Antiviral Research 123 (2015) 15–21

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Resveratrol inhibits rhinovirus replication and expression of inflammatory mediators in nasal epithelia

Paola Mastromarino [a,*], Daniela Capobianco [a], Federica Cannata [a], Chiara Nardis [a], Elena Mattia [a], Alessandra De Leo [a], Rossella Restignoli [b], Antonio Francioso [c], Luciana Mosca [c]

[a] Department of Public Health and Infectious Diseases, Section of Microbiology, Sapienza University, I-00185 Rome, Italy
[b] NOOS s.r.l., I-00181 Rome, Italy
[c] Department of Biochemical Sciences, Sapienza University, I-00185 Rome, Italy

ARTICLE INFO

Article history:
Received 15 June 2015
Revised 31 July 2015
Accepted 17 August 2015
Available online 19 August 2015

Keywords:
Resveratrol
Human rhinovirus
Inflammatory mediators
Nasal epithelia

ABSTRACT

Human rhinoviruses (HRV), the cause of common colds, are the most frequent precipitants of acute exacerbation of asthma and chronic obstructive pulmonary disease, as well as causes of other serious respiratory diseases. No vaccine or antiviral agents are available for the prevention or treatment of HRV infection. Resveratrol exerts antiviral effect against different DNA and RNA viruses. The antiviral effect of a new resveratrol formulation containing carboxymethylated glucan was analyzed in H1HeLa cell monolayers and *ex vivo* nasal epithelia infected with HRV-16. Virus yield was evaluated by plaque assay and expression of viral capsid proteins by Western blot. IL-10, IFN-β, IL-6, IL-8 and RANTES levels were evaluated by ELISA assay. ICAM-1 was assessed by Western blot and immunofluorescence. Resveratrol exerted a high, dose-dependent, antiviral activity against HRV-16 replication and reduced virus-induced secretion of IL-6, IL-8 and RANTES to levels similar to that of uninfected nasal epithelia. Basal levels of IL-6 and RANTES were also significantly reduced in uninfected epithelia confirming an anti-inflammatory effect of the compound. HRV-induced expression of ICAM-1 was reversed by resveratrol. Resveratrol may be useful for a therapeutic approach to reduce HRV replication and virus-induced cytokine/chemokine production.

1. Introduction

Human rhinoviruses (HRV) – the most prevalent respiratory viruses – are responsible for at least 50% of the common colds (Makela et al., 1998; Rollinger and Schmidtke, 2011) a mild, self-limiting upper respiratory tract illness, nonetheless with major economic impact through loss of productivity (Fendrick et al., 2003). HRV infection is also associated with acute otitis media, sinusitis, bronchiolitis, pneumonia, and severe infections, especially in immunocompromised patients (Henquell et al., 2012; Lieberman et al., 2010; Ruuskanen et al., 2013). HRV are also the major cause of exacerbations in both chronic obstructive pulmonary disease (COPD) and asthma (Busse et al., 2010; Papi et al., 2006).

HRV are positive-sense RNA viruses of the *Picornaviridae* family. The coding region of the viral genome contains sequences for non-structural and structural proteins divided into three primary precursor molecules (P1, P2, and P3). The four structural proteins (VP1, VP2, VP3 and VP4), which form the viral capsid, are derived from the P1 portion of the polyprotein by sequential proteolytic cleavages (Bedard and Semler, 2004).

There are no approved antiviral agents for the prevention or treatment of HRV infection. Several drug candidates have been progressed into clinical trials, including a viral 3C protease inhibitor, and different compounds that prevent virus attachment and entry into cells by binding to the viral capsid (Gunawardana et al., 2014). However, none of these candidates were commercialized due to unacceptable side effects or lack of efficacy when applied to the natural setting (Docherty et al., 2005). Currently, capsid binder vapendavir is under clinical development (Feil et al., 2012). Prevention of HRV infection through vaccination is not feasible since more than 100 different rhinovirus types with

Abbreviations: HRV, human rhinovirus; COPD, chronic obstructive pulmonary disease; CMG, carboxymethylated-(1,3/1,6)-β-D-glucan.

* Corresponding author at: Dipartimento di Sanità Pubblica e Malattie Infettive, Sezione di Microbiologia, Università "Sapienza", Piazzale Aldo Moro 5, I-00185 Roma, Italy.

E-mail address: paola.mastromarino@uniroma1.it (P. Mastromarino).

http://dx.doi.org/10.1016/j.antiviral.2015.08.010
0166-3542/

low antibody cross-reactivity have been described (Papi and Contoli, 2011).

Resveratrol is a polyphenol produced by several plants in response to stress or injury induced by microorganisms or environmental hazards and protects fruits and vegetables against fungal infections (Pervaiz, 2003; Sanders et al., 2000). Resveratrol displays a wide range of biological and pharmacological activities including anti-inflammatory, antioxidative, anticancer, antibacterial, antiviral, cardioprotective and neuroprotective effects (Campagna and Rivas, 2010; Chan, 2002; Fremont, 2000; Pervaiz, 2003). However, in spite of its multiple beneficial effects on human health, resveratrol use as a drug has been limited by its poor solubility, low bioavailability, and the tendency to become unstable due to autooxidation and photosensitivity (Francioso et al., 2014a). A recent paper describes the development of an aqueous formulation of resveratrol in combination with a modified glucan, the carboxymethylated-(1,3/1,6)-β-D-glucan (CMG) (Francioso et al., 2014b). CMG confers stability to resveratrol in solution without affecting its biological activity. This new formulation provided as a nasal spray was recently proved to be safe and effective in reducing the severity and recurrence of respiratory infections in children (Miraglia Del Giudice et al., 2014a,b; Varricchio et al., 2014).

Several studies have demonstrated that resveratrol exerts antiviral effect against different DNA and RNA viruses, including herpes simplex virus, human cytomegalovirus, varicella-zoster virus, Epstein Barr virus, influenza A virus, respiratory syncytial virus, and HIV both *in vitro* and *in vivo* (Beach et al., 2014; Chen et al., 2012; De Leo et al., 2012; Docherty et al., 2005, 2006; Evers et al., 2004; Faith et al., 2006; Lin et al., 2015; Palamara et al., 2005; Xie et al., 2012).

