Antiviral Research 96 (2012) 181–186

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Eight flavonoids and their potential as inhibitors of human cytomegalovirus replication

Sébastien Cotin [a], Claude-Alain Calliste [b], Marie-Christine Mazeron [d], Sébastien Hantz [a,c], Jean-Luc Duroux [b], William D. Rawlinson [a,c,e,f], Marie-Cécile Ploy [a], Sophie Alain [a,c,*]

[a] Univ. Limoges, Inserm UMR 1092, Faculté de médecine, Limoges, France
[b] Univ. Limoges, EA1069, Faculté de pharmacie, Limoges, France
[c] National Reference Center for Cytomegaloviruses, Bacteriology-Virology-Hygien Department, CHU Limoges, France
[d] Associate Laboratory for the National Reference Center for Cytomegaloviruses, Bacteriology-Virology Department, Saint Louis Hospital, Paris, France
[e] Virology Division, SEALS Microbiology, Prince of Wales Hospital, Randwick 2031, Australia
[f] University of NSW SOMS and BABS, Kensington 2052, Australia

ARTICLE INFO

Article history:
Received 8 June 2012
Revised 7 September 2012
Accepted 11 September 2012
Available online 21 September 2012

Keywords:
Cytomegalovirus
Flavonoids
Antiviral
Toxicity

ABSTRACT

The drugs currently available for treatment of severe human cytomegalovirus (HCMV) infections suffer from many drawbacks, particularly toxicity, and potential teratogenicity contraindicating their use in target populations such as pregnant women. The emergence of drug-resistant strains is still a problem for disease management, particularly in immunosuppressed populations where antivirals are used for extended periods of time. The flavonoid family of drugs contains promising candidates as they have low toxicity and inhibit different targets to currently available antivirals. We report here that, unlike their chalcon homologs, four flavonoids (baicalein, quercetin, quercetagetin and naringenin) inhibit various stages of HCMV replication, the most active anti-HCMV compound being baicalein and the less active and less selective being quercetagetin. These drugs could provide potential inhibitors of virus replication alone or in combination, without increased toxicity.

1. Introduction

Human cytomegalovirus (HCMV) is a widespread opportunistic pathogen in immunocompromised individuals such as allograft recipients and patients with AIDS, and remains the leading viral cause of birth defects (Haun et al., 2007). Antiviral drugs currently available in clinical practice are restricted to the polymerase inhibitors ganciclovir (and the prodrug valganciclovir), foscarnet and cidofovir (Balfour, 1999). They cannot be used during pregnancy and have not been approved for the treatment of congenital infections (Nassetta et al., 2009). They are virustatic, often requiring administration for long periods of time in immunocompromised patients, they are toxic particularly to the bone marrow and kidneys, and resistance emerges with prolonged use (Baldanti et al., 2004). In the French transplant cohort, non-response to therapy affects 10% of transplant recipients, half of them harboring HCMV strains resistant to one or more current antiviral drugs (Hantz et al., 2010). Therapeutic alternatives are limited by the similarity of toxicity between antivirals, and the occurrence of cross resistance. There is an increasing need for new antiviral compounds, and the initial events of the virus replication cycle represent attractive targets for the development of such compounds (Mocarski et al., 2007).

Flavonoids are natural plant metabolites that are widespread in nature. They have numerous biological activities and generally low toxicity. More than 5000 naturally occurring flavonoids have been identified in dietary or botanical sources and many have potential health benefits (Beecher, 2003). Major flavonoids that show well-categorized structures and well-defined structure–function relationships are flavans, flavanones, flavones, flavanonols, flavonols, catechins, anthocyanidins, isoflavones and chalcons. The biological properties of flavonoids include antioxidative, anti-inflammatory, antitumoral, antiviral and antibacterial effects, as well as a direct cytoprotective effect on coronary systems, vascular systems, the pancreas and the liver (Cazarolli et al., 2008). These characteristics place them among the most attractive natural substances able to enrich the current antiviral therapy options. Indeed, different flavonoids inhibit replication of a wide array of viruses. In fact, activity against several herpesviruses particularly herpes simplex virus has been shown for kaempferol (Amoros et al., 1992) and a series of biflavonoids (Lin et al., 1999). Concerning HCMV, baicalein and genistein have been shown to interact with the first events of

* Corresponding author. Address: Inserm UMR 1092, Centre National de Référence des Cytomégalovirus, Laboratoire de Virologie, CHU Limoges, 2 avenue Martin Luther King, 87000 Limoges, France. Tel.: +33 5 55 05 67 28/67 24; fax: +33 5 55 05 65 21.
E-mail address: sophie.alain@unilim.fr (S. Alain).

