Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 63 (2004) 85–95
Antiviral Research
www.elsevier.com/locate/antiviral

3,4′,5-Trihydroxy-*trans*-stilbene (resveratrol) inhibits human cytomegalovirus replication and virus-induced cellular signaling

David L. Evers [a], Xin Wang [a], Shu-Mei Huong [a], David Y. Huang [b], Eng-Shang Huang [a,c,d,e,*]

[a] *Lineberger Comprehensive Cancer Center, Rm 32-026, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295, USA*
[b] *Department of Neurology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7025, USA*
[c] *Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295, USA*
[d] *Curriculum of Genetics and Molecular Biology, Rm 32-026, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295, USA*
[e] *Department of Microbiology and Immunology, Rm 32-026, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295, USA*

Received 31 December 2003; accepted 15 March 2004

Abstract

Resveratrol is a polyphenolic natural product that is present in red wine and peanuts and has inhibitory activity against inflammation, heart disease, and cancer. Here we describe its inhibition of human cytomegalovirus replication ($IC_{50} = 1–2\ \mu M$). At least 50-fold higher concentrations of compound were required to produce cytotoxicity against growing or stationary human embryonic lung fibroblasts. Mechanism of action studies determined that resveratrol blocked virus-induced activation of the epidermal growth factor receptor (EGFR) and phosphatidylinositol-3-kinase signal transduction as well as NF-κB and Sp1 transcription factor activation shortly following infection. Resveratrol prevented the appearance of immediate-early, early, and late viral proteins. Human cytomegalovirus DNA replication was reduced to undetectable levels by treatment with resveratrol, as were the second (late) phases of virus-induced phosphatidylinositol-3-kinase signaling and transcription factor activation. Resveratrol lost substantial antiviral activity when its addition was delayed until 4 h postinfection. Compound reversibility and preincubation studies were inconsistent with a virucidal mechanism of action. These data indicated that this compound likely operated during attachment and entry. We hypothesize that the primary molecular target for resveratrol may be blockage of epidermal growth factor receptor activation and its downstream effectors.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Resveratrol; Epidermal growth factor receptor; Cytomegalovirus

1. Introduction

At least 60% of the population is seropositive for human cytomegalovirus (HCMV; Huang and Kowalik, 1993). While it is possible that the ubiquitous prevalence of HCMV prevents epidemiological correlations with diseased states, the morbidities and mortalities caused by this β-herpesvirus are normally limited to immunocompromised individuals. HCMV is the number one viral cause of birth defects, often causing mental retardation and deafness (Plotkin, 1999). This virus is a common opportunistic pathogen in transplant recipients and AIDS patients, and is associated with allograft rejection (Balfour, 1979). Additionally, HCMV is an important factor in the etiologies of atherosclerosis and coronary artery restenosis (Speir et al., 1994), and inflammatory bowel diseases (Berk et al., 1985).

The most widely used clinical therapies for the prophylaxis and treatment of HCMV infections are viral DNA polymerase inhibitors: foscarnet, cidofovir, and ganciclovir (and its valine ester pro-drug valganciclovir) (Griffiths, 2002). These virostatic drugs block viral DNA synthesis and subsequently the late stages of HCMV replication, however, they do not prevent viral induction of multiple signal transduction events (Huang, 1975b; Mar et al., 1983, 1985). Additional limitations include the clinical prevalence of drug-resistant and cross-resistant strains of HCMV (Baldanti and Gerna, 2003), as well as dose limiting renal (foscarnet and cidofovir) and bone marrow (ganciclovir) toxicities (Griffiths, 2002). HCMV hyperimmune globulin (pooled human antisera to HCMV) and phosphorothioate oligonucleotide fomivirsen are also employed as anti-HMCV agents.

We have screened numerous compounds in our efforts to discover and validate novel treatments for HCMV infections. Minimal antiviral activity against HCMV for

* Corresponding author. Tel.: +1 919 966 4323; fax: +1 919 966 4303.
E-mail address: eshuang@med.unc.edu (E.-S. Huang).

0166-3542/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2004.03.002

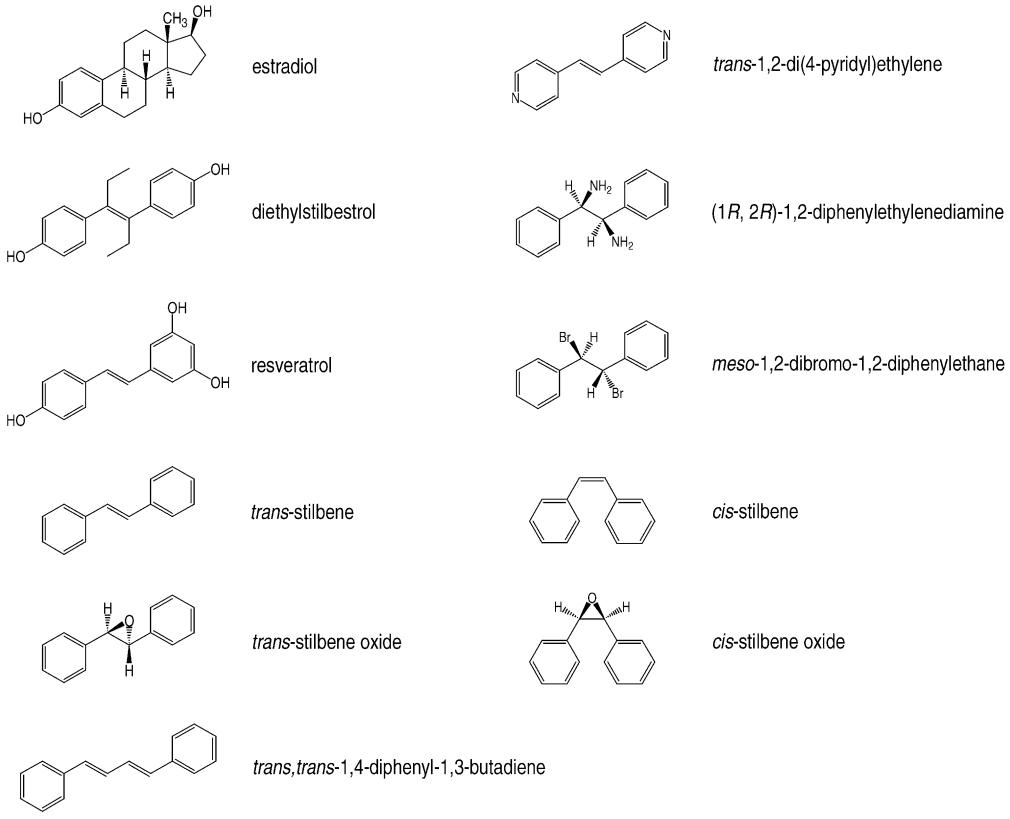

Fig. 1. The structures of compounds evaluated in this study.

estradiol and the synthetic estrogen mimetic diethylstilbestrol motivated us to examine the anti-HCMV properties of a panel of stilbenes and stilbene-like compounds. Here we report that one such compound, resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; Fig. 1), is a potent and selective inhibitor of HCMV replication. It was recently brought to our attention that credit for this discovery belongs to others (Atreides et al., 2002).