The benefits of resveratrol may be associated with its general activity as a modulator of the transcription factor NF-kB, of the cell cycle, apoptosis, and possibly as activator of SIRT1 (Campagna and Rivas, 2010). NF-κB transcription mediates the production of cytokines and chemokines in response to toll-like receptor recognition of intermediate dsRNA during viral infections (Majde, 2000; Rudd et al., 2006).

Human airway epithelial cells (HAECs) are the principal sites of HRV infection in both upper and lower airways (Holgate, 2011). HAECs not only serve as a target and possible reservoir for the infecting virus, but also are the site and source of a wide range of mediators that drive subsequent immune and physiological responses specific to HRV (Holgate, 2013). Indeed, rhinovirus infection induces the production of cytokines and chemokines including interleukin-6 (IL-6), interleukin-8 (IL-8), regulated on activation normal T cell expressed and secreted (RANTES), interleukin-10 (IL-10) and interferon-β *in vivo* and *in vitro* (Bartlett et al., 2008; Message et al., 2008; Subauste et al., 1995; Zhu et al., 1996). The production of most cytokines and chemokines is HRV replication dependent (Bartlett et al., 2008), has pro-inflammatory effects and correlates with the severity of cold symptoms (Gern et al., 2002). Moreover, rhinovirus infection of both primary bronchial epithelial cells and a respiratory epithelial cell line markedly increases cell surface expression of intercellular adhesion molecule-1 (ICAM-1) (Papi and Johnston, 1999) the cellular receptor for the major group (90%) of rhinoviruses (Greve et al., 1989; Uncapher et al., 1991).

To date, no studies investigated the effects of resveratrol on inflammatory mediator production in HRV-infected airway epithelia. In this study, we examined the effect of resveratrol on chemokine and cytokine production in HRV-infected nasal epithelia *ex vivo*, and evaluated the ability of resveratrol to suppress production of infectious virus in nasal epithelia and in H1HeLa cells. Nasal epithelia were cultured at air–liquid interface using a two-chamber trans-well tissue culture system to reproduce the structural and functional phenotype of differentiated airway epithelium. Resveratrol was tested alone or in combination with CMG to examine possible synergistic activity of the compounds. The effect of resveratrol on the production of ICAM-1 in both cell systems was also analyzed.

2. Materials and methods

2.1. HeLa cells

H1HeLa cells, particularly susceptible to rhinovirus infection, were used. Cells were cultured at 37 °C in 5% CO_2 atmosphere in Eagle's Minimum Essential Medium (MEM, HyClone) with 10% fetal bovine serum (FBS). For viral infection the serum concentration was lowered to 2% (maintenance MEM).

2.2. Human nasal epithelia

Fully differentiated human nasal epithelial models reconstituted using primary nasal cells isolated from healthy donors (MucilAir™ Epithelix Sarl) were cultured on a Transwell of 6.5 mm of diameter, with a pore size of 0.4 μm (Costar) at the air–liquid interface, using MucilAir™ culture medium at 37 °C in 5% CO_2 atmosphere. Each insert, consisting of about 400,000 cells, mimics the tissue of the human nasal epithelium and contains, from the bottom to the top, basal, goblet, ciliated cells and mucus.

2.3. Virus

Rhinovirus serotype 16 (HRV-16, ATCC VR-283) was grown in H1HeLa cells inoculated with virus at a multiplicity of infection (MOI) of 0.1 PFU/cell in maintenance medium.

2.4. Plaque assay

Plaque assay was performed on H1HeLa cells inoculated with ten-fold serial dilutions of HRV-16. After 48 h of incubation at 33 °C, plaques were stained with 0.1% crystal violet solution.

2.5. Cytotoxicity assays

Non cytotoxic concentrations of CMG or resveratrol for H1HeLa cells and nasal epithelia were determined by MTT and resazurin assay, respectively. See Supplementary Materials.

2.6. Antiviral assays

The antiviral effect of resveratrol and/or CMG in H1HeLa cells was evaluated before, during and after viral adsorption. Antiviral effects of the compounds were also evaluated in human nasal epithelia. HRV-16 infection was performed on the apical side of epithelia. After 7 h viral adsorption at 37 °C in 5% CO_2 atmosphere, cells were washed 3 times with MucilAir™ culture medium to remove unabsorbed virus. The compounds were then added in the culture medium and on the apical surface (30 μL). Cells from duplicate wells were scraped with cold PBS, centrifuged at 400*g* for 5 min at 4 °C and resuspended in Laemmli sample buffer for Western blot analysis.

2.7. Western blot analysis

Cell extracts prepared as above described were resolved on 8% SDS-polyacrylamide gels. After blotting, membranes were probed with ICAM-1 (1:4000; Santa Cruz), VP2 (1:8000; QED Bioscience

inc.) and β2-microglobulin (1:500; Sigma) antibodies. The specific signals were detected by chemiluminescence (ECL; Amersham).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Culture media of human nasal epithelia were analyzed for various cytokines and chemokines. Human IL-6, IL-8, IL-10, RANTES Quantikine immunoenzymatic kits (R&D Systems Inc.), and IFN-β ELISA kit (PBL Biomedical Laboratories) were used following the manufacturer's instructions. All the experiments were performed in duplicate.

2.9. Immunofluorescence

H1HeLa cells were grown in micro-tissue chamber slides, infected with HRV-16 (1 PFU/cell) in the presence or absence of resveratrol and incubated for 6 h at 33 °C in 5% CO_2 atmosphere. Cells were fixed and stained by indirect immunofluorescence with ICAM-1 antibodies (CD54, immunotools, 1:25). Slides were observed using a Leica DM 4000 fluorescence microscope with a 340 digital camera.

2.10. Statistical analysis

Data were expressed as mean ± SD and the effect of resveratrol and/or CMG was evaluated using the Student's *t* test. Differences were considered significant for values of $p < 0.05$.

3. Results

3.1. Cytotoxicity of resveratrol and CMG on H1HeLa cells and human nasal epithelia

Preliminary investigations were carried out to verify the maximum non-cytotoxic concentration of resveratrol and CMG in the two different cellular models. Resveratrol up to 50 μM did not significantly affect cell viability neither in proliferating cells nor in subconfluent monolayers up to 48 h, while nasal epithelia viability was not affected by resveratrol concentrations up to 300 μM within 72 h (Supplementary Fig. 1). CMG was not toxic neither for H1HeLa cells nor for epithelia.