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.09.010

viral infection (Hayashi et al., 1997; Mitrocotsa et al., 2000; Evers et al., 2005).

We assayed the *in vitro* activity against HCMV of eight natural flavonoids chosen to represent various families: baicalein, naringenin, quercetin and quercetagetin (flavones, flavanones and flavonols, respectively), chalcones (2′,3′,4′-trihydroxychalcone, 2,2′,4′-trihydroxychalcone, naringenin chalcone and butein) and the closely related monocycle compound gallic acid. We compared their activity with that of ganciclovir, and showed four of them are potent inhibitors of CMV, with low toxicity. For the most active of them we identified the potential target stages inhibited and described their additive effects when used together.

2. Materials and methods

2.1. Compounds

Flavonoids and gallic acid (Table 1) were produced by EA4021 (Limoges, France) and stock solutions of compounds (10 mg/ml) were prepared in dimethylsulfoxide (DMSO) and stored at −80 °C. Ganciclovir was purchased from Roche Pharmaceutical (Neuilly, France) and stored at 200 mM in water at −80 °C.

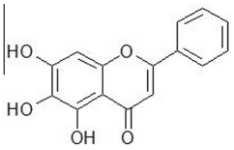
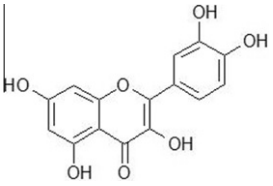
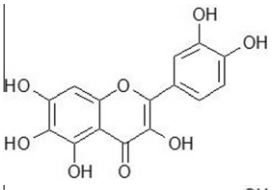
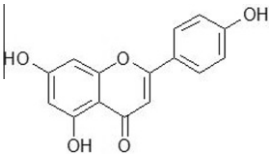
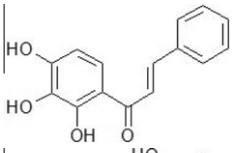
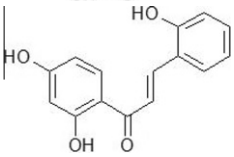
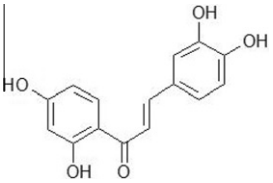
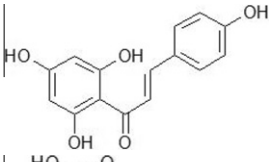
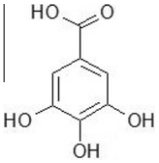
2.2. Viruses and cell culture

HCMV reference strain AD169 ATCC (VR-538) and four clinical isolates, which contain a full-length genome (two naïve isolates from newborns, one ganciclovir-resistant, BOU, with a A594V UL97 substitution and one multi-drug resistant CHA with an UL97 C592G and UL54 N408K and G841A substitution) were used for antiviral assays. Recombinant virus HCMV VR2356 that has the *Escherichia coli* β-galactosidase gene under the control of the HCMV major early β gene promoter integrated into the viral genome by homologous recombination was purchased from ATCC (Spaete and Mocarski, 1987). In addition a naive HSV-1 isolate was tested. Human embryonic lung (HEL) fibroblasts (MRC-5) purchased from Biomérieux (Lyon, France) were cultured in modified Eagle's media GlutamaX (MEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and used between passages 25 and 35. Stocks of HCMV cell-free virus were prepared by infecting confluent cells at a multiplicity of infection (MOI) of 0.1 plaque-forming units (pfu)/cell, and collecting supernatants from infected cell cultures three days after 100% cytopathic effect was reached. Human epithelial cells (HEp2) were purchased from the ATCC and were cultured in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum. Clinical isolates were cell-associated isolates with 3 (for naïve) to 7 passages in MRC-5 cells.

