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Resveratrol inhibits Epstein Barr Virus lytic cycle in Burkitt's lymphoma cells by affecting multiple molecular targets

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ABSTRACT

Resveratrol (RV), a polyphenolic natural product present in many plants and fruits, exhibits anti-inflammatory, cardio-protective and anti-proliferative properties. Moreover, RV affects a wide variety of viruses including members of the Herpesviridae family, retroviruses, influenza A virus and polyomavirus by altering cellular pathways that affect viral replication itself. Epstein Barr Virus (EBV), the causative agent of infectious mononucleosis, is associated with different proliferative diseases in which it establishes a latent and/or a lytic infection. In this study, we examined the antiviral activity of RV against the EBV replicative cycle and investigated the molecular targets possibly involved. In a cellular context that allows *in vitro* EBV activation and lytic cycle progression through mechanisms closely resembling those that *in vivo* initiate and enable productive infection, we found that RV inhibited EBV lytic genes expression and the production of viral particles in a dose-dependent manner. We demonstrated that RV inhibited protein synthesis, decreased reactive oxygen species (ROS) levels, and suppressed the EBV-induced activation of the redox-sensitive transcription factors NF–kB and AP-1.

Further insights into the signaling pathways and molecular targets modulated by RV may provide the basis for exploiting the antiviral activity of this natural product on EBV replication.

1. Introduction

The vast majority of people carry latent Epstein Barr Virus (EBV) infection for their lifetimes without any symptoms, but the clinical feature of infectious mononucleosis (IM) may arise in adolescents and young adults. The primary cellular targets are resting B lymphocytes that are induced to proliferate by the virus. In an immune-competent host, the virus-induced proliferation is limited by a strong T cell response which allows spontaneous resolution of EBV primary infection. However, the virus maintained in a pool of latently infected memory B cells, may be reactivated in the immune-deficient host (Rickinson and Kieff, 2007). Immunodeficiency-related B cell lymphoma, including post transplant lymphoproliferative disorders (PTLD), are directly caused by EBV. In AIDS patients EBV causes hairy leucoplakia (HLP) of the tongue whose lesions produce large amount of virus (Greenspan et al., 1985). Moreover, EBV is associated with a variety of tumors including Burkitt's lymphoma (BL), Hodgkin's lymphoma, T-cell lymphoma and nasopharyngeal carcinoma (Kutok and Wang, 2006).

Except for IM and HLP, all the other EBV diseases are malignancies characterized by latent infection. However, also in the latter, the EBV productive cycle allows horizontal spread of the virus and favors B cell tumors development by promoting lytically-infected B cell secretion of several growth factors and cytokines (Cayrol and Flemington, 1995; Hong et al., 2005; Hsu et al., 2008; Jones et al., 2007; Mahot et al., 2003; Miyazaki et al., 1993). Moreover, recent studies carried out in a mouse model, found that EBV lymphoma formation is less frequent in animals infected with a lytic replication-defective virus than the control virus, thus supporting an important role for lytic EBV infection in the development of B cell lymphoma (Ma et al., 2011).

Treatments of EBV infection typically include (alone or in combination) antivirals, radiotherapy, chemotherapy, CD20 antibodies and adoptive T-cell therapy (De Paoli, 2010; Villegas et al., 2010). Generally, the use of antiviral compounds is limited by toxic side effects, poor oral bioavailability and the risk for the emergence of drug-resistent virus strains.

Resveratrol (trans-3,4′,5 trihydroxy–stilbene, RV), a polyphenolic phytoalexin produced by a variety of plants, has gained

Abbreviations: RV, resveratrol; EBV, Epstein Barr Virus; IE, immediate early; E, early; NF–kB, nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1, activator protein 1.

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considerable attention as a cancer chemopreventive agent and to control fungal, bacterial and viral infections (Bhat et al., 2001; Yu et al., 2012). We have recently shown that RV is able to induce apoptosis of EBV infected Burkitt's lymphoma cells with an efficacy inversely related to the restriction pattern of viral latent gene expression (De Leo et al., 2011). Moreover, the antiviral activity of RV has also been reported for several members of the Herpesviridae family (Docherty et al., 2005, 2006; Evers et al., 2004; Faith et al., 2006; Yiu et al., 2010), HIV (Zhang et al., 2009), influenza A virus (Palamara et al., 2005) and polyomavirus (Berardi et al., 2009).