3.2. Inhibition of HRV-16 replication in H1HeLa cells by resveratrol and CMG

The antiviral effect of resveratrol on HRV-16 multiplication in H1HeLa cells was studied under single cycle (MOI 3) or multiple cycles (MOI 0.3) of viral replication (Fig. 1). The addition of 50 μM resveratrol to the cell culture during HRV-16 replication produced a significant inhibition of single cycle viral multiplication ($p = 0.012$). A stronger inhibitory effect was observed in H1HeLa cells infected under conditions of multiple cycles of viral replication as resveratrol was able to reduce HRV-16 multiplication by more than one log as compared to untreated control ($p = 0.0026$). CMG was devoid of any inhibitory effect either under one cycle or multiple cycles of HRV-16 multiplication. In the presence of CMG, resveratrol showed an inhibiting activity similar to that observed in the presence of the compound alone. To verify whether the reduced virus yield observed in the presence of resveratrol was due to a virucidal effect on HRV-16 released from infected cells, high (3×10^7) and low (3×10^3) amounts of HRV-16 were incubated (1 h, 37 °C) with concentrations of resveratrol up to 2.2 mM before titration by plaque assay. The results obtained demonstrated that resveratrol did not inactivate extracellular viral particles (data not shown). Moreover to determine whether resveratrol affected the attachment/entry of HRV-16 to H1HeLa cells, cells were treated with 200 μM of the polyphenol before and during the adsorption phase (1 h, 37 °C; MOI 3 and 0.3). Results demonstrated that resveratrol did not inhibit early steps of virus infection (data not shown).

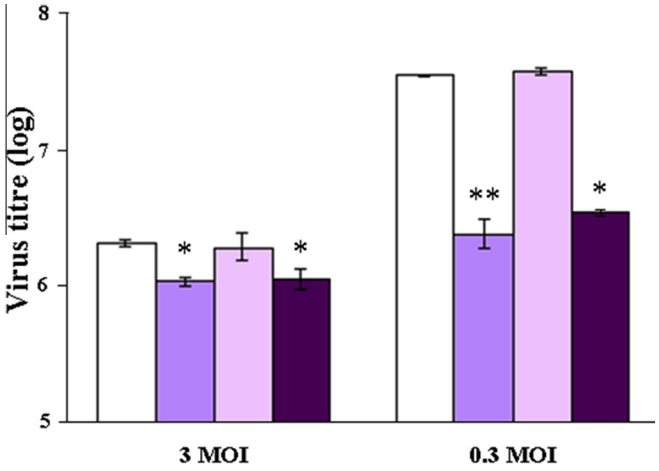

Fig. 1. Inhibition of HRV-16 replication in H1HeLa cells. H1HeLa cells were infected with HRV-16 under single cycle (3 MOI, 9 h) or multiple cycles (0.3 MOI, 36 h) of viral replication, in the absence (□) or in the presence of 50 μM resveratrol (■), 0.023 mg/mL CMG (■), 0.023 mg/mL CMG + 50 μM resveratrol (■). Data represent the mean of duplicate samples. Each experiment was repeated three times with similar results. Viral yield was determined by plaque assay. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* the respective control (□), Student's *t* test.

3.3. Inhibition of HRV-16 replication in human nasal epithelia

The antiviral effect of resveratrol on HRV-16 replication was also assessed in human nasal epithelia which are known to produce chemokines and cytokines. Preliminary experiments were performed to verify the optimal conditions of HRV-16 infection since no data are present in literature. For this purpose nasal epithelia were infected at both low and high multiplicity of infection and the virus produced from the apical side or in basal medium was quantified at various times after infection up to 96 h. Data obtained showed that infectious viral particles were present only in the apical surfaces of epithelia indicating a polarity of virus release. Maximal levels of virus yield were obtained after 20 h and 40 h at high and low multiplicity of infection, respectively (data not shown).

Infection of nasal epithelia with HRV-16 caused reduction of ciliary motility and cytopathic effect (CPE) detected by microscopic observation. In nasal epithelia infected with high MOI of HRV-16 the ciliary motility was greatly reduced and the cells appeared highly damaged after 20 h. However, in the presence of 100 μM and 300 μM resveratrol ciliary motility was partially recovered and CPE was reduced (Fig. 2A). The amount of infectious viral particles released from infected epithelia was significantly reduced by 100 μM and 300 μM resveratrol in comparison to the untreated control.

The infection of epithelia with low MOI HRV-16 did not produce any type of CPE within 24 h, while ciliary motility and integrity of epithelia were strongly compromised after 40 h. Increasing amounts of resveratrol determined a dose-dependent reduction of virus yield. Indeed, a statistically significant reduction of HRV-16 titer was observed starting from the concentration of 75 μM, while ciliary motility was partially restored with resveratrol concentration of 100 μM and higher (Fig. 2A). A 3 log reduction in the amount of infectious virus released from nasal epithelia was