Resveratrol is a polyphenolic natural product that is found in some plants, such as grape skins, plums, and peanuts. It is a phytoalexin; an antibiotic produced by a plant in response to the entry of a disease-producing substance or organism. This compound is proposed to be the source of the “French paradox”; the apparent healthiness of those who consume regular but moderate amounts of red wine, despite a diet rich in fats and cholesterol (St. Leger et al., 1979; Arichi et al., 1982). There is evidence that resveratrol inhibits inflammation, coronary heart disease, and cancer (reviewed in Pervaiz, 2003; Soleas et al., 1997). This phytoalexin appears to inhibit these processes through multiple mechanisms. Resveratrol acts as an antioxidant (Jang et al., 1997), inhibits cyclooxygenases (COX; Subbaramaiah et al., 1998) and protein kinase C (PKC; Stewart et al., 1999), and prevents NF-κB and AP-1 transcription factor induction (Manna et al., 2000), as well as cytokine release (Culpitt et al., 2003). It has been suggested that resveratrol inhibits NF-κB by blocking the activity of IKK, the stress-induced kinase that facilitates the degradation of NF-κB’s homeostatic inhibitor, IκB (Holmes-McNary and Baldwin, 2000). Other effects of resveratrol include increasing high-density lipoprotein cholesterol, and inhibition of platelet aggregation and lipid peroxidation (Frankel et al., 1993). Resveratrol also inhibits mammalian ribonucleotide reductase (Fontecave et al., 1998) and DNA polymerases (Sun et al., 1998). In several types of cancer cells, treatment with resveratrol resulted in cell cycle arrest, induction of the p53 tumor suppressor protein, and apoptosis (Jang et al., 1997; She et al., 2003). Antiviral activity against herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus (HIV-1) has been reported for resveratrol (Docherty et al., 1999, 2004; Heredia et al., 2000). However, it was not a very potent inhibitor of these viruses (see discussion). Nonetheless, resveratrol acted early in the replication cycle of HSV-1 and reduced levels of ICP4, a major immediate-early viral regulatory protein (Docherty et al., 1999).

The molecular targets by which resveratrol probably inhibits inflammation, coronary heart disease, and cancer closely parallel a number of cellular signaling and stress activation events that are apparently required for productive HCMV infection. Antioxidants (Speir et al., 1996), non-steroidal anti-inflammatory drugs that inhibit COX (Tanaka et al., 1988; Speir et al., 1998; Zhu et al., 2002), and inhibitors of PKC (Slobbe-van Drunen et al., 1997)

displayed antiviral activity against HCMV. Pharmacological inhibition of cellular p38 mitogen-activated protein kinase demonstrated selective inhibition of HCMV replication (Johnson et al., 1999). Initial events in the HCMV replication cycle involve entry and/or signal transduction via phosphorylation of the epidermal growth factor receptor (EGFR; Wang et al., 2003) and downstream activation of phosphatidylinositol 3-kinase (PI3-K) and effectors Akt and p70S6K (Johnson et al., 2001). Inhibition of this receptor tyrosine kinase pathway with EGFR kinase inhibitor AG1478 and PI3-K inhibitors wortmannin and LY294002 blocked productive HCMV infection. Resveratrol also inhibited the activation of a variety of stress-induced, pro-inflammatory transcription factors such as NF-κB, Sp1, and AP-1, that are induced in HCMV-infected cells (Boldogh et al., 1990; Kowalik et al., 1993; Yurochko et al., 1995, 1997a,b; Yurochko and Huang, 1999).

2. Materials and methods

2.1. Antiviral compounds, reagents, and antibodies

Estradiol, stilbenes, stilbene-like compounds, and ganciclovir were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were prepared as 10–20 mg/ml stocks in dimethylsulfoxide (DMSO), and stored at –70 °C.

Primary monoclonal antibodies specific for HCMV IE1, IE2, UL84, and UL94 proteins were prepared in our laboratories as described previously (He et al., 1992; Kowalik et al., 1994; Wing et al., 1996). Antibody to β-actin (CP01) was purchased from Oncogene (San Diego, CA). Antibody to EGFR (SC528) was purchased from Santa Cruz (Santa Cruz, CA). Antibody to Akt (9272) and p70S6K (9202), and antibodies specific for Akt phosphorylated at Ser 473 (9275) and p70S6K phosphorylated at Thr 389 (9205) were purchased from Cell Signaling (Beverly, MA). Antiobdy to PI3-K p85α (06-195) was purchased from Upstate Biotech (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated antibody to phosphotyrosine (RC20H) was purchased from BD Biosciences/Pharmingen (Lexington, KY). Appropriate secondary HRP-conjugated antibodies were purchased from Sigma or Oncogene.

2.2. Cell culture and viral infection

Primary human embryonic lung fibroblasts (HEL 299) were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All experiments were performed using cells that were between passages 13–21. Stocks of the Towne strain of HCMV (passages 36–42; obtained from Dr. S. Plotkin) were prepared as described previously, and purified through a sucrose cushion to eliminate cytokines and growth factor contamination (Huang et al., 1973). The RC256 variant of the HCMV AD169 strain (Spaete and Mocarski, 1987) was purchased from ATCC and the supernatant from previously infected cells was used to directly infect cell cultures. In all experiments, drugs were present from the time they were added until cells were harvested.

2.3. Titer reduction assay

To determine the effects of compounds upon viral pathology, confluent HEL fibroblasts in 24-well plates were incubated at 37 °C in an atmosphere of 6% CO_2 for 1 h in the presence or absence of various concentrations of drugs in DMEM. Without changing media, cells were infected with an aliquot of concentrated HCMV (Towne strain) at approximately 2–5 PFU/cell. Six days postinfection, 10 μl of the supernatant from each well was transferred to the corresponding wells of a new 24-well plate. Alternatively, eight 1:3 dilutions of supernatant were performed in 96-well plates and data were plotted on a log scale. Following a 1 h adsorption, the inoculant was removed, and a 1% methylcellulose (methocell) overlay containing DMEM with heat-inactivated 4% FBS was added. Seven days postinfection, plaques were scored by inverted light microscopy (Andreoni et al., 2002). Data are presented as the percentage of plaques in the wells lacking drug (positive controls). In these experiments, some 150–200 plaques were present in each positive control well.

2.4. Cytotoxicity assays

To determine drug cytotoxicity in static HEL monolayers ("cell viability"), cells were grown to confluence in 96-well plates and serum-starved for 48 h prior to the addition of various concentrations of compounds, as described for titer reduction assays, except that virus innoculum and methocell were omitted. Cytotoxicity against growing HEL monolayers ("cell growth") was determined similarly, except that cells were not serum-starved and were approximately 10% confluent when drug was added. Seventy-two hours posttreatment with selected drug concentrations, viable cell number was assessed with a tetrazolium colorimetric dye assay kit (G1780, Promega; Madison, WI), according to the manufacturer's instructions. At each concentration of drug, the percentage of absorbance at 470 nm was determined as compared to control wells where an equivalent amount of DMSO had been added. Data were plotted comparing percent inhibition to drug concentration, and linearly regressed to arrive at 50% inhibitory concentration values (IC_{50}). IC_{50} are presented as the mean ± standard deviation of experiments performed in quadruplicate.