In this study, we have examined the effects of RV on EBV replication in two Burkitt's lymphoma cell lines that allow EBV lytic cycle induction through different treatments. We show that, independently of the method used to trigger EBV activation, RV strongly inhibits lytic cycle initiation. Moreover, in cross-linked Akata cells, a system for EBV induction that most likely mimics the mechanism of viral reactivation *in vivo*, we demonstrate that RV inhibited EBV lytic genes expression and viral particles production in a dose-dependent manner. We provide evidences that the down-regulation of EBV gene expression occurs at the post-transcriptional level, involving the inhibition of protein synthesis, the reduction of reactive oxygen species (ROS) and the suppression of redox sensitive NF–kB and AP-1 activities stimulated by EBV lytic cycle activation.

2. Materials and methods

2.1. Reagents

Resveratrol (Sigma), prepared as 50 mg/ml stock solution in ethanol and kept at −20 °C protected from light, was diluted to final concentrations in RPMI 1640 medium.

Cycloheximide (CHX) purchased from Sigma, was dissolved in DMSO at 100 mg/ml and used at 50 μg/ml.

2.2. Cell lines, EBV lytic cycle induction and resveratrol treatment

EBV-positive Raji cells, characterized by a latency III pattern and an EBV abortive lytic cycle and Akata cells showing a latency I phenotype, are Burkitt's lymphoma (BL)-derived cell lines. Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and antibiotics, in a 5% CO_2 atmosphere and maintained at a cell density of 3.5×10^5 /ml.

To induce EBV lytic cycle, Raji cells (1.5×10^7) were electroporated with 10 μg of pCMVgenZ (a kind gift of Dr. G. Miller, Yale University School of Medicine, USA) using a Bio-Rad Gene Pulser (0.26 kV and 960 μF) and thereafter diluted to $5x10^5$ /ml. Control Raji cells were electroporated with 10 μg of the CMV vector. To activate the virus in Akata cell line, cells were diluted to 10^6 /ml and treated with 10 μg/ml of goat anti-human IgG (Sigma, St. Louis, MO, USA). The efficiency of EBV lytic cycle induction was evaluated, in both cell lines, by counting positive cells after immunofluorescence staining with FITC-labeled antibodies specific for EBV early antigens (Matusali et al., 2009).

Statistical analysis of the cells expressing BZLF1 revealed that in Raji as well as in Akata cells, EBV lytic cycle was activated in about 30% of the cell population, regardless of the method used to initiate the process.

2.3. Cell counts and flowcytometry

Following a 24 h period of incubation with IgG and RV at the concentrations of 10, 20 and 40 μM, Akata cells were counted in a Burker chamber by Trypan blue exclusion assay. The percentage of proliferation was determined as proliferation of treated cells × 100/proliferation of untreated cells. Apoptosis was evaluated by Annexin V-FITC apoptosis detection kit (BD Pharmingen) which measures Annexin V binding to phosphatidylserine in conjunction with propidium iodide (PI) staining, according to the accompanying procedure.

2.4. Immunoblots

Raji and Akata cells, treated as described, were incubated in the absence or in the presence of either resveratrol or CHX, collected at different times and analyzed by Western blot, as previously described (Matusali et al., 2009). Equal amounts of proteins, as determined by RC-DC Protein Assay (Bio-Rad, Hercules, CA, USA), were resolved by 4–20% Bio-Rad TGX gels and electroblotted to an Amersham Hybond PVDF membrane (GE Healthcare, Milan, Italy). The following primary antibodies were used: BZLF1(1:100 Argene); BRLF1 (1:2000 Argene), BFRF1(1: 1000) and BFLF2 (1: 10), kindly provided by Dr. A. Farina, Dept. of Experimental Medicine, Univ. of Rome "Sapienza", Italy; BALF5 (1: 40) was a kind gift of Dr. F. Graesser, GSF-Forschungszentrum, Munich, Germany; NF–kb RelA/p65 (1: 1000) and NF–kBp50 (1:3000) were purchased from Millipore; β-actin (1:1500 Sigma). Membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) and signals were visualized by ECL detection kit (Amersham).