2.3. Antiviral plaque reduction assay

A plaque reduction assay was used to measure the concentration of drug required to reduce the number of plaques by 50% (50% inhibitory concentration IC_{50}) as compared to controls without drug. Confluent HEL fibroblasts in 24-well plates were treated with the drugs diluted in culture medium at various concentrations and infected one hour later by addition of cell-free virus at a MOI of 0.1 pfu/cell for reference strains or to reach some 100 plaques within two wells, for cell-associated virus in the positive control well. The concentration of each compound was calculated and adjusted by volume such that it was constant throughout the experiment. Three hours post-infection, media was replaced by fresh media with the same drug concentrations. Five days post infection, plaques were counted and IC_{50} was calculated by graphic extrapolation.

Table 1
Chemical structures of compounds assayed in this study.

Subfamily	Chemical structure	Name
Flavone		Baicalein
Flavonol		Quercetin
		Quercetagetin
Flavonone		Naringenin
Chalcone		2′,3′,4′-trihydroxychalcone
		2,2′,4′-trihydroxychalcone
		Butein
		Naringenin chalcone
Phenolic acid		Gallic acid

2.4. Antiviral colorimetric assays

HCMV antiviral colorimetric assay was performed as previously reported (Hippenmeyer and Dilworth, 1996; Evers et al., 2005). Various concentrations of drugs (2×) in MEM were added to confluent, serum-starved HEL fibroblasts in 96-well plates. Plates were incubated for 1 h at 37 °C in 5% CO_2. Cells were then infected with an equal volume of MEM containing cell-free HCMV (VR2356

Table 2
Cytotoxicity and antiviral activity of flavonoids. The number of independent experiments performed is indicated into brackets.

Compounds	Cytotoxicity (on confluent fibroblasts) CC_{50}[a] (μM)	Cytotoxicity (on confluent fibroblasts) CC_{50}[a] (μM)	AD169 IC_{50}[a] (μM)	Selectivity index Confluent/growing fibroblasts
Baicalein	610 ± 30 (3)	210 ± 10 (2)	2.2 ± 0.5 (6)	277.27/95.45
Quercetin	820 (3)	625 ± 25 (2)	4.8 ± 1.2 (6)	170.8/130.21
Quercetagetin	250 ± 50 (3)	135 ± 5 (2)	23 ± 3 (5)	10.87/5.87
Naringenin	600 (2)	>200 (1)	6 (2)	100/>33.33
2′,3′,4′-trihydroxychalcone	60 (2)	5 (1)	>50 (3)	0
2,2′,4′-trihydroxychalcone	30 (2)	nd[b]		0
Butein	55 ± 5 (2)	40 (1)	>50 (3)	0
Naringenin chalcone	550 (1)	nd[b]	>100 (1)	0
Gallic acid	105 ± 5 (2)	40 (1)	>50 (3)	0
Ganciclovir	110	110	3[c]	36,67/36.67

[a] CC_{50} and IC_{50} are drug concentrations producing 50% of cell death and 50% of virus plaque reduction, respectively.
[b] Not determined.
[c] Concentrations for ganciclovir are the mean of those currently found in our laboratory using the same conditions.

(0.1pfu/cell)). Seventy-two hours later, supernatants were removed and cell monolayers were rinsed with phosphate-buffered saline (PBS). After addition of 50 μl of lysis solution (10% glycerol, 1% Triton X-100, 2 mM DTT, 2 mM EDTA, 25 mM Tris pH 7,8) plates were incubated 30 min at 37 °C. Then 50 μl of a freshly prepared and gravity filtered β-galactosidase substrate solution (33 mg ortho-nitrophenyl-β-D-galactopyranoside (ONPG) in 25 ml of buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, and 100 mM β-mercaptoethanol)) was added, and plates were incubated at room temperature for 30 min. Reactions were stopped by adding 100 μl/well of 1 M Na_2CO_3. Optical densities were measured at 415 nm using a micro plate ELISA reader. Following the subtraction of absorbance values determined for negative controls, data were quantified as percentages of the average absorbance determined for positive controls.