2.5. Time of addition study

This assay was modified from a procedure described elsewhere (Hippenmeyer and Dilworth, 1996). To the wells

of 96-well plates containing confluent and serum-starved HEL fibroblasts was added HCMV (RC256; approximately 1.2 PFU/cell). Plates were incubated 37 °C in an atmosphere of 6% CO_2 until 72 h postinfection. At various times postinfection, an equal volume of 2× 20 μM resveratrol was added to wells. Seventy-two hours postinfection, supernatants were removed and cell monolayers were rinsed with phosphate-buffered saline (PBS). To each well was added 50 μl of a lysis solution (10% glycerol, 1% Triton X-100, 2 mM DTT, 2 mM EDTA, and 25 mM Tris pH 7.8) and plates were incubated at 37 °C for 30 min. To each well was then added 50 μl of a freshly prepared and gravity filtered β-galactosidase solution (33 mg ONPG added to 25 ml of a buffer consisting of 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, and 100 mM 2-mercaptoethanol) and plates were incubated at room temperature for 30 min. Reactions were stopped by adding 100 μl/well 1 M Na_2CO_3. Optical densities were determined at 415 nm using a microplate reader. Following the subtraction of absorbance values determined for negative controls (uninfected cells, 16 wells/plate), data were quantified as percentages of the average absorbance determined for positive controls (infected cells, 16 wells/plate). The absorbances for positive controls were between 1.6 and 1.9.

2.6. Western blot analyses

Resveratrol was added to the indicated concentrations in 100 mm dishes of confluent, serum-starved fibroblasts 1 h prior to infection. Cells were infected with HCMV (2–5 PFU/cell) and incubated at 37 °C in an atmosphere of 6% CO_2 until harvesting. Monolayers were harvested at the indicated times by scraping dishes in 2× sodium dodecyl sulfate (SDS) sample loading buffer (Laemmli, 1970). Samples were boiled, loaded onto 8% SDS–polyacrylamide gels, and proteins were separated by polyacrylamide gel electrophoresis (PAGE) then transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). Blots were blocked for 1 h in 5% (w/v) non-fat dry milk in PBS with 0.1% Tween-20 (PBS-T). Blots were probed with primary antibodies overnight at 4 °C. Blots were then washed with PBS-T (4 × 60 ml × 10 min) at room temperature then probed with secondary horse radish peroxidase-conjugated antibodies, washed and developed by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Amersham Biosciences Corp., Piscataway, NJ).

2.7. Cell nuclear extract isolations

Nuclear and cytoplasmic extracts were prepared as described previously (Yurochko et al., 1995, 1997a,b). Briefly, mock-infected and infected HEL fibroblasts (with and without resveratrol) were collected by scraping the cell monolayers of 100 mm dishes in ice-cold PBS and centrifuging. Cells were washed twice with PBS and resuspended and incubated for 10 min on ice in 50 μl of a cytoplasmic isolation buffer, consisting of 10 mM HEPES (pH 7.6), 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 2 mM phenanthroline, 250 μM dichloroisocoumarin, 100 μM E-64, and 10 μM pepstatin A (Sigma). Samples were centrifuged and the cytoplasmic supernatants were removed. The nuclear pellets were washed with cytoplasmic extraction buffer (minus NP-40) and then incubated for 10 min on ice in 25 μl of a nuclear isolation buffer, consisting of 20 mM Tris (pH 8.0), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, the above mentioned protease inhibitors, and 25% v/v glycerol. Samples were centrifuged and the desired supernatant extracts were stored at −70 °C.

2.8. Electrophoretic mobility shift assays (EMSA)

EMSA were performed as described previously (Yurochko et al., 1997a,b). Briefly, equal amounts of nuclear extracts (as determined by colorimetric protein content assays, Bio-Rad, Hercules, CA) were incubated for 10 min at room temperature in binding buffer (10 mM Tris–HCl, 50 mM NaCl, 7.5 mM $MgCl_2$, 0.5 mM EDTA, 10% v/v glycerol, 1 mM DTT) with 2 μg poly(dI-dC). ^{32}P-labeled double-stranded deoxy oligonucleotide probes were added, and incubated for 10 min at room temperature. Samples were then electrophoresed on a 5% polyacrylamide gel, dried, and analyzed by autoradiography. Probes were labeled by annealing complimentary deoxy oligonucleotides and filling in overhanging ends with [α-^{32}P]dATP. A wild-type GC box (5′-CCTTTTTAAGGGGCGGGGCTT-3′) probe was used for the experiments examining Sp1 activity, and a wild-type major histocompatibility complex κB binding site (5′-CCTTTTTTTTTGGGGATTCCCCA-3′) probe was used for experiments examining NF-κB activity (Yurochko et al., 1997a,b).

2.9. Immunoprecipitations

HEL cells in 100 mm dishes were infected or mock-infected with HCMV following 60 min treatment with 20 μM resveratrol (or none) and harvested at various times postinfection as described above. Cytoplasmic extracts were prepared from ice-cold PBS scraped cell monolayers by a 10-min incubation at 0 °C in 0.5 ml of immunoprecipitation (ip) buffer containing 240 mM NaCl, 100 mM NaF, 200 mM Na-orthovanadate, 0.5% NP-40, 50 mM Tris, pH 8.0 with protease inhibitor cocktail (Sigma) in Eppendorf tubes. Equivalent input protein content of samples was determined by colorimetric assays (Bio-Rad). Samples were incubated with 10 μg antibody to EGFR or PI3-K p85 with rocking at 4 °C for 3 h, then 20 μl of a suspension of protein G-sepharose beads (Amersham) was added, and samples were incubated for an additional 8 h at 4 °C with gentle agitation. Samples were centrifuged, the supernatant

was removed, and the pellets were washed four times with 4 °C PBS-T. Beads were boiled in 2× Laemmli buffer and the supernatant was loaded onto polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. The same membranes were analyzed first with an antibody specific for phosphotyrosine then stripped and examined with an antibody specific for total EGFR or PI3-K p85 by Western blotting, as described above.

2.10. HCMV DNA assay (dot blot)

Quantification of HCMV DNA synthesis was performed as described previously (Johnson et al., 1999). HEL cells were grown to confluence in 24-well plates, serum-starved for 48 h, then infected with HCMV (Towne, 2–5 PFU/cell in the presence or absence of various concentrations of resveratrol). At various times postinfection, individual wells were harvested by removing the cell culture medium and replacing it with 0.4 ml of a denaturation buffer consisting of 1.5 M NaCl and 1 M NaOH. After 10 min, 0.4 ml of a neutralization buffer consisting of 1 M NaCl and 1 M Tris–HCl pH 7.0 was added to wells. After 5 min samples were adjusted to 6× SCC and transferred to an Immobilion-NC membrane (Millipore) with a minifold apparatus (Schleicher and Schuell; Keene, NH) and immobilized under vacuum by baking at 80 °C for 2 h. Non-specific binding was blocked by incubation in 5× Denhardt's, 5× SSC, 50% formamide, 1% SDS, with 100 μg/ml salmon sperm DNA at 42 °C for 3 h. The membrane was probed in blocking buffer for 14 h at 42 °C with denatured Towne genomic DNA, that had been labeled with [α-^{32}P]dATP (NEN; Boston, MA) by nick translation according to kit instructions (Invitrogen). Following washes (twice with 2× SSC + 1% SDS; twice with 0.5× SSC + 1% SDS; once with 0.1× SSC + 1% SDS; rinsed with 6× SSC), the membrane was developed by autoradiography.