2.5. Semiquantitative RT-PCR assay

Total RNA was extracted from 10^6 cells by NucleoSpin RNAII columns and treated with DNase I (Macherey–Nagel, Duren, Germany). A total of 3 μg of the RNA was subjected to MMLV reverse transcriptase (Invitrogen) with 2.5 μM oligo$(dT)_{23}$ primers (Sigma) in a 50 μl reaction mixture according to the manufacturers' protocols. 3 μl of RT products were analyzed undiluted or 1/10 and 1/20 diluted, by conventional PCR. As controls, PCR of RNA samples not subjected to RT and of RT products in the absence of primers, were used. Amplifications (25 cycles) were carried out with the following primers: BZLF1-F, 5′agaagcacctcaacctggagacaa; BZLF1-R, 5′cagc gattctggctgttgtggttt; BRLF1-F, 5′tcactacacaaacagacgcagcca; BRLF1-R, 5′aatctccacactcccggctgtaaa; BALF5-F, 5′cggaagccctctggacttc; BALF5-R, 5′ccctgtttatccgatggaatg; BHRF1-F, 5′gtcaaggtttcgtctgtgtg; BHRF1-R, 5′ttctcttgctgctagctcca; and GAPDH-F, 5′ttcgacagtcagccg-catcttctt; GAPDH-R, 5′gcccaatacgaccaaatccgttga. Amplification products were resolved on 1.5% agarose gels and stained by GelRed (Biotium).

2.6. Real-time PCR

Akata cells were treated for 24 h with IgG in the absence or in the presence of RV at 10, 20 and 40 μM. Cells were collected by centrifugation for 5 min at 300×*g* and culture supernatant used to determine EBV DNA copies by real-time PCR. The assay was performed essentially as described (Gaeta et al., 2009). Briefly, extracted DNA was analyzed by a commercially available kit (Nanogen Advanced Diagnostics S.r.l.) based on TaqMan technology.A5′ reporter dye 6-carboxyfluorescein (FAM)-labeled probe for the viral EBNA1gene was used for PCR reactions. Dilutions of plasmid carrying the specific viral gene were used to construct the reference curve and quantify viral DNA in the samples. PCR reactions were carried out with ABI PRISM 7000 (Applied Biosystem, USA) according to the manufacturer's protocol. Computer software quantified the viral DNA load referred to the sample extracted volume.

2.7. Production of reactive oxygen species (ROS)

Akata cells were treated with anti-human IgG in the absence or in the presence of 10, 20 or 40 μM RV. Aliquots (2.5×10^5cells),

incubated for the last 15 min with 40 μM 5,6-carbossy-2′,7′-dicl-orohydro fluoresceine diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) were collected at 30, 60 and 120 min. ROS production was analyzed by measuring the oxidized fluorescent derivative DCF with a flow-cytometer as previously described (Matusali et al., 2009). The levels of ROS detected in H_2O_2 treated cells have been used as positive controls.

2.8. Electrophoretic mobility shift assay (EMSA)

The DNA binding activity of transcription factors was measured as previously reported (Matusali et al., 2009). Aliquots of cells treated with IgG in the absence or in the presence of 40 μM RV, were lyzed in a high salt extraction buffer. 20 μg of proteins were incubated with 30 fmol of a DIG-labeled (DIG oligonucleotide 3′ end-labeling kit, Roche Applied Science) NF–kB (Zabel et al., 1993)and AP-1 (Saatian et al., 2006) DNA probe. Binding specificity was verified by competition with the unlabeled oligonucleotides. DNA–protein complexes were resolved by non denaturing 4% polyacrylamide gel electrophoresis, transferred to nylon membrane and detected by chemiluminescence (DIG luminescent detection kit, Roche Applied Science). Densitometric analysis of the specific signals was carried out by ImageJ Freeshare software (http://rsbweb.nih.gov/ij).

2.9. Statistical analysis

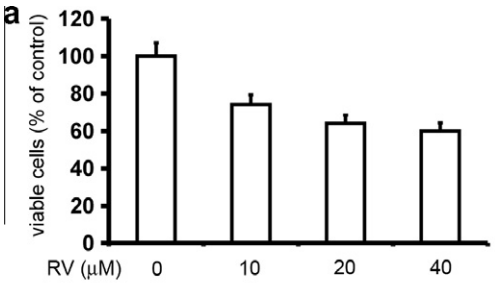
Statistical analysis was carried out using the ANOVA followed by post hoc tests (Bonferroni/Dunn). The level of significance was set at $P < 0.05$.

3. Results

3.1. Evaluation of the cytotoxicity of resveratrol on Akata cells upon EBV lytic cycle induction

Previous studies which evaluated the antiproliferative effects of RV on EBV-infected BL cells with different forms of latency, allowed us to determine the concentrations of the polyphenol causing a 50% reduction in the proliferation of Raji and Akata cells at 48 h (De Leo et al., 2011). These concentrations, equal to 300 and 40 μM, respectively, were chosen as the highest values of a range used to examine the effects of RV on EBV replicative cycle in the two cell lines. EBV lytic cycle was induced in Raji cells by electroporation of the plasmid expressing the immediate early gene BZLF1 and in Akata cells by IgG cross-linking.