HSV1 antiviral assays were performed using colorimetric analysis, which measures the quantity of cells not killed by HSV1 infection adapted the assay published by Danve et al. (2002). Confluent HEp2 cells in 96 well plates were infected with 250 μl of 20 pfu/ml HSV1 in the presence of increasing concentrations of drug. Thirty-six hours later supernatant was removed and after two washes with physiologic saline, cells were fixed with 10% formaldehyde in PBS for 10 min, washed again in borate buffer and dried. Cells were stained with 100 μl per well of 1% methylene blue borate buffer (30 min). After one last wash with water, elution was performed with 200 μl of 0.1 N HCl and the absorbance read at 650 nm.

2.5. Cytotoxicity assays

Cellular toxicity was measured using the CytoTox96® Non-Radioactive cytotoxicity assay (Promega, Charbonnières, France) which determines the lactate dehydrogenase (LDH) activity in the residual cells after incubation with the test-drug. Cells were grown to confluence or were approximately 10% confluent in 96-well plates prior to the addition of various concentrations of compound. Seventy-two hours post treatment with selected drug concentrations, LDH activity was determined by measuring color reaction with an ELISA reader. At each concentration of drug, the absorbance at 490 nm was compared to control wells where an equivalent concentration of DMSO had been added (Weislow et al., 1989). The 50% cell cytotoxicity (CC_{50}) was determined graphically as the concentration of molecule, which causes 50% cellular death.

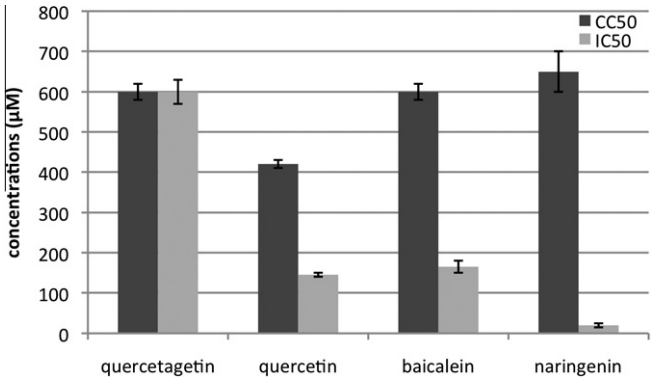


Fig. 1. Cytotoxicity on HEp2 cells and anti-HSV-1 activity of four flavonoids. Graphs show CC50 in black and IC_{50} in grey. Each molecule was assayed three times.

2.6. Western blot analysis

Baicalein, quercetin and quercetagetin were added at concentrations of 10, 20 and 80 μM respectively to AD169 infected cells in 25 cm^2 flasks under the same conditions as used for titer reduction assays. The concentration values used were four fold higher than the IC_{50} to ensure strong inhibition of HCMV replication, at a MOI allowing detection of HCMV proteins and no toxicity. After 48 h incubation, global antigen extraction was performed as described (Goldstein et al., 1982). Protein extracts were quantified by NanoDrop® ND-1000, mixed with equal volumes of Laemmli buffer, heated 10 min at 94 °C with 1% DTT and 1% β-mercaptoethanol. 20 μg of protein extracts were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels. After electrophoresis and transfer western blots were probed with E13 mouse monoclonal antibody to HCMV immediate early IE1-72, and IE2-86 proteins for 1 h and then with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (both from Argene, Verniolle, France). Protein levels were estimated by semi-quantitative measurement of intensity of the bands and comparison with the control and with the β-actin.

2.7. Polymerase activity inhibition measurement

This assay was realized as previously reported for foscarnet (Ducancelle et al., 2007). Briefly, recombinant wild-type AD169