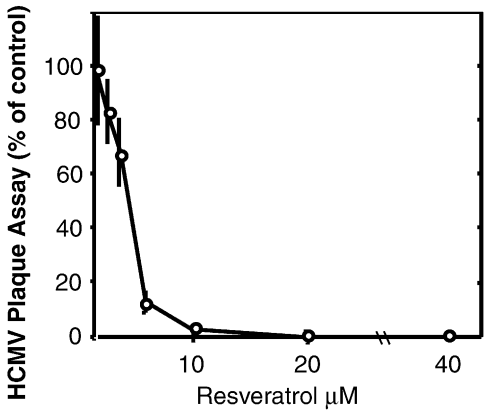

Fig. 2. Dose-dependent antiviral activity of resveratrol as measured by titer reduction assay. Data are presented as the mean ± standard deviation of five independent experiments.

3. Results

3.1. Antiviral activity and cytotoxicity of stilbenes and related compounds

We examined estradiol and the estrogen mimetic diethylstilbestrol for their abilities to inhibit HCMV replication. Neither compound was a particularly good inhibitor (Table 1), however, the data suggested that similar compounds might more potently inhibit HCMV. We then tested a panel of stilbenes and stilbene-like compounds. Resveratrol potently inhibited HCMV replication in a dose-dependent manner (Fig. 2). Titer reduction assays revealed little or no antiviral activity at 50 μM concentrations of eight similar compounds lacking phenolic hydroxyl groups: *trans*-stilbene, *cis*-stilbene, *trans*-stilbene oxide, *cis*-stilbene oxide, *meso*-1,2-dibromo-1,2-diphenylethane, *trans,trans*-1,4-diphenyl-1,3-butadiene, (1*R*,2*R*)-1,2-diphenylethylen-

Table 1
Antiviral activity against HCMV and cellular cytotoxicity of stilbenes and related compounds

Compound	IC_{50}: concentration producing 50% inhibition (μM)			In vitro selectivity index[a]
	Antiviral activity (titer assay)	Cytotoxicity (cell growth)	Cytotoxicity (cell viability)	
Estradiol	>20[b]	nd	>20	nd
Diethylstilbestrol	>30[b]	nd	>30	nd
Resveratrol	1.7 ± 0.2	85 ± 9	>400	≥50
trans-Stilbene	>50	nd	>50	nd
cis-Stilbene	>50	nd	>50	nd
trans-Stilbene oxide	>50	nd	>50	nd
cis-Stilbene oxide	>50	nd	>50	nd
meso-1,2-Dibromo-1,2-diphenylethane	>50	nd	>50	nd
trans,trans-1,4-Diphenyl-1,3-butadiene	>50	nd	>50	nd
(1*R*,2*R*)-1,2-Diphenylethylenediamine	>50	nd	>50	nd
trans-1,2-Di(4-pyridyl)ethylene	>50	nd	>50	nd

nd: not determined.

[a] In vitro selectivity index: cytotoxic IC_{50} divided by antiviral IC_{50}.

[b] Estradiol displayed 25% titer inhibition at 20 μM, diethylstilbestrol displayed 30% titer inhibition at 30 μM.

ediamine, and *trans*-1,2-di(4-pyridyl)ethylene (Table 1). We determined an antiviral IC_{50} of 1.7 μM for resveratrol against the Towne strain (Table 1). Significantly higher concentrations of resveratrol were necessary to produce cellular cytotoxicity in both static and growing HEL cells (Table 1). Resveratrol inhibited HCMV replication with an in vitro selectivity index of at least 50 (Table 1). In order to determine the antiviral mode of action for resveratrol, we chose a drug concentration that unambiguously inhibited viral replication but did not appear to display cytotoxicity in cell culture: 20 μM (4.6 μg/ml) resveratrol.

3.2. Effects of resveratrol upon HCMV proteins

In order to identify the stage(s) of HCMV replication that were specifically inhibited by resveratrol, we determined its ability to block the expression of viral proteins that were representative of immediate-early, early, and late gene expression. Western blot analyses revealed that HCMV IE1-72 and IE2-86 were reduced to undetectable levels at 4 h postinfection by treatment of infected cells with resveratrol (Fig. 3). Resveratrol treatment also reduced HCMV early (UL84; He et al., 1992) and true late (UL94; Wing et al., 1996) proteins to background levels. These results suggested that resveratrol might block early and late gene expression by inhibiting HCMV replication at or prior to the onset of immediate-early gene expression.

3.3. Effect of resveratrol upon HCMV DNA

Since resveratrol appeared to inhibit immediate-early, early, and late HCMV proteins, and HCMV DNA replication occurs directly following the synthesis of viral early proteins (Huang, 1975a,b), we hypothesized that this drug would also block viral DNA replication. We measured viral DNA levels in HCMV-infected cells treated with resveratrol by dot blotting. Fig. 4 shows that HCMV DNA levels were potently inhibited by resveratrol in a dose-dependent manner. Consistent with previously reported results, HCMV DNA levels were minimal prior to 72 h postinfection (Huang, 1975a,b). Positive control viral DNA polymerase inhibitor ganciclovir (GCV; Mar et al., 1983, 1985) produced similar inhibition (Fig. 4, lane 6).

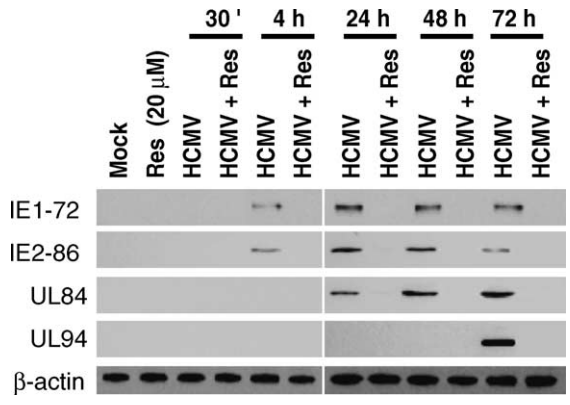

Fig. 3. The effects of resveratrol on HCMV protein levels. HCMV protein levels at various time points in the presence and absence of 20 μM resveratrol were determined by Western blotting. Control β-actin levels indicated that similar levels of protein were loaded in each lane. Uninfected control samples were harvested at the last time point of these experiments.

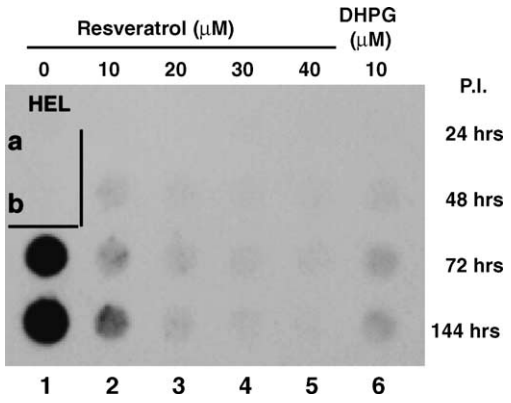

Fig. 4. Resveratrol reduces HCMV DNA levels in a dose-dependent manner. HCMV DNA levels in the presence and absence of 10–40 μM resveratrol were determined by dot (Southern) blotting. Controls were performed for uninfected cells (lane 1, HEL a and b), HCMV-infected cells without drug treatment (lane 1, 72 and 84 h), and HCMV-infected cells treated with 10 μM GCV (lane 6). Uninfected control samples were harvested at the last time point of these experiments.