In the latter cellular system for EBV replication, we evaluated a putative cytotoxic effect of RV by Trypan blue exclusion assay and by cytofluorimetric determination of apoptotic/necrotic cells after FITC-Annexin V and propidium iodide (PI) staining. The data reported in Fig. 1 show that cell counts, determined after 24 h incubation of Akata cells with 40 μM RV, were reduced by about 40% with respect to control cells incubated with IgG in the absence of the compound, while only a 10–20% decrement was measured after exposure of the cells to RV concentrations of 10 and 20 μM respectively. In agreement with these data, in the presence of 40 μM RV, the percentage of the cell population stained by Annexin V was about 30% higher than that measured in the absence of the polyphenol, while an increment of 10–20% was detected with RV concentrations of 10 and 20 μM (Fig. 1b). Interestingly, the percentages of necrotic cells (stained with PI), and those of late apoptotic cells (Annexin V/PI-positive) detected after 24 h incubation, were substantially low and not significantly affected by the treatment with RV. These results clearly show that at the concentrations used for our experiments, RV inhibits the proliferation of Akata cells without increasing cell death.



b

	AnnV (%)	PI (%)	AnnV + PI (%)
RV 0 μM	27	1,7	0,9
RV 10 μM	34	1,5	1,1
RV 20 μM	47	1,2	2
RV 40 μM	58	1	3

Fig. 1. Antiproliferative effect of RV on Akata cells upon EBV lytic cycle induction. IgG cross-linked Akata cells were treated with RV at the concentrations indicated for 24 h. (a) cell counts were assessed by Trypan blue staining. The values represent the mean ±SD of three similar experiments. (b) Samples of cells treated as in (a) were subjected to Annexin V (AnnV) and PI staining and analyzed by flowcytometry as described in the Methods. The table reports the results of a representative experiment as percentages of apoptotic cells (AnnV-positive), necrotic cells (PI) and late apoptotic cells (AnnV and PI positive).

3.2. Down-regulation of EBV lytic antigens expression by resveratrol

To examine the effects of RV on EBV lytic antigens expression, Raji cells were treated with concentrations of the polyphenol varying from 50 to 300 μM for 24 h, while Akata cells with concentrations varying from 10 to 40 μM for six hours. Cell lysates were analyzed by Western blot with antibodies specific for the two EBV immediate early (IE) antigens BZLF1 and BRLF1 and for the early (E) proteins, BFRF1, BFLF2 and the viral DNA polymerase BALF5.

Fig. 2 shows that 24 h after electroporation of Raji cells with the BZLF1 plasmid, EBV immediate early and early antigens were efficiently expressed. In contrast, viral lytic antigens were not expressed in Raji cells electroporated with the CMV vector (data not shown). When 300 μM RV was added to the cell culture, BZLF1 and BRLF1 signals, as well as those representing BFRF1, BFLF2 and BALF5 antigens, were barely detectable. Moreover, a dose-dependent effect on the expression of EBV early antigens and BRLF1

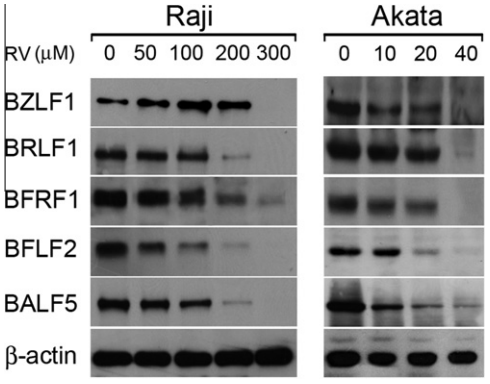


Fig. 2. Expression of EBV lytic antigens in Raji and Akata RV-treated cells. Raji and Akata cells treated as described in the Methods to induce EBV lytic cycle, were incubated in the absence or in the presence of RV at the indicated concentrations for 24 and 6 h, respectively. Cell lysates (40 μg) were resolved by SDS–PAGE, blotted on membrane and probed with antibodies for EBV antigens. Specific signals were visualized by ECL. β-Actin was used as the internal marker. The data are representative of those obtained in at least 3 independent experiments with similar results.

was observed with lower concentrations of the polyphenol. In contrast, concentrations of RV lower than 300 μM increased BZLF1 levels suggesting a stimulatory effect on the expression of the BZLF1 plasmid.