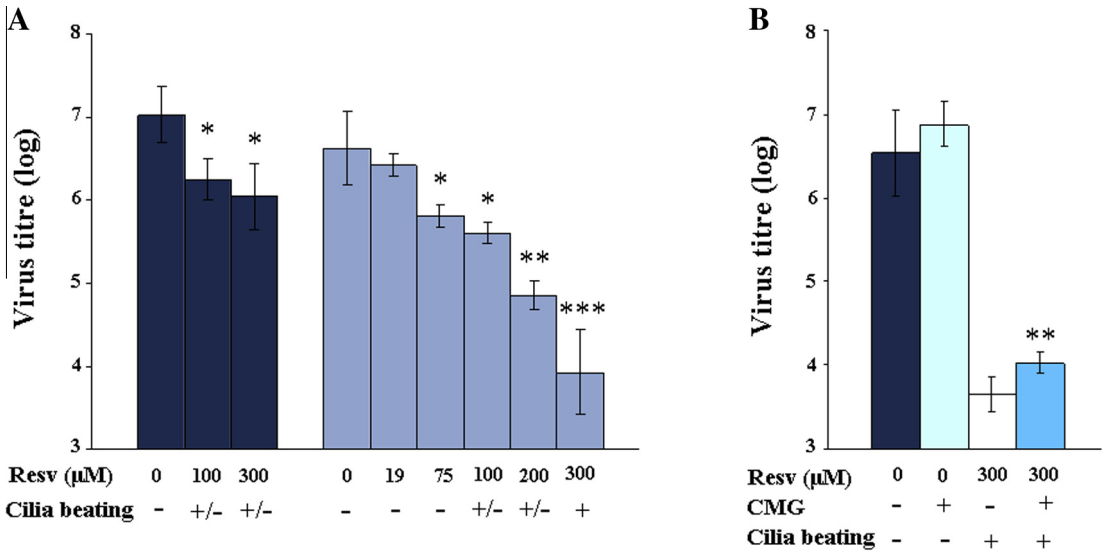

Fig. 2. Inhibition of HRV-16 replication in human nasal epithelia. (A), Epithelia were infected with HRV-16 under single cycle (■, 3 MOI, 20 h) or multiple cycles (■, 0.3 MOI, 40 h) of viral replication in the absence or in the presence of the indicated concentrations of resveratrol. (B), Epithelia infected under multiple cycles of viral replication were treated with 0.136 mg/mL CMG (□), 300 μM resveratrol (□) or 0.136 mg/mL CMG + 300 μM resveratrol (■). Data represent the mean of two independent experiments; each experiment was conducted in duplicate. The infectious viral particles released from the apical side, collected by washing with 100 μL of MucilAir™ culture medium, were quantified by plaque assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* the respective control, Student's *t* test.

observed in the presence of 300 μM resveratrol as compared to the untreated infected epithelia ($p = 0.0002$), indicating that the antiviral effect of resveratrol is highly increased when HRV-16 replication occurs under multiple cycles as compared to single cycle conditions ($p = 0.033$).

CMG did not produce any effect on HRV-16 multiplication and on ciliary damage induced by the virus. In addition, the antiviral activity of resveratrol toward HRV-16 infection was unaffected by the presence of CMG (Fig. 2B).

3.4. Cytokines and chemokines modulation

Symptoms of rhinovirus infection are due in part to the production of proinflammatory cytokines induced by the virus. To verify the potential anti-inflammatory effect of resveratrol, alone or in combination with CMG, the levels of IL-6, IL-8 and RANTES were evaluated in uninfected or HRV-16 infected nasal epithelia (Fig. 3A–C). Under basal conditions, IL-6, IL-8 and RANTES were already expressed in epithelial cells. Both resveratrol and CMG were found to modulate basal cytokine expression. Resveratrol caused a significant reduction of IL-6 and RANTES levels, whereas it showed an opposite effect on IL-8 production, with a significant increase compared with the untreated control. CMG did not induce proinflammatory effect per se compared to control cells, conversely a significant reduction in the production of IL-6 was observed. Simultaneous treatment of epithelia with both resveratrol and CMG caused a significant reduction in the production of all the cytokines. Notably, IL-6 production was further significantly reduced compared to resveratrol treatment ($p = 0.0077$).

As expected, HRV-16 infection induced a strong increase in the secretion of all cytokines in comparison to uninfected epithelia. In the presence of CMG an even more massive cytokine production was observed. Resveratrol, either alone or in combination with CMG, reversed the increased release of cytokines induced by virus infection. Similar to what observed in uninfected epithelia, the simultaneous presence of both compounds further significantly decreased IL-6 secretion compared to resveratrol alone ($p = 0.00028$), and to uninfected control epithelia ($p = 0.00049$). Although not significant, a similar trend of reduction was observed for the other cytokines in infected epithelia treated with resveratrol and CMG compared to uninfected controls.

In order to verify whether the reduction of cytokine production induced by resveratrol in HRV-16 infected epithelia correlated with the inhibition of virus replication, experiments were carried out to compare the effect of increasing amounts of resveratrol both on virus and cytokine production. Results obtained (Fig. 3D) demonstrated that the reduction of RANTES production reflects the inhibition of HRV-16 replication induced by resveratrol. Similar results were observed for IL-6 and IL-8 (data not shown).

Evaluation of IL-10 and IFN-β showed no production of these cytokines in uninfected or infected nasal epithelia, both in the presence or in the absence of resveratrol and/or CMG (data not shown).

3.5. Modulation of ICAM-1 and viral protein VP2 levels

As above mentioned, HRV increases cell surface expression of its own receptor ICAM-1. Since resveratrol was able to inhibit HRV-16 replication as well as the production of pro-inflammatory cytokines, we next investigated whether the polyphenol would alter the expression of ICAM-1. To this end, human nasal epithelia, uninfected or infected with HRV-16, were treated with CMG and/or resveratrol and cell lysates analyzed by Western blot. As shown in Fig. 4 A, a 40 h treatment of the uninfected epithelia with 300 μM resveratrol increased the signal for ICAM-1 that appeared as a broader and somewhat faster migrating form. In the untreated infected epithelia, ICAM-1 level was higher than that observed in the absence of the virus, indicating a stimulatory effect of HRV-16 on its receptor. However, exposure of the infected epithelia to increasing concentrations of resveratrol determined a progressive decrement of ICAM-1 levels. Notably, at 300 μM resveratrol the signal for ICAM-1 was slightly lower in molecular weight than that detected in the untreated control, similarly to what was observed in the uninfected cells treated with the same resveratrol concentration. Both in uninfected or infected epithelia, CMG did not affect ICAM1 levels while resveratrol/CMG appeared to reduce the intensity and broadening of ICAM-1 signal induced by resveratrol alone. Blots were then analyzed for the expression of the viral antigen