3.4. Resveratrol acts very early in the HCMV replication cycle

Next we performed a time of addition study—delaying drug treatment to virus-infected cells to various time points. The replication cycle of HCMV is modeled as a sequential cascade of discrete temporal events, each requiring the completion of the previous step (Mocarski and Tan Courcelle, 2001). If antiviral drugs are present at times during and prior to their targeted replicative step, they are active. When drugs are present only at times after their targeted replicative step, they lose antiviral activity (Krueger and Mayer, 1970). Delaying the addition of resveratrol to times as early as 4 h postinfection resulted in approximately 50% less antiviral activity, and this compound was essentially inactive when added at 6 or more hours postinfection (Fig. 5). This indicated that resveratrol probably targeted a viral replication step that occurred prior to 4 h postinfection.

We used two approaches to rule out the possibility that resveratrol directly inactivated virus particles. Upon changing the cell culture media, thus presumably removing drug, at times as late as 72 h postinfection, viral cytopathic effects were restored (data not shown). This was indicative of reversibility. We also pre-treated our virus stock with resveratrol for 1 h prior to infecting cells, and compared those viral titers to that from cells that had been pre-treated with a similar aliquot of resveratrol and infected with virus stock. Fig. 6 demonstrates that pre-treatment of cells for 1 h with an

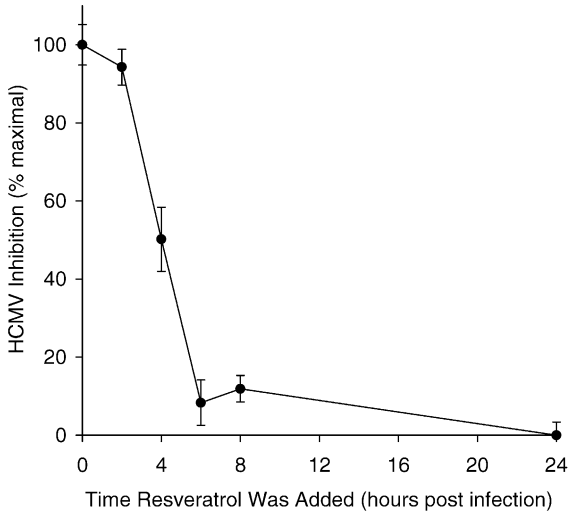

Fig. 5. Time of addition study. Cells were infected with RC256, a transgenic variant of HCMV expressing β-galactosidase, and treated with 20 μM resveratrol at various times postinfection. Drug was present from the indicated time points until harvested at 72 h postinfection, when β-galactosidase activity was determined. Results are presented as the mean ± standard deviation of four experiments.

effective concentration of 0.4 μM resveratrol had little effect upon viral replication, nor did pre-treatment of virions with an effective concentration of 20 μM resveratrol. In contrast, virus replication was decreased some two to three orders of magnitude in cells treated with 20 μM resveratrol. We employed laboratory ethanol as a virucidal control to demonstrate that pre-treatment of cells with 10 μl ethanol (1:50 dilution) did little to block HCMV replication. In contrast, pre-treatment of virus with 10 μl ethanol reduced HCMV titers to undetectable levels. These results suggested that resveratrol did not directly inactivate infectious viral particles and do not support a virucidal mechanism of action.

3.5. Effects of resveratrol upon HCMV-mediated receptor tyrosine kinase activation

We examined the ability of resveratrol to block HCMV activation of EGFR and PI3-K signal transduction. EGFR was isolated by immunoprecipitation and relative amounts of tyrosine phosphorylated (activated) species under different treatments were determined by Western blotting, as described previously (Wang et al., 2003). Consistent with previously reported results (Wang et al., 2003), HCMV-induced EGFR tyrosine hyperphosphorylation was detected directly following infection. Resveratrol inhibited EGFR autophosphorylation, reducing its activation to background levels (Fig. 7). Longer film exposures determined that resveratrol also reduced the phosphorylation of unstimulated, hypophosphorylated EGFR (data not shown).

To determine the effect(s) of resveratrol on HCMV activation of PI3-K we compared the phosphorylated (activated) forms of PI3-K and downstream kinases Akt and p70S6K, to total protein levels of each kinase, in the presence and absence of drug, at various times postinfection (Fig. 8). Similar to results reported previously (Johnson

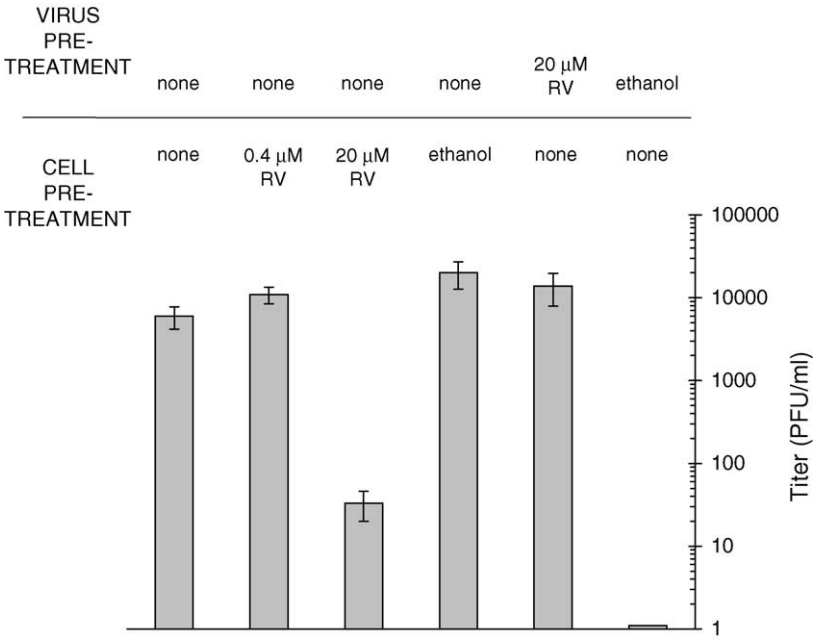

Fig. 6. Virucidal evaluation. Ten microliters of stock Towne virus was incubated with an equal volume of media ("none"), ethanol, or 40 μM resveratrol ("RV," 20 μM diluted concentration). HEL cells in a total volume of 1 ml media were incubated with 10 μl media ("none"), 10 μl ethanol, 10 μl of 40 μM resveratrol (0.4 μM final concentration), or 10 μl of 2 mM resveratrol (20 μM final concentration) in 24-well plates. After 1 h, virus preparations were added to cells (1:50 dilution). After 6 days, viral titers were measured. Results are presented as the mean ± standard deviation of experiments performed in triplicate. Since data were plotted on a log scale, titers below detection limits were assigned a value of 1.05 PFU/ml.

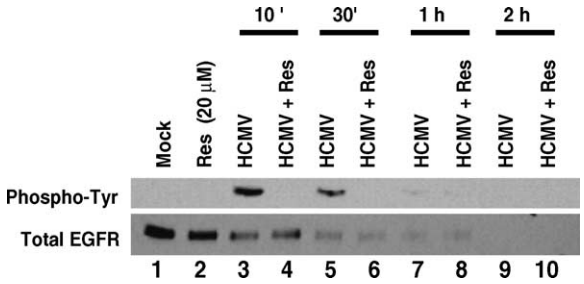

Fig. 7. HCMV-specific EGFR activation in the presence and absence of 20 μM resveratrol. Immunoprecipitation of EGFR was followed by Western blotting for phosphotyrosine. The same membranes were stripped and re-immunoblotted to determine total EGFR levels. Uninfected control samples were harvested at the last time point of these experiments.

et al., 2001), infection with HCMV did not alter the total protein levels of PI3-K, Akt, and p70S6K but resulted in their hyperphosphorylation. Treatment with resveratrol reduced the first (10 min to 4 h postinfection) and second (4–72 h postinfection) phases of PI3-K activation to undetectable/mock-infected levels (Fig. 8).