The results obtained in Akata cells show that EBV IE and E genes were highly expressed after 6 h incubation with IgG. The addition of RV to the cell cultures inhibited the expression of all EBV lytic antigens in a concentration-dependent manner so that at 40 μM, the reduction of protein levels, measured by densitometric evaluation of the specific signals, was close to 90%. Because RV similarly inhibited EBV lytic antigens expression in Raji and Akata cells, the effects of the polyphenol appeared to be independent of the method used to trigger viral reactivation. Therefore, further experiments have been performed on Akata cells that allow full completion of EBV lytic cycle.

3.3. Modulation of EBV lytic gene expression occurs at the post-transcriptional level

To gain insights into the regulation of EBV lytic gene expression by RV, we measured mRNA levels of the viral IE genes in untreated and RV-treated cells by semi-quantitative RT-PCR. As shown in Fig. 3a, even at the highest concentration, RV did not significantly affect the transcription of BZLF1 and BRLF1 genes. Similar results were obtained when the levels of EBV early genes BHRF1 and BALF5 were evaluated (data not shown). Therefore, the down-regulation exerted by the polyphenol on EBV lytic gene expression appeared to occur at the post-transcriptional level.

To investigate whether inhibition of EBV mRNAs translation could account for the decrement of the viral lytic antigens observed after RV administration, EBV lytic cycle was induced in the presence of either the protein synthesis inhibitor cycloheximide (CHX) or RV, each added at time zero (together with the IgG), 2 or 4 h later. The levels of EBV lytic antigens were measured by Western blot of cells harvested at 8 h. As shown in Fig. 3b, inclusion of CHX or RV for the 8 h duration of the experiment strongly inhibited the synthesis of BZLF1, BRLF1 and BALF5 induced by IgG (lanes 0–8). The addition of the two compounds 2 and 4 h after EBV induction, completely abolished their inhibitory effect on EBV lytic antigens expression. In fact, similar levels of the viral products were detected upon addition of either CHX or RV at 2 or 4 h after EBV induction. All together these results suggest that down-regulation of EBV lytic antigens expression observed in the cells simultaneously exposed to IgG and RV, might involve a block in the synthesis of protein factors required in the early phases of EBV productive cycle.

We also measured the abundance of BZLF1 mRNA by RT-PCR in the samples harvested at 8 h. Fig. 3c shows that the mRNA levels were similar in the cells treated with either RV or CHX, implying that the expression of BZLF1 mRNA was independent of new protein synthesis.

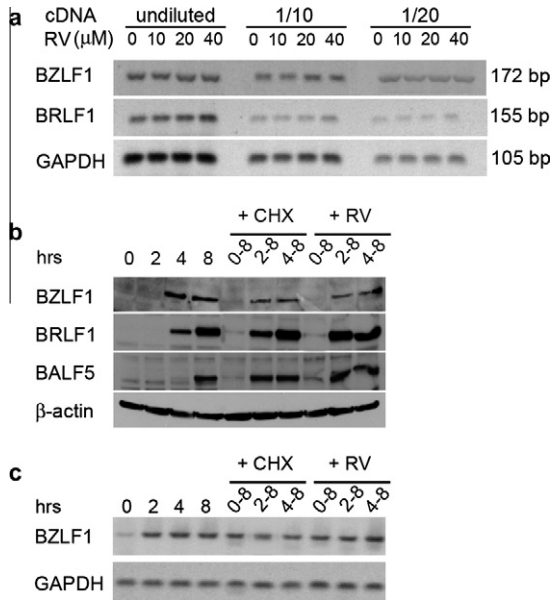


Fig. 3. EBV lytic genes expression in RV-treated cells. (a) EBV lytic cycle was induced in Akata cells in the absence or in the presence of 10, 20 or 40 μM RV for 6 h. Total RNA was purified and subjected to reverse transcription as described in the Methods. Undiluted and diluted cDNA were analyzed by PCR with the specific primers for BZLF1 and BRLF1 genes. GAPDH amplification was used to verify equal amounts of total RNA. (b) Akata cells treated with IgG were harvested at 0, 2, 4, and 8 h. CHX or 40 μM RV were added with the inducing agent (lane 0–8) or at 2 h (lane 2–8) and at 4 h (lane 4–8) after EBV lytic activation. Cell lysates were prepared from all treated cells 8 h after starting the experiment. Expression of EBV antigens was analyzed by immunoblotting. β-actin was used as loading control. (c) Total RNA was extracted from Akata cells treated as in (b) and RT-PCR was performed as described in the Methods. Representative results of 3 independent experiments are shown.