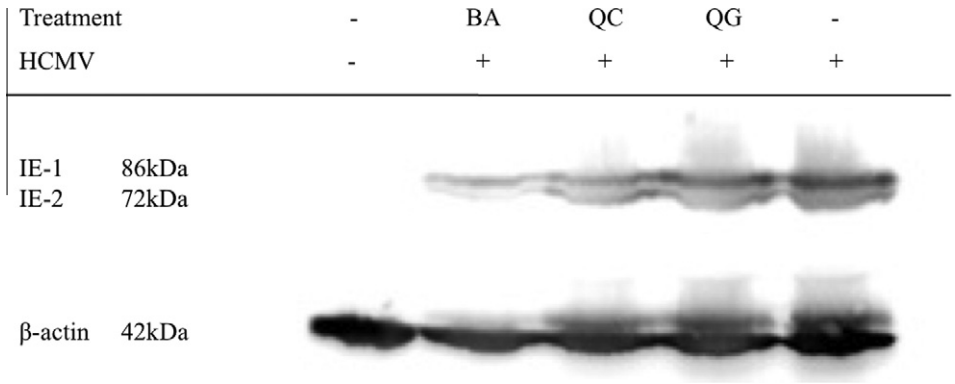


Fig. 2. HCMV immediate-early protein production in the presence of flavonoids. Western-blot analysis was performed at 48 h post infection. QC: HEL cells were treated with 20 μM of quercetin; QG: HEL cells were treated with 80 μM of quercetagetin; BA: HEL cells were treated with 10 μM of baicalein. Protein levels can be estimated by semiquantitative measurement of intensity of the bands and comparison with the control and with the beta actin. Experiments were performed three times with similar results.

polymerase pUL54, cloned in pGEM11 Zf(+) vector system (Promega, Charbonnieres-les-Bains, France) under the control of T7 RNA polymerase promoter interspesed with the 5_UTR from alfalfa mosaic virus, was produced using the TNT T7 reticulocyte lysate system coupled *in vitro* transcription/translation assay (Promega, Charbonnières-les-Bains, France). The non-radioactive DNA polymerase assay quantified DNA polymerase activity by measuring the incorporation of digoxigenin- and biotin-labeled nucleotides (Roche Diagnostics, Meylan, France) into the growing DNA chain. DNA polymerase activities were measured in the absence and presence of serial dilutions of drugs from 10 μl of transcription/translation products. The IC_{50s} were calculated by graphic extrapolation as previously.

3. Results

3.1. Antiviral activity against cytomegalovirus and cytotoxicity

The results are reported in Table 2. Four of the eight compounds tested (baicalein, quercetin, quercetagetin and naringenin) showed antiviral activity towards HCMV reference strain AD169 at concentrations below 25 μM. However, antiviral activity of quercetagetin was moderate. Selectivity indexes (SI), measured on static (confluent) and growing fibroblasts were high except for quercetagetin with a SI of 10,87 and 5,87 on static and growing fibroblasts, respectively. Flavonoids 2′,3′,4′-trihydroxychalcone, butein, naringenin chalcone and gallic acid had no antiviral activity, whereas 2,2′,4′-trihydroxychalcone was highly cytotoxic. On the basis of these results, further studies were then limited to baicalein, quercetin, quercetagetin and naringenin.

3.2. Activity against HSV-1

The four molecules were tested against the clinical isolate of HSV-1. Antiviral activity was assessed in HEp2 cells by a colorimetric titer reduction assay. To rule out the possibility that antiviral activity was an artifact of cytotoxicity, cytotoxicity of the compounds was determined for confluent HEp2 cells as described above. Results presented in Fig. 1 show quercetagetin had no inhibitory effect on HSV-1, whereas naringenin was highly active. Quercetin and baicalein demonstrated very low antiviral activity against HSV-1 (IC_{50} values of 165 μM for baicalein and 145 μM for quercetin) and were less selective for HSV than for HCMV (SI = 3.63 and 2.89, respectively).

3.3. Inhibition of HCMV Immediate Early gene expression

To further study the mechanism of inhibition of HCMV replication by flavonoids, HCMV *Immediate Early* gene expression was analyzed by western-blot. As naringenin was much less efficient on HCMV and on HSV in Evers study than in our work, suggesting its activity may vary depending on the cell-line and on the HCMV strain tested, we therefore chose to analyze the mechanism of action of the drugs combining the best SI and specificity: baicalein, quercetin and quercetagetin.

The results are shown in Fig. 2. In the presence of 80 μM quercetagetin, the production of proteins IE-1 and IE-2 was similar to that of the control. In the presence of 20 μM quercetin a very small reduction can be seen in IE-1 and IE-2, but as expected in the presence of baicalein there was a very marked reduction of IE-1 protein and a complete disappearance of IE-2 expression. So, the primary mechanisms of action of quercetagetin and quercetin are different from that of baicalein, which was previously described as an inhibitor of *Immediate Early* stage of the viral cycle (Evers et al., 2005).