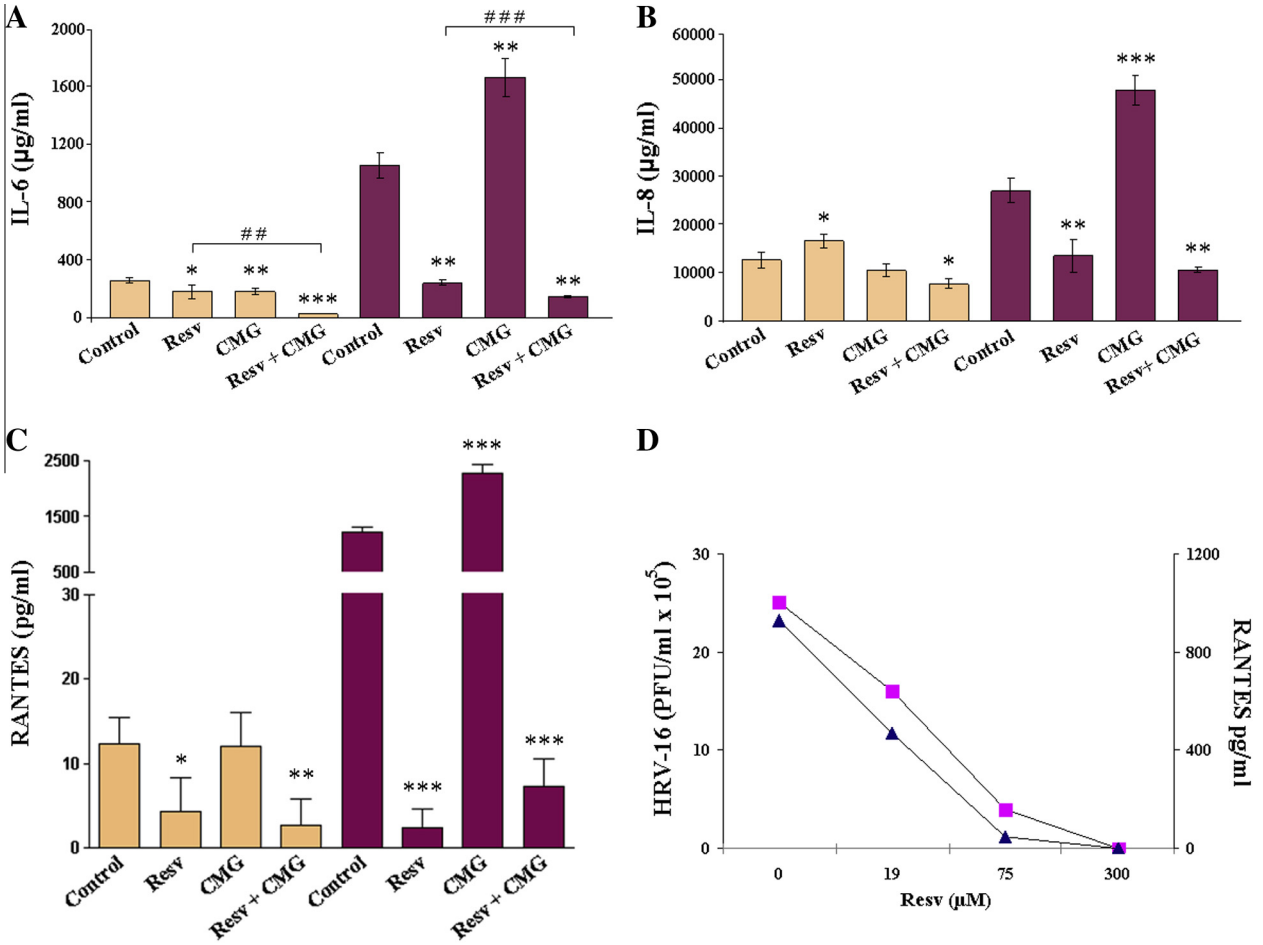

Fig. 3. Cytokines and chemokines secretion following HRV-16 infection of human nasal epithelia in the presence or in the absence of resveratrol and/or CMG. IL-6 (A), IL-8 (B), and RANTES (C) concentrations were evaluated by ELISA assay in the culture medium of human nasal epithelia uninfected (□) or HRV-16 infected (■) (0.3 MOI, 40 h) and treated or not with 300 µM resveratrol, 0.136 mg/mL CMG or a combination of both compounds. (D) HRV-16 replication (■) and RANTES production (▲) as a function of increasing concentrations of resveratrol. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* the respective control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, Student's *t* test.

VP2 the last viral capsid protein derived from the proteolytic cleavage of the precursor protein VP0 (VP4 + VP2). As shown in the lower panel of Fig. 4A, treatment of the infected epithelia with resveratrol, even at the lowest concentration, dramatically reduced VP2 as well as VP0 levels indicating a high efficacy of the polyphenol on HRV-16 antigen expression.

In order to confirm the modulatory effects exerted by resveratrol on ICAM-1 levels, we carried out immunofluorescence experiments on H1HeLa cells. Fig. 4B shows that treatment of the uninfected cells with the polyphenol did not considerably alter the fluorescent signal corresponding to ICAM-1 as compared to that detected in the untreated ones. However, a dramatic increment of the protein, decorating the surface and the cytoplasm of H1HeLa cells, was observed upon infection with HRV-16. Interestingly, the fluorescent signal was barely detectable when the infected cells were exposed to resveratrol, this result further confirming the inhibitory effect of the polyphenol on HRV-16-induced ICAM-1 expression.

4. Discussion

The antiviral activity of resveratrol has been investigated by several groups, and these studies identified broad-spectrum antiviral effects of resveratrol that include inhibition of DNA and RNA viruses (Campagna and Rivas, 2010). However, the effects of resveratrol on HRV, the most common cause of respiratory illness, have not been investigated. Our results indicate that resveratrol exerts a high, dose-dependent, antiviral activity against HRV-16 replication, both in cell monolayers and in *ex vivo* nasal epithelia. The amount of infective viral particles released from infected cells resulted significantly reduced in the presence of non-cytotoxic concentrations of resveratrol.

The inhibition of virus multiplication in the presence of resveratrol was not attributed to direct inactivation or inhibition of virus attachment to the host cells since treatment of HRV-16 with the compound or treatment of cells before or during virus adsorption did not influence infection. Indeed, the strong reduction of the viral capsid protein VP2 observed in cells treated with resveratrol concentrations that hardly reduced the viral titer, leads to hypothesize that, at least in part, the antiviral effect of the polyphenol on HRV-16 is exerted on viral protein synthesis.