3.6. *Effects of resveratrol upon HCMV-mediated induction of transcription factors*

Since resveratrol inhibited EGFR activation/autophosphorylation and PI3-K signaling, we suspected that it might also inhibit HCMV-induced transcription factor activation. Transcription factors NF-κB and Sp1 activate the major IE gene promoter (MIEP) of HCMV and are probably necessary for continuation of the viral gene expression cascade (Cherrington and Mocarski, 1989; Yurochko et al., 1995, 1997a). Analysis of the effects of resveratrol upon HCMV-induced transcription factor activation by EMSA revealed that this compound fully inhibited the initial phase of NF-κB and Sp1 activation, reducing the DNA-binding activities of these proteins to uninfected levels (Fig. 9).

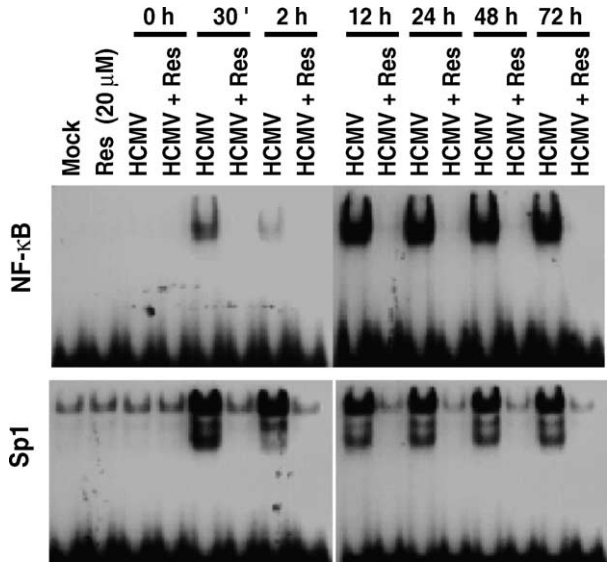

Fig. 9. Effects of resveratrol (20 μM) upon initial and secondary HCMV activation of transcription factors as measured by EMSA. (a) NF-κB binding to an MHC class I probe. (b) Sp1 binding to a GC box probe.

Resveratrol treatment also reduced the second phase of NF-κB and Sp1 induction to background levels. These data indicated that resveratrol inhibited the first and second tiers of HCMV-specific NF-κB and Sp1 induction in a manner similar to its inhibition of the phosphorylation of EGFR, PI3-K p85, Akt, and p70S6K.

4. Discussion

The first aim of this study was to determine whether or not antiviral compound(s) active against HCMV could be found within the stilbene pharmacophore. Our data indicate potent and selective antiviral activity for resveratrol against

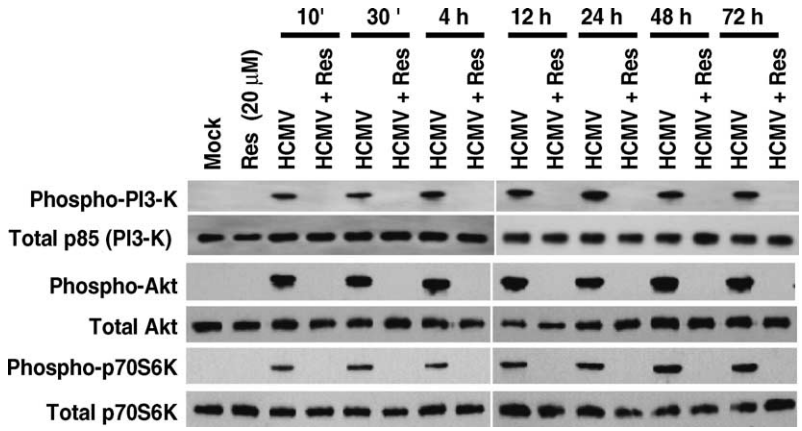

Fig. 8. Effects of resveratrol (20 μM) upon first and second phases of PI3-K activation by HCMV were determined by Western blots of whole cell lysates for total and phosphorylated Akt (Ser 473) and p70S6K (Thr 389); total and phosphorylated PI3-K p85 were determined by immunoprecipitation of p85, followed by Western blotting for phosphotyrosine and total PI3-K p85. Uninfected control samples were harvested at the last time point of these experiments.

HCMV (Table 1). The lack of antiviral activity for other stilbenes and stilbene-like compounds suggests that the phenolic hydroxyl group(s) of resveratrol are important for such activity. This is consistent with a report describing the 4′ hydroxyl of resverastrol derivatives as required for genotoxic activities (Matsuoka et al., 2002). Resveratrol was much more cytotoxic against growing cells than it was against stationary cells. Our cytotoxicity data against stationary cells was consistent with a report of 82% viability for Vero cells at 440 μM (Docherty et al., 1999). Resveratrol was considerably more potent an inhibitor of HCMV than was previously reported against other viruses. However, different experimental conditions in different laboratories provide for less than ideal comparisons. In particular, many laboratories wait to add test compounds until 1 h or more postinfection. Nonetheless, 10 μM resveratrol reduced HIV-1 replication by 20–30% and was only indicated for combination therapies against this retrovirus (Heredia et al., 2000). It required 110 μM resveratrol to reduce HSV-1 replication by 95% and 219 μM resveratrol to reduce HSV-2 replication by 99% (Docherty et al., 1999). However, topical efficacy against HSV for resveratrol was shown in a mouse model of infection (Docherty et al., 2004).

Resveratrol has demonstrated anticancer activity in cell culture and in animals (Jang et al., 1997). In general, anticancer chemotherapeutics tend to make poor antivirals due to their intrinsic poor selectivities and high toxicities (Evers et al., 2002). Resveratrol seems to have remarkable selectivity between cancerous and primary cells (Jang et al., 1997). This compound did not produce detectable toxicity when administered daily to rats at 20 mg/kg, 1000 times the normal therapeutic oral human dose, for 1 month (Juan et al., 2002). This is interesting, considering that the related compound diethylstilbestrol was implicated as a human (and rodent) carcinogen (LaBarthe et al., 1978). This suggests at least some promise for the possible development of resveratrol or proprietary derivatives as anti-HCMV drug(s). We make no claims regarding the potential antiviral benefits of drinking red wine (or any alcoholic beverages) and urge the general public to exercise caution with any such interpretations.

The second aim of this study was to determine how resveratrol inhibited HCMV replication. Previous studies indicated a requirement for EGFR phosphorylation in productive HCMV infection (Wang et al., 2003), as well as subsequent PI3-K activation (Johnson et al., 2001). Resveratrol inhibited HCMV-induced phosphorylation of EGFR, as well as inhibition of HCMV-mediated PI3-K signaling and Sp1 and NF-κB activation. Interestingly, it was reported that resveratrol inhibited tumor necrosis factor (TNF)-mediated activation of NF-κB, but not Sp1, in macrophages (Manna et al., 2000). Western blot analyses indicated that resveratrol blocked the appearance of HCMV immediate-early, early, and late viral proteins. Viral DNA replication was inhibited, as was the second (sustained) phase of PI3-K signal transduction and transcription factor activation. These experiments implicate a mode of antiviral action that is very early in the HCMV replication cycle, acting no later than 4 h postinfection. We have previously shown that inhibition of EGFR kinase activity by AG1478 blocked HCMV entry and replication (Wang et al., 2003). Similarly, we hypothesize that the primary mode of action for resveratrol against HCMV is to block EGFR activation, thus blocking HCMV entry. The data do not prove this hypothesis, and this compound may alternatively (or secondarily) act upon numerous anti-inflammatory (and/or additional) targets that have been previously described for resveratrol (reviewed in Pervaiz, 2003; Soleas et al., 1997).