3.4. Inhibition of viral particles production by resveratrol

The strong down-regulation of EBV lytic gene expression observed after exposure of the cells to RV led us to investigate the effects of the polyphenol on EBV replication. To this end, EBV copies were measured in the medium of Akata cells exposed for 24 h to IgG with or without different RV concentrations. The results, illustrated in Fig. 4a, show that EBV particles production was modulated by RV in a dose-dependent manner. In the presence of 10 μM RV, EBV copies released in the medium were about 65% of those measured in the absence of the polyphenol. A progressive decrement of EBV particles was observed when the induction of the EBV lytic cycle was carried out in the presence of either 20 or 40 μM RV as the EBV copies measured under these conditions were about 45 and 30% respectively, of those released in the absence of the compound.

3.5. Resveratrol inhibition of EBV lytic cycle is associated with reduced ROS generation

It has been well established that many pharmacological actions of RV are due to its anti-inflammatory and anti-oxidant properties. To clarify whether RV down-regulation of EBV lytic cycle was associated with a decrement of ROS, the cellular redox state was analyzed in RV-treated or untreated Akata cells after EBV activation. After 30, 60 and 120 min, cell samples were subjected to flow-cytometric analysis to quantify ROS levels by the ROS-sensitive fluorescent product DCF. We found that ROS markedly decreased in the cells exposed to RV during the induction of EBV lytic cycle (Fig. 4b). After 30 min, ROS levels measured in RV-treated cells were about 45% of those measured in the absence of the polyphenol. In addition, the values obtained after 60 and 120 min of incubation, were about 55 to 40% and 60 to 42% of the control, respectively, depending on RV concentrations and the time elapsed from EBV lytic cycle activation.

3.6. Effects of resveratrol on EBV-mediated transcription factors activation

Since RV has been reported to inhibit the activity of several transcription factors, we measured NF–kB and AP-1 DNA binding capacity after activating EBV in Akata cells exposed or not to RV. As shown in Fig. 5, within three hours following induction of EBV lytic cycle, NF–kB DNA-binding activity rapidly increased reaching a three fold higher level than that measured at time zero; there after it declined and by 6 h returned to the levels measured before

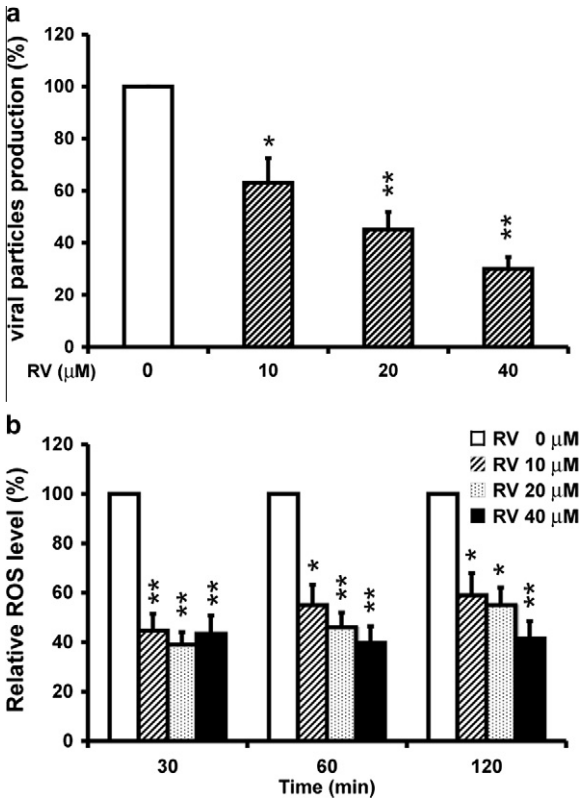


Fig. 4. (a) Inhibition of viral particles release from RV-treated cells. Akata cells were treated for 24 h with IgG in the absence or in the presence of RV at the concentrations reported. The culturing medium was collected and EBV DNA measured by real-time PCR, as described in the Methods. The values in the bar-graph are means ±SD of 3 different experiments and are presented as the percentage of control (in the absence of RV). $^{*}P < 0{,}001$ or $^{**}P < 0{,}0001$. (b) Inhibition of ROS production in RV-treated cells. Akata cells were exposed to IgG in the absence or in the presence of RV at 10, 20 or 40 μM. At the indicated times, samples were analyzed to determine ROS levels as described in the Methods; values in the bar-graph are means ±SD of 3 different experiments and are presented as the percentage of control (in the absence of RV). $^{*}P < 0.05$ or $^{**}P < 0.01$.