The activation of inflammatory response in infected nasal epithelia, which are the target host cells for HRV replication in humans, was reversed in the presence of resveratrol. Our results demonstrated that resveratrol reduced HRV-16-induced secretion of inflammatory mediators to levels similar to that of uninfected epithelia (IL-6 and IL-8) or even significantly lower (RANTES). Similar effects were observed for the combination resveratrol/CMG but in this case also the level of IL-6 was found to be significantly lower in comparison to that of uninfected epithelia. These results are of particular interest since the

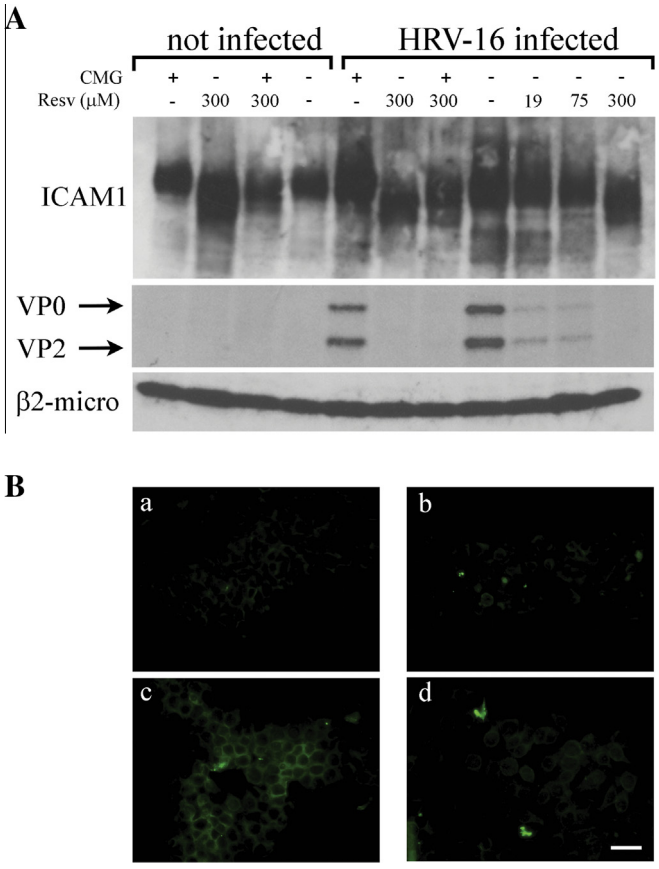

Fig. 4. Modulation of ICAM-1 and HRV-16 VP2 protein levels by resveratrol. (A) Cell extracts, obtained from human nasal epithelia uninfected or infected with HRV-16 (MOI 0.3, 40 h) in the absence or in the presence of CMG and/or different concentrations of resveratrol, were analysed by Western blot with ICAM-1 and VP2 antibodies. β2-microglobulin was used as a loading control. The data are representative of those obtained in three independent experiments with similar results. (B) Mock or HRV-16 infected H1HeLa cells in the presence or in the absence of 50 μM resveratrol, were analyzed with ICAM-1 antibody by indirect immunofluorescence: (a) control, (b) treated with resveratrol, (c) infected with HRV-16 (1 MOI), (d) infected with HRV-16 in the presence of resveratrol. Bar length corresponds to 40 μm.

production of HRV infection-induced pro-inflammatory cytokines and chemokines may be involved in the pathogenesis of rhinovirus infection. The mechanisms by which rhinovirus infection influences asthma are not fully established, but current evidence indicates that the immune response to this virus is critical in this process. Indeed, a variety of mediators, including RANTES, IL-6 and IL-8, are involved in airway inflammation in exacerbations of COPD and bronchial asthma caused by HRV infections (Pacifico et al., 2000; Seemungal et al., 2000).

Notably, CMG produced a significant increase in the expression levels of all the inflammatory mediators in infected epithelia, suggesting a synergistic effect on virus-induced inflammation.

Basal levels of IL-6 and RANTES were also significantly reduced by resveratrol in uninfected epithelia confirming an anti-inflammatory effect of the compound. The combination resveratrol/CMG exerted a further reduction in the IL-6 level in comparison to that showed by resveratrol alone.

Our results indicate that resveratrol inhibits HRV-16 induced expression of ICAM-1 in cell monolayers and in nasal epithelia. Moreover, resveratrol at the highest concentration increased ICAM-1 levels in uninfected epithelia, while both in the uninfected as well as in the infected ones, caused broadening of the signal detected on immunoblots with faster migrating components. It has been reported that membrane-bound and soluble ICAM-1 isoforms arise from alternative splicing in the same and in different cell types; moreover, ICAM-1 isoforms might be differentially glycosylated and N-glycosylation of the molecule has been shown to affect human rhinovirus binding (Bella et al., 1999; Jimenez et al., 2005). From our results, seems likely that resveratrol is able to reduce ICAM-I glycosylation. Because resveratrol at the highest concentration causes a clear reduction of ICAM-1 molecular weight and greatly affects HRV replication, it is conceivable that a lower degree of glycosylation might reduce cell viral binding. This effect might explain the higher inhibition of HRV-16 under multiple cycles replication condition in comparison to single cycle replication exerted by similar concentrations of resveratrol (Fig. 2A). Indeed in multiple cycles the presence of an altered receptor induced by resveratrol could hinder cell infection by virions produced during the first round of multiplication.

Interestingly, CMG was found to modulate resveratrol effect on ICAM-1 expression by reducing the band shift of this protein, which results to be less evident for the combination resveratrol + CMG compared to resveratrol alone. These data further support the evidence of a biological interaction between the two compounds as already observed in the modulation of IL-6 production both in infected and in uninfected epithelia.

The underlying molecular and cellular mechanisms of resveratrol antiviral activity towards rhinovirus are still to be elucidated. However, it can be hypothesized that a central role is played by the transcription factor NF-kB. Indeed, it is well known that resveratrol is able to modulate NF-kB, which is also known to be involved in rhinovirus infection and in cytokines production (Campagna and Rivas, 2010; Majde, 2000; Papi and Johnston, 1999; Rudd et al., 2006).

In conclusion, the present study provides evidence for an inhibitory effect of resveratrol on the production of several HRV-induced inflammatory mediators in nasal epithelia likely depending on the ability of resveratrol to suppress viral replication. Our results support the clinical observation that a spray containing resveratrol plus CMG is capable of relieving nasal symptoms and respiratory infections sequelae in children with allergic rhinitis (Miraglia Del Giudice et al., 2014b).