Resveratrol appears to affect a plethora of molecular targets (Pervaiz, 2003; Soleas et al., 1997). It has been proposed that those “targets” that require concentrations of resveratrol in excess of 100 μM to exhibit effects should be summarily discarded (Soleas et al., 1997). The physiological steady-state plasma concentrations of resveratrol obtained from its various sources may force this constraint, but this limitation conflicts with in vivo activity against HSV (Docherty et al., 2004). Resveratrol inhibited purified PKC with an IC_{50} of 90 μM (Stewart et al., 1999) but 15 μM was sufficient to reduce phorbol ester-induced PKC activity by 80% in cancer cells (Subbaramaiah et al., 1998). Similarly, 8–10 μM resveratrol inhibited tumor cell DNA synthesis by 50%, but 100 μM was required to inhibit ribonucleotide reductase activity by 50% (Fontecave et al., 1998) and 10 μM resveratrol produced only a small minority of abortive transcripts from mammalian replicative DNA polymerases (Sun et al., 1998). While these experiments were performed in different laboratories under different conditions, they do suggest a possible trend. In general, resveratrol is a poorer inhibitor of the activity of its putative targets than of their activation. Others determined that EGFR was required for mediating phorbol ester-induced signal transduction and tumor promotion (Chen et al., 2001). This indicates the possibility that resveratrol may exhibit its biological effects primarily via modulation of cellular signal transduction, perhaps directed at protein tyrosine kinase activities, and, in some cases, specific to EGFR.

There is literature precedent for inhibitors of cellular stress or signaling pathways displaying antiviral activity against HCMV and other viruses (Albrecht et al., 1990; Johnson et al., 1999, 2001). Apparently a number of inflammatory/excitatory cellular responses to HCMV infection that a casual observer might assume to be intrinsically antiviral, are beneficial if not required for productive HCMV replication (Slobbe-van Drunen et al., 1997). The inhibition of cellular inflammatory stress and signal transduction mediators (Albrecht et al., 1990) remains an under-utilized strategy for antiviral drug development.

Acknowledgements

We thank Haobin Cai, Chih-Fang Chao, and Zhigang Zhang for helpful comments and useful advice. This study

was funded by AI47678 and CA19014 grants from the National Institutes of Health, USA (to E.-S. Huang). D.L. Evers was supported by NIH Training Grant CA09156.

References

Albrecht, T., Boldogh, I., Fons, M., AbuBakar, S., Deng, C.Z., 1990. Cell activation signals and the pathogenesis of human cytomegalovirus. Intervirology 3, 68–75.

Andreoni, K.A., Wang, X., Huong, S.M., Huang, E.-S., 2002. Human cytomegalovirus hyperimmune globulin not only neutralizes HCMV infectivity, but also inhibits HCMV-induced intracellular NF-κB, Sp1, and PI3-K signaling pathways. J. Med. Virol. 67, 33–40.

Arichi, H., Kimura, Y., Okuda, H., Baba, K., Kozawa, M., Arichi, S., 1982. Effects of stilbene components of the roots of *Polygonum cuspidatum Sieb. et Zucc.* on lipid metabolism. Chem. Pharm. Bull. 30, 1766–1770.

Atreides, S.-P.A., Wilkins, C., Ekworomadu, C.O., Docherty, J.J., Dix, R.D., 2002. Does resveratrol exhibit antiviral properties against human cytomegalovirus replication? Invest. Ophthalmol. Vis. Sci. 43, E-Abstract 4325.

Baldanti, F., Gerna, G., 2003. Human cytomegalovirus resistance to antiviral drugs: diagnosis, monitoring and clinical impact. J. Antimicrob. Chemother. 52, 324–330.

Balfour, H.H., 1979. Cytomegalovirus: the troll of transplantation. Arch. Intern. Med. 139, 279–280.

Berk, T., Gordon, S.J., Choi, H.Y., Cooper, H.S., 1985. Cytomegalovirus infection of the colon: a possible role in the exacerbations of inflammatory bowel disease. J. Gastroenterol. 80, 355–360.

Boldogh, I., AbuBakar, S., Albrecht, T., 1990. Activation of proto-oncogenes: an immediate early event in human cytomegalovirus infection. Science 247, 561–564.

Chen, N., Ma, W.-Y., She, Q.-B., Wu, E., Liu, G., Bode, A.M., Dong, Z., 2001. Transactivation of the epidermal growth factor receptor is involved in 12-*O*-tetradecanoylphorbol-13-acetate-induced signal transduction. J. Biol. Chem. 276, 46722–46728.

Cherrington, J.M., Mocarski, E.S., 1989. Human cytomegalovirus IE1 transactivates the α promoter-enhancer via an 18-base-pair repeat element. J. Virol. 63, 1435–1440.

Culpitt, S.V., Rogers, D.F., Fenwick, P.S., Shah, P., De Matos, C., Russell, R.E., Barnes, P.J., Donnelly, L.E., 2003. Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. Thorax 58, 942–946.

Docherty, J.J., Fu, M.M., Stiffler, B.S., Limperos, R.J., Pokabla, C.M., DeLucia, A.L., 1999. Resveratrol inhibition of herpes simplex virus replication. Antiviral Res. 43, 145–155.

Docherty, J.J., Smith, J.S., Fu, M.M., Booth, T., 2004. Effect of topically applied resveratrol on cutaneous herpes simplex virus infections in hairless mice. Antiviral Res. 61, 19–26.

Evers, D.L., Breitenbach, J.M., Borysko, K.Z., Townsend, L.B., Drach, J.C., 2002. Inhibition of cyclin-dependent kinase 1 by purines and pyrrolo[2,3-*d*]pyrimidines does not correlate with antiviral activity. Antimicrob. Agents Chemother. 46, 2470–2476.

Fontecave, M., Lepoivre, M., Elleingand, E., Gerez, C., Guittet, O., 1998. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. FEBS Lett. 421, 277–279.

Frankel, E.N., Waterhouse, A.L., Kinsella, J.E., 1993. Inhibition of human LDL oxidation by resveratrol. Lancet 341, 1103–1104.

Griffiths, P.D., 2002. The treatment of cytomegalovirus infection. J. Antimicrob. Chemother. 49, 243–253.

He, Y.S., Xu, L., Huang, E.-S., 1992. Characterization of human cytomegalovirus UL84 early gene and identification of its putative protein product. J. Virol. 66, 1098–1108.

Heredia, A., Davis, C., Redfield, R., 2000. Synergistic inhibition of HIV-1 in activated and resting peripheral blood mononuclear cells, monocyte-derived macrophages, and selected drug-resistant isolates with nucleoside analogues combined with a natural product, resveratrol. J. Acquir. Immune Defic. Syndr. 25, 246–255.

Hippenmeyer, P.J., Dilworth, V.M., 1996. A rapid assay for determination of antiviral activity against human cytomegalovirus. Antiviral Res. 32, 35–42.