EBV reactivation. Addition of RV during EBV induction rapidly inhibited the initial phase of NF–kB activation. In fact, the signals corresponding to the DNA–protein complexes were similarly high in untreated and RV-treated cells after one hour of treatment but had declined to base levels already after three hours exposure of the cells to the polyphenol. Similar results were obtained when cells extracts were assessed for AP-1 DNA binding activity, clearly indicating that RV treatment inhibited the boosted activity of transcription factors induced by EBV lytic switch. To assess whether the decrement of transcription factors activities could be ascribed to decreased amounts of the constituting subunits, the levels of p65 and p50 NF–kB components were measured by Western blot analysis in the cell extracts used for EMSA. The data illustrated in Fig. 1S show that treatment of Akata cells with IgG in the absence or in the presence of 40 μM RV did not alter the levels of NF–kB protein components, indicating that the polyphenol mainly affected the binding activity of the transcription factor.

4. Discussion

We report here that RV is able to inhibit EBV lytic cyclein Burkitt's lymphoma cells when induced either by the ectopic expression of the immediate early gene BZLF1 as in Raji cells, or by activation of the B-cell receptor signaling pathway as in Akata cells. In the latter that support the complete replicative cycle, we showed that RV treatment reduced EBV particles production in a concentration dependent manner, without significantly affecting cell viability.

We have previously studied the effects of RV on latently infected Burkitt's lymphoma cells and found that the polyphenol is able to arrest the cell cycle progression, ultimately leading the cells to apoptosis. The data reported here, indicate that upon EBV lytic cycle induction, RV antiproliferative effects are even more pronounced since, in the two cell lines, the concentrations able to reduce by 50% the proliferation of EBV latently-infected cells at 48 h, caused similar decrements 24 h after induction of EBV lytic cycle. However, despite the increment of early apoptic (AnnV-positive) cells with increasing RV concentrations, the percentages of late apoptotic and necrotic Akata cells did not represent any relevant value, indicating that cell viability was not affected even by the highest concentration of the drug. Moreover, because EBV induction blocks the cell cycle at G1/S (Wu et al., 2004), the effects of RV on EBV lytic cycle have to be considered independent of the arrest of cellular proliferation.

We demonstrate that down-regulation of EBV genes expression by RV occurs without variation of the corresponding mRNA levels, indicating that the polyphenol acts at a post-transcriptional level. We also show that in the absence of significant alterations in the amounts of the transcripts, the expression of EBV lytic products is largely suppressed when lytic cycle is carried out in the presence of either RV or CHX, and that the inhibitory effect of the two compounds is lost when they are added to the cells 2 or 4 h after IgG stimulation. This result, while strongly suggesting a similar mechanism for the two compounds, indicates that both exert their effects only in the very early phases of EBV induction. Because, at least CHX, is known to block protein synthesis, it was surprising to find that cells exposed to the drugs either two or four hours after EBV induction, showed levels of EBV lytic antigens higher than those detected in the control cells, collected at two and four hours after addition of IgG. A likely explanation for this result could rely on a decreased availability and/or activity of the two drugs within the 8 h period of incubation, as previously reported for CHX (Martin et al., 1990).

Recently, an effect of RV on protein synthesis through the modulation of mTOR-dependent and independent pathways has also been reported in hepatic cells (Villa-Cuesta et al., 2011). Further investigations will be required to assess the involvement of mTOR pathways in EBV lytic cycle suppression by RV.

Our data are apparently in contrast with those obtained by Yiu et al. who showed that in P3HR1 cells transfected with BRLF1 or BZLF1 plasmids and treated with RV and sodium butyrate, the activity of the two promoters was inhibited by the polyphenol (Yiu et al., 2010). However, because sodium butyrate activates EBV gene expression by favoring histone acetylation and RV has been reported to stimulate the deacetylase activity of SIRT1 (Borra et al., 2005), the polyphenol might annihilate the effect of sodium butyrate on EBV immediate early antigens promoters. In this respect, we consider the activation of EBV lytic cycle by IgG cross-linking in a cellular context that allows the coordinated expression pattern of EBV lytic genes, a more suitable model to study the possible targets/mechanisms of RV antiviral activity *in vivo*.