3.4. Inhibition of early stage of viral cycle

The β-galactosidase activity reduction assay was performed after one single viral cycle after infection and reveals the ability of each molecule to inhibit events during or before early stage of the viral cycle. Ganciclovir was used as a control. Fig. 3 shows that, as expected, ganciclovir did not inhibit early events whereas concentrations inhibiting β-galactosidase activity by 50% were 5 μM for baicalein, 12 μM for quercetin and 62 μM for quercetagetin.

3.5. Inhibition of DNA polymerase activity

The HCMV DNA polymerase activity was assayed and drug concentrations inhibiting enzymatic activity by 50% (IC_{50}) were reported in Fig. 4. Cidofovir-di phosphate IC_{50} was used as a reference. Baicalein and quercetin had no effect on polymerase activity whereas a moderate inhibition of viral DNA polymerase activity by quercetagetin was observed (IC_{50} of 12 μM).

3.6. Effect of flavonoids upon clinical isolates

The potential of baicalein and quercetin on HCMV clinical isolates, which contain a full-length genome, was examined by determining the IC_{50} (Fig. 5). Experimentation with quercetagetin was not performed on clinical isolates because of less favorable SI. The IC_{50} values of both flavonoids, although slightly higher for

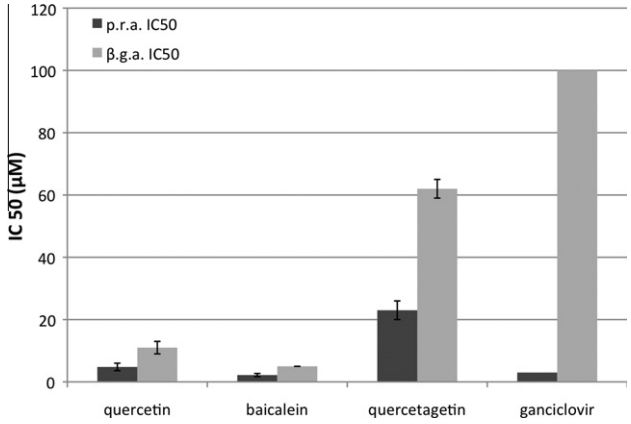


Fig. 3. Effect of three flavonoids upon early gene expression. Early gene expression was measured by quantification of β-galactosidase activity 72 h after infection (one single viral cycle) of HEL fibroblasts with HCMV recombinant VR2356 that harbors the *Escherichia coli* β-galactosidase gene under the control of the major early promoter. The compound concentrations inhibiting by 50% β-galactosidase activity are indicated in grey columns. The results of the phenotypic assay were also reported as a comparison reference (in dark). Ganciclovir was assayed as negative control. p.r.a.: plaque reduction assay (in μM). β.g.a.: β-galactosidase activity assay (in μM).

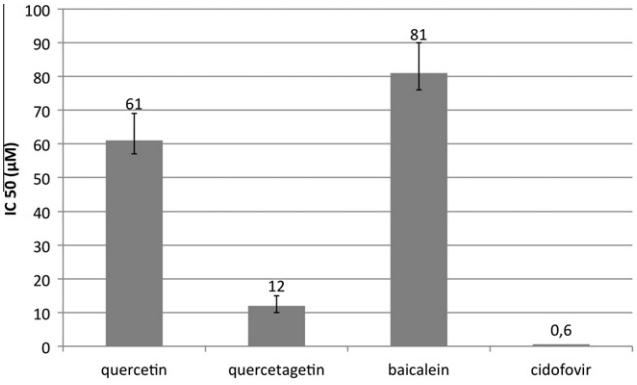


Fig. 4. Effect of three molecules assayed upon reduction of DNA polymerase activity. IC_{50} were found by colorimetric enzymatic reduction assay performed with HCMV DNA polymerase produced *in vitro* in reticulocytes lysate with variable drugs concentrations. Results obtained with cidofovir in the same assays were reported as positive control.

clinical isolates than for AD169, were well below CC_{50} values and similar to those of ganciclovir.

3.7. Effect of combination of two flavonoids

To determine if combination of baicalein and quercetin have additive or synergistic effects, a checkerboard was performed, with baicalein tested with constant concentrations of quercetin, and quercetin with constant concentrations of baicalein. Results are reported in Table 3 and showed an additive effect of these two compounds.