Holmes-McNary, M., Baldwin Jr., A.S., 2000. Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the IκB kinase. Cancer Res. 60, 3477–3483.

Huang, E.-S., 1975a. Human cytomegalovirus. III. Virus-induced DNA polymerase. J. Virol. 16, 298–310.

Huang, E.-S., 1975b. Human cytomegalovirus IV. Specific inhibition of virus induced DNA polymerase activity and viral DNA replication by phosphonoacetic acid. J. Virol. 16, 1560–1565.

Huang, E.-S., Kowalik, T.F., 1993. The pathogenicity of human cytomegalovirus: an overview. In: Becker, Y., Darai, G., Huang, E.-S. (Eds.), Molecular Aspects of Human Cytomegalovirus. Springer-Verlag, Berlin, pp. 1–45.

Huang, E.-S., Chen, S.T., Pagano, J.S., 1973. Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. 12, 1473–1481.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C., Pezzuto, J.M., 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275, 218–220.

Johnson, R.A., Huong, S.-M., Huang, E.-S., 1999. Inhibitory effect of 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole on HCMV DNA replication and permissive infection. Antiviral Res. 41, 101–111.

Johnson, R.A., Wang, X., Ma, X.L., Huong, S.-M., Huang, E.-S., 2001. Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. J. Virol. 75, 6022–6032.

Juan, M.E., Vinardell, M.P., Planas, J.M., 2002. The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. J. Nutr. 132, 257–260.

Kowalik, T.F., Yurochko, A.D., Rinehart, C.A., Lee, C.Y., Huang, E.-S., 1994. Productive infection of human endometrial stromal cells by human cytomegalovirus. Virology 202, 247–257.

Kowalik, T.F., Wing, B., Haskill, J.S., Azizkhan, J.C., Baldwin Jr., A.S., Huang, E.-S., 1993. Multiple mechanisms are implicated in the regulation of NF-kappa B activity during human cytomegalovirus infection. Proc. Natl. Acad. Sci. U.S.A. 90, 1107–1111.

Krueger, R.F., Mayer, G.D., 1970. Tilorone hydrochloride: mode of action. Science 169, 1214–1215.

LaBarthe, D., Adam, E., Noller, K.L., O'Brien, P.C., Robboy, S., Tilley, B.C., et al., 1978. Design and preliminary observation of the National Cooperative Diethylstilbestrol Adenosis (DESAD) project. Obstet. Gynecol. 51, 453–458.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Manna, S.K., Mukhopadhyay, A., Aggarwal, B.B., 2000. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-κB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J. Immunol. 164, 6509–6519.

Mar, E.C., Cheng, Y.C., Huang, E.-S., 1983. Effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine on human cytomegalovirus replication in vitro. Antimicrob. Agents Chemother. 24, 518–521.

Mar, E.C., Chiou, J.F., Cheng, Y.C., Huang, E.-S., 1985. Inhibition of cellular DNA polymerase alpha and human cytomegalovirus-induced DNA polymerase by the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine. J. Virol. 53, 776–780.

Matsuoka, A., Takeshita, K., Furuta, A., Ozaki, M., Fukuhara, K., Miyata, N., 2002. The 4′-hydroxy group is responsible for the in vitro cytogenetic activity of resveratrol. Mutat. Res. 26, 29–35.

Mocarski, E.S., Tan Courcelle, C., 2001. Cytomegaloviruses and their replication. In: Knipe, D.M., Howley P.M. (Eds.), Fields Virology, 4th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 2629–2673.

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

Plotkin, S.A., 1999. Vaccination against cytomegalovirus, the changeling demon. Pediatr. Infect. Dis. J. 18, 313–326.

She, Q.B., Ma, W.Y., Wang, M., Kaji, A., Ho, C.T., Dong, Z., 2003. Inhibition of cell transformation by resveratrol and its derivatives: differential effects and mechanisms involved. Oncogene 22, 2143–2150.

Slobbe-van Drunen, M.E., Vossen, R.C., Couwenberg, F.M., Hulsbosch, M.M., Heemskerk, J.W., van Dam-Mieras, M.C., Bruggeman, C.A., 1997. Activation of protein kinase C enhances the infection of endothelial cells by human cytomegalovirus. Virus Res. 48, 207–213.

Soleas, G.J., Diamandis, E.P., Goldberg, D.M., 1997. Resveratrol: a molecule whose time has come? And gone? Clin. Biochem. 30, 91–113.

Spaete, R.R., Mocarski, E.S., 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. U.S.A. 84, 7213–7217.

Speir, E., Yu, Z.-X., Ferrans, V.J., Huang, E.-S., Epstein, S.E., 1998. Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. Circ. Res. 83, 210–216.

Speir, E., Shibutani, T., Yu, Z.-X., Ferrans, V.J., Epstein, S.E., 1996. Role of reactive oxygen intermediates in cytomegalovirus gene expression and in the response of human smooth muscle cells to viral infection. Circ. Res. 79, 1143–1152.

Speir, E., Modali, R., Huang, E.-S., Leon, M.B., Shawl, F., Finkel, T., Epstein, S.E., 1994. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 265, 391–394.

St. Leger, A.S., Cochrane, A.L., Moore, F., 1979. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. Lancet 1, 1017–1020.

Stewart, J.R., Ward, N.E., Ioannides, C.G., O'Brian, C.A., 1999. Resveratrol preferentially inhibits protein kinase C-catalyzed phosphorylation of a cofactor-independent, arginine-rich protein substrate by a novel mechanism. Biochemistry 38, 13244–13251.

Subbaramaiah, K., Chung, W.J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J.M., Dannenberg, A.J., 1998. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem. 273, 21875–21882.

Sun, N.J., Woo, S.H., Cassady, J.M., Snapka, R.M., 1998. DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. J. Nat. Prod. 61, 362–366.

Tanaka, J., Iida, H., Sato, H., Hatano, M., 1988. Inhibitors of prostaglandin synthesis inhibit growth of human cytomegalovirus and reactivation of latent virus in a productively and latently infected human cell line. Virology 163, 205–208.

Wang, X., Huong, S.-M., Chiu, M.L., Raub-Traab, N., Huang, E.-S., 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature 424, 456–461.

Wing, B.A., Lee, G.Y.C., Huang, E.-S., 1996. Human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. J. Virol. 70, 3339–3345.

Yurochko, A.D., Kowalik, T.F., Huong, S.-M., Huang, E.-S., 1995. Human cytomegalovirus upregulates NF-κB activity by transactivating the NF-κB p105/p50 and p65 promoters. J. Virol. 69, 5391–5400.

Yurochko, A.D., Mayo, M.W., Poma, E.E., Baldwin Jr., A.S., Huang, E.-S., 1997a. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-κB promoters. J. Virol. 71, 4638–4648.

Yurochko, A.D., Hwang, E.S., Rasmussen, L., Keay, S., Periera, L., Huang, E.-S., 1997b. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-κB during infection. J. Virol. 71, 5051–5059.

Yurochko, A.D., Huang, E.-S., 1999. Human cytomegalovirus binding to human monocytes induces immunoregulatory gene expression. J. Immunol. 162, 4806–4816.

Zhu, H., Cong, J.-P., Yu, D., Bresnahan, W., Shenk, T.E., 2002. Inhibition of cyclooxygenase 2 blocks human cytomegalovirus replication. Proc. Natl. Acad. Sci. U.S.A. 99, 3932–3937.