The studies here reported aimed to examine the possible mechanisms by which RV inhibits EBV replication. The polyphenol has proven to exert potent antiviral activity against various families of DNA and RNA viruses by altering molecular pathways within cells affecting the viral life cycle (Campagna and Rivas, 2010). ROS production is considered as one of the host defense reaction against microbes. We and others have reported an increment of ROS levels following EBV lytic cycle activation (Gargouri et al., 2011; Matusali et al., 2009). Moreover, it has been shown that oxidative stress contributes to the reactivation of the EBV lytic cycle through the induction of BZLF1 expression (Lassoued et al.,

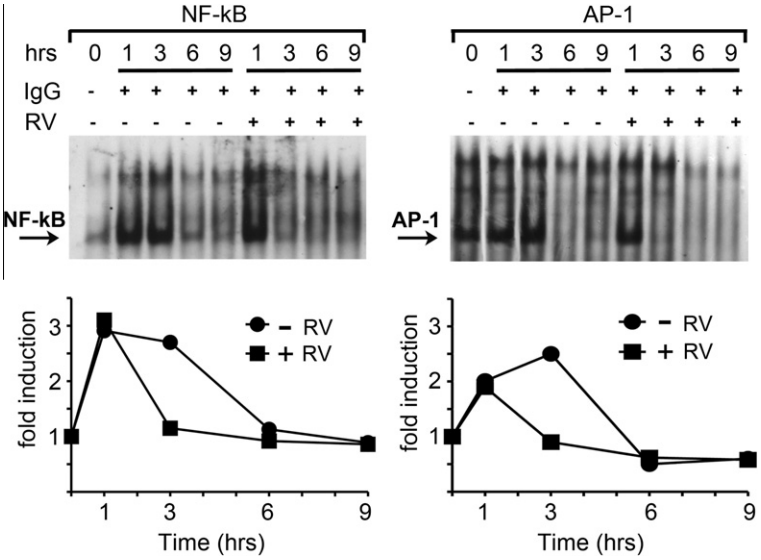


Fig. 5. NF–kB and AP-1 activities in RV-treated cells. Akata cells were exposed to IgG and 40 μM RV. At the times indicated, whole-cell extracts were obtained and equal amounts of proteins incubated with the NF–kB or AP-1 probe. Transcription factors activities were evaluated by EMSA (see Section 2). 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.

2010). Here we demonstrate that RV caused a strong reduction of ROS levels generated upon EBV activation. The anti-oxidative effect of the polyphenol was slightly reduced with time when concentrations lower than 40 μM were used, these results suggesting that increased concentration of RV can prolong the inhibitory effect on the burst of ROS levels generated during EBV lytic cycle activation.

Following B-cell receptor cross-linking, activated signaling pathways lead cell-encoded transcription factors to bind to the promoter region of EBV immediate early gene BZLF1 and initiate the lytic replication process (Amon and Farrell, 2005). We report here that RV-mediated EBV lytic cycle inhibition involves the rapid suppression of NF–kB and AP-1 activation. In a similar cellular context, RV was found to inhibit KSHV reactivation by lowering the level of Egr-1, a transcription factor mediating the expression of RTA, the analog of BZLF1 (Dyson et al., 2012). The impairment in the activities of NF–kB and AP-1 might similarly affect both upstream, as well as downstream events of BZLF1 expression. However, our data showing that RV does not alter BZLF1 transcription, suggest a prominent inhibitory effect on the transactivating function of the viral product.

NF–kB and AP-1 are known to be redox-sensitive transcription factors, positively regulated by ROS levels during stress response, inflammation and cellular proliferation (Angel and Karin, 1991; Lin and Karin, 2003). Numerous anti-oxidative and anti-inflammatory phytochemicals have been used to alter abnormal cellular signaling mediated via NF–kB or AP-1 and prevent tumor promotion or progression (Ralhan et al., 2009; Shu et al., 2010). RV is demonstrated to modulate the expression and/or activity of transcription factors involved in critical pathways of carcinogenesis (Manna et al., 2000; Whitlock and Baek, 2012) and to inhibit COX2-induced AP-1 DNA binding activity in TPA-treated murine skin (Kundu et al., 2006). In addition, RV was found to block NF–kB activation in HCMV-infected cells (Evers et al., 2004), HSV-1, HSV-2 (Faith et al., 2006) and VZV (Docherty et al., 2006). Therefore, it is conceivable that the RV-mediated inhibition of the EBV lytic cycle occurs, at least in part, through the down-regulation of critical transcription factors involved in the initial phases of EBV reactivation.

5. Conclusion

Taken together, our data indicate that RV is able to inhibit EBV lytic cycle initiation and particles release in Burkitt's lymphoma cells. RV treatment causes down-regulation of EBV lytic genes at the post-transcriptional level by affecting multiple cellular targets. The molecular mechanisms involved include: (i) inhibition of protein synthesis; (ii) a strong decrement of ROS levels and (iii) rapid suppression of NF–kB and AP-1 activities, boosted by EBV lytic cycle reactivation.

Although we still lack *in vivo* experimental data, the activity study of RV on EBV replication and underlying molecular targets, will provide the experimental basis for further research to evaluate the antiviral activity of this natural product against EBV productive infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.09.003.

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