRNA targeting LMP1 (siRNA LMP1) and, at the time indicated, LMP1 protein levels analyzed by Western blots; (b) LCLs treated as in (a) and incubated for 24 hours were exposed to 130 μM (pointed columns), 220 μM (dashed columns) RV or to diluent (empty columns) for 24 hours. Cell counts were determined by trypan blue staining and the values expressed as percentage of control cells treated with the diluent. Each value represents the mean ± SD of three 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 RV or the diluent (+24). 20 μg of cell lysates were resolved on a 8-16% acrylamide gel and analyzed by Western blot with PARP antibodies. Data shown are representative of three independent experiments.
Figure 1

The graph shows the percentage of viable cells over time for different cell lines under varying concentrations of a substance. The cell lines include Raji, Jijoye M13, Akata, 2A8, and Ramos. Each graph plots the percentage of viable cells against time (24, 48, 72 hours) for different concentrations of the substance (130 μM, 220 μM, 360 μM, 20 μM, 40 μM, 60 μM).
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7