The possibility that an inhibitor can be used to affect viral replication and cytokine production represents an ideal therapeutic approach. Such therapy, particularly if topical, could have obvious implications for the treatment of rhinovirus infections and the prevention of complications.

Potential conflicts of interest

A.F. and L.M. serve as consultants to NOOS srl. R.R. is an employee of Noos srl. L.M. is coinventor of the relevant patent ≪Association of resveratrol and carboxymethyl glucan≫, EP2674155A1.

All other authors have no potential conflicts.

Acknowledgments

This work was supported by MIUR (Ministero Istruzione Università Ricerca, Italy); and NOOS srl.

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.2015.08.010.

References

Bartlett, N.W., Walton, R.P., Edwards, M.R., Aniscenko, J., Caramori, G., Zhu, J., Glanville, N., Choy, K.J., Jourdan, P., Burnet, J., Tuthill, T.J., Pedrick, M.S., Hurle, M. J., Plumpton, C., Sharp, N.A., Bussell, J.N., Swallow, D.M., Schwarze, J., Guy, B., Almond, J.W., Jeffery, P.K., Lloyd, C.M., Papi, A., Killington, R.A., Rowlands, D.J., Blair, E.D., Clarke, N.J., Johnston, S.L., 2008. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. Nat. Med. 14, 199–204.

Beach, L.B., Rawson, J.M., Kim, B., Patterson, S.E., Mansky, L.M., 2014. Novel inhibitors of human immunodeficiency virus type 2 infectivity. J. Gen. Virol. 95, 2778–2783.

Bedard, K.M., Semler, B.L., 2004. Regulation of picornavirus gene expression. Microbes Infect. 6, 702–713.

Bella, J., Kolatkar, P.R., Marlor, C.W., Greve, J.M., Rossmann, M.G., 1999. The structure of the two amino-terminal domains of human intercellular adhesion molecule-1 suggests how it functions as a rhinovirus receptor. Virus Res. 62, 107–117.

Busse, W.W., Lemanske Jr., R.F., Gern, J.E., 2010. Role of viral respiratory infections in asthma and asthma exacerbations. Lancet 376, 826–834.

Campagna, M., Rivas, C., 2010. Antiviral activity of resveratrol. Biochem. Soc. Trans. 38, 50–53.

Chan, M.M., 2002. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. Biochem. Pharmacol. 63, 99–104.

Chen, X., Qiao, H., Liu, T., Yang, Z., Xu, L., Xu, Y., Ge, H.M., Tan, R.X., Li, E., 2012. Inhibition of herpes simplex virus infection by oligomeric stilbenoids through ROS generation. Antiviral Res. 95, 30–36.

De Leo, A., Arena, G., Lacanna, E., Oliviero, G., Colavita, F., Mattia, E., 2012. Resveratrol inhibits Epstein Barr Virus lytic cycle in Burkitt's lymphoma cells by affecting multiple molecular targets. Antiviral Res. 96, 196–202.

Docherty, J.J., Fu, M.M., Hah, J.M., Sweet, T.J., Faith, S.A., Booth, T., 2005. Effect of resveratrol on herpes simplex virus vaginal infection in the mouse. Antiviral Res. 67, 155–162.

Docherty, J.J., Sweet, T.J., Bailey, E., Faith, S.A., Booth, T., 2006. Resveratrol inhibition of varicella-zoster virus replication in vitro. Antiviral Res. 72, 171–177.

Evers, D.L., Wang, X., Huong, S.M., Huang, D.Y., Huang, E.S., 2004. 3,4′,5-Trihydroxy-trans-stilbene (resveratrol) inhibits human cytomegalovirus replication and virus-induced cellular signaling. Antiviral Res. 63, 85–95.

Faith, S.A., Sweet, T.J., Bailey, E., Booth, T., Docherty, J.J., 2006. Resveratrol suppresses nuclear factor-kappaB in herpes simplex virus infected cells. Antiviral Res. 72, 242–251.

Feil, S.C., Hamilton, S., Krippner, G.Y., Lin, B., Luttick, A., McConnell, D.B., Nearn, R., Parker, M.W., Ryan, J., Stanislawski, P.C., Tucker, S.P., Watson, K.G., Morton, C.J., 2012. An orally available 3-ethoxybenzisoxazole capsid binder with clinical activity against human rhinovirus. ACS Med. Chem. Lett. 3, 303–307.

Fendrick, A.M., Monto, A.S., Nightengale, B., Sarnes, M., 2003. The economic burden of non-influenza-related viral respiratory tract infection in the United States. Arch. Intern. Med. 163, 487–494.

Francioso, A., Mastromarino, P., Masci, A., d'Erme, M., Mosca, L., 2014a. Chemistry, stability and bioavailability of resveratrol. Med. Chem. 10, 237–245.

Francioso, A., Mastromarino, P., Restignoli, R., Boffi, A., d'Erme, M., Mosca, L., 2014b. Improved stability of trans-resveratrol in aqueous solutions by carboxymethylated (1,3/1,6)-beta-D-glucan. J. Agric. Food Chem. 62, 1520–1525.

Fremont, L., 2000. Biological effects of resveratrol. Life Sci. 66, 663–673.

Gern, J.E., Martin, M.S., Anklam, K.A., Shen, K., Roberg, K.A., Carlson-Dakes, K.T., Adler, K., Gilbertson-White, S., Hamilton, R., Shult, P.A., Kirk, C.J., Da Silva, D.F., Sund, S.A., Kosorok, M.R., Lemanske Jr., R.F., 2002. Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. Pediatr. Allergy Immunol. 13, 386–393.

Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E., McClelland, A., 1989. The major human rhinovirus receptor is ICAM-1. Cell 56, 839–847.

Gunawardana, N., Finney, L., Johnston, S.L., Mallia, P., 2014. Experimental rhinovirus infection in COPD: implications for antiviral therapies. Antiviral Res. 102, 95–105.

Henquell, C., Mirand, A., Deusebis, A.L., Regagnon, C., Archimbaud, C., Chambon, M., Bailly, J.L., Gourdon, F., Hermet, E., Dauphin, J.B., Labbe, A., Peigue-Lafeuille, H., 2012. Prospective genotyping of human rhinoviruses in children and adults during the winter of 2009–2010. J. Clin. Virol. 53, 280–284.

Holgate, S.T., 2011. The sentinel role of the airway epithelium in asthma pathogenesis. Immunol. Rev. 242, 205–219.

Holgate, S.T., 2013. Mechanisms of asthma and implications for its prevention and treatment: a personal journey. Allergy Asthma Immunol. Res. 5, 343–347.

Jimenez, D., Roda-Navarro, P., Springer, T.A., Casasnovas, J.M., 2005. Contribution of N-linked glycans to the conformation and function of intercellular adhesion molecules (ICAMs). J. Biol. Chem. 280, 5854–5861.

Lieberman, D., Shimoni, A., Shemer-Avni, Y., Keren-Naos, A., Shtainberg, R., Lieberman, D., 2010. Respiratory viruses in adults with community-acquired pneumonia. Chest 138, 811–816.

Lin, C.J., Lin, H.J., Chen, T.H., Hsu, Y.A., Liu, C.S., Hwang, G.Y., Wan, L., 2015. Polygonum cuspidatum and its active components inhibit replication of the influenza virus through toll-like receptor 9-induced interferon beta expression. PLoS One 10, e0117602.

Majde, J.A., 2000. Viral double-stranded RNA, cytokines, and the flu. J. Interferon Cytokine Res. 20, 259–272.

Makela, M.J., Puhakka, T., Ruuskanen, O., Leinonen, M., Saikku, P., Kimpimaki, M., Blomqvist, S., Hyypia, T., Arstila, P., 1998. Viruses and bacteria in the etiology of the common cold. J. Clin. Microbiol. 36, 539–542.

Message, S.D., Laza-Stanca, V., Mallia, P., Parker, H.L., Zhu, J., Kebadze, T., Contoli, M., Sanderson, G., Kon, O.M., Papi, A., Jeffery, P.K., Stanciu, L.A., Johnston, S.L., 2008. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. Proc. Natl. Acad. Sci. U.S. A. 105, 13562–13567.

Miraglia Del Giudice, M., Maiello, N., Capristo, C., Alterio, E., Capasso, M., Perrone, L., Ciprandi, G., 2014a. Resveratrol plus carboxymethyl-beta-glucan reduces nasal symptoms in children with pollen-induced allergic rhinitis. Curr. Med. Res. Opin. 30, 1931–1935.

Miraglia Del Giudice, M., Maiello, N., Decimo, F., Capasso, M., Campana, G., Leonardi, S., Ciprandi, G., 2014b. Resveratrol plus carboxymethyl-beta-glucan may affect respiratory infections in children with allergic rhinitis. Pediatr. Allergy Immunol. 25, 724–728.

Pacifico, L., Iacobini, M., Viola, F., Werner, B., Mancuso, G., Chiesa, C., 2000. Chemokine concentrations in nasal washings of infants with rhinovirus illnesses. Clin. Infect. Dis. 31, 834–838.

Palamara, A.T., Nencioni, L., Aquilano, K., De Chiara, G., Hernandez, L., Cozzolino, F., Ciriolo, M.R., Garaci, E., 2005. Inhibition of influenza A virus replication by resveratrol. J. Infect. Dis. 191, 1719–1729.

Papi, A., Bellettato, C.M., Braccioni, F., Romagnoli, M., Casolari, P., Caramori, G., Fabbri, L.M., Johnston, S.L., 2006. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. Am. J. Respir. Crit. Care Med. 173, 1114–1121.

Papi, A., Contoli, M., 2011. Rhinovirus vaccination: the case against. Eur. Respir. J. 37, 5–7.

Papi, A., Johnston, S.L., 1999. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappaB-mediated transcription. J. Biol. Chem. 274, 9707–9720.

Pervaiz, S., 2003. Resveratrol: from grapevines to mammalian biology. FASEB J. 17, 1975–1985.

Rollinger, J.M., Schmidtke, M., 2011. The human rhinovirus: human-pathological impact, mechanisms of antirhinoviral agents, and strategies for their discovery. Med. Res. Rev. 31, 42–92.

Rudd, B.D., Smit, J.J., Flavell, R.A., Alexopoulou, L., Schaller, M.A., Gruber, A., Berlin, A. A., Lukacs, N.W., 2006. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. J. Immunol. 176, 1937–1942.

Ruuskanen, O., Waris, M., Ramilo, O., 2013. New aspects on human rhinovirus infections. Pediatr. Infect. Dis. J. 32, 553–555.

Sanders, T.H., McMichael Jr., R.W., Hendrix, K.W., 2000. Occurrence of resveratrol in edible peanuts. J. Agric. Food Chem. 48, 1243–1246.

Seemungal, T.A., Harper-Owen, R., Bhowmik, A., Jeffries, D.J., Wedzicha, J.A., 2000. Detection of rhinovirus in induced sputum at exacerbation of chronic obstructive pulmonary disease. Eur. Respir. J. 16, 677–683.

Subauste, M.C., Jacoby, D.B., Richards, S.M., Proud, D., 1995. Infection of a human respiratory epithelial cell line with rhinovirus. Induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. J. Clin. Invest. 96, 549–557.

Uncapher, C.R., DeWitt, C.M., Colonno, R.J., 1991. The major and minor group receptor families contain all but one human rhinovirus serotype. Virology 180, 814–817.

Varricchio, A.M., Capasso, M., Della Volpe, A., Malafronte, L., Mansi, N., Varricchio, A., Ciprandi, G., 2014. Resveratrol plus carboxymethyl-beta-glucan in children with recurrent respiratory infections: a preliminary and real-life experience. Ital. J. Pediatr. 40, 93.

Xie, X.H., Zang, N., Li, S.M., Wang, L.J., Deng, Y., He, Y., Yang, X.Q., Liu, E.M., 2012. Resveratrol Inhibits respiratory syncytial virus-induced IL-6 production, decreases viral replication, and downregulates TRIF expression in airway epithelial cells. Inflammation 35, 1392–1401.

Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M.L., Gwaltney Jr., J., Elias, J. A., 1996. Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activation. J. Clin. Invest. 97, 421–430.