4. Discussion

Flavonoids constitute a large family of compounds with numerous and various biological properties. Some of them display antiviral activity with anti-HCMV activity reported for genistein, baicalein and kaempferol. The large variety of compounds within the family makes the selection of the best candidates as anti-HCMV agents difficult. The eight compounds studied here belong to different structural families. Three of them (baicalein, quercetin and naringenin) had anti-HCMV activity at concentrations well below those producing cytotoxicity. A fourth (quercetagetin) had a modest antiviral activity associated with a weak selectivity index. The most active compound was baicalein. In contrast with the report from Evers et al., who found an $IC_{50} > 40$ μM for naringenin, we showed significant antiviral activity for naringenin with an IC_{50} of 6 μM in the system used here, which could be explained by the viral strains we used (AD169 reference strain instead of Towne strain) or the 10-fold lower multiplicity of infection we chose in order to more closely approach physiological conditions. However, naringenin remains a less attractive anti-HCMV candidate with a less favorable selectivity index. As described before (Lyu et al., 2005), naringenin was found active against HSV-1. In contrast quercetin exhibited a very low activity against HSV-1. This discrepancy could be due to the use of HEp2 cells in the present study instead of Vero cells in the study of Lyu.

Taken together, IE western blotting, β-galactosidase assay and DNA polymerase assay represent a first approach to understand the mechanism of action of new compounds. The inhibition and the absence of inhibition of early antigens production by baicalein (inhibitor of HCMV entry), and ganciclovir (inhibitor of DNA polymerase) respectively confirmed the usefulness of the β-galactosidase assay to study early stages of HCMV infection. Our results for baicalein are consistent with previous findings (Evers et al., 2005) where baicalein was shown to inhibit the tyrosin kinase

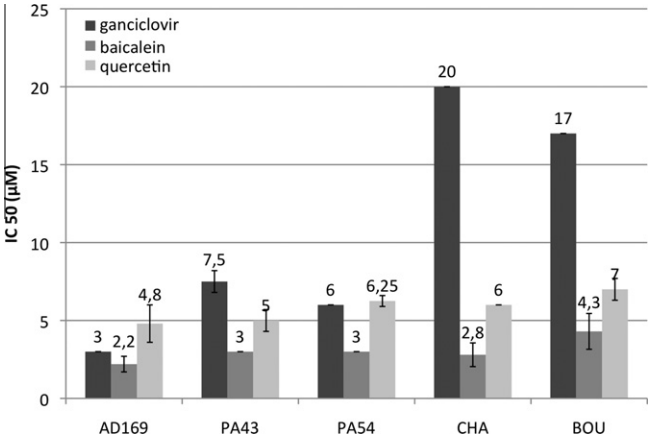


Fig. 5. Inhibitory effect of flavonoids upon clinical isolates. Baicalein, quercetin and ganciclovir were assayed upon four isolates. PA43 and PA54 were isolated from children saliva and are naïve from antiviral. Molecules were also assayed upon two isolates from transplant patients non responding to therapy: a multidrug resistant isolate CHA which was resistant to GCV (IC_{50} 20 μM-AD169 IC_{50}: 3,5 μM), with low-level resistance to CDV (IC_{50} 0.78 μM-AD169 IC_{50}: 0,25 μM) and resistance to FOS (IC_{50} 400 μM-AD169 IC_{50}: 71 μM) and harbor mutations in both the kinase UL97 (C592G) and the polymerase UL54 (N408K + G841A) conferring GCV, CDV, and FOS resistance, and another isolate, BOU resistant to GCV only (IC_{50} 17 μM-AD169 IC_{50}: 3,5 μM) with a single A594 V mutation within the UL97 kinase.

Table 3
Antiviral effect of combination of flavonoids. Activity of baicalein or quercetin against HCMV AD169 was tested in the absence and in the presence of fixed concentrations of the other flavonoid by plaque reduction assay. The number of independent experiments performed is indicated into brackets.

Assayed compound	Added compound	IC_{50} (μM)
Baicalein	None	2.2 ± 0.5 (3)
	Quercetin 0,5 μM	1.6 ± 0.2 (3)
	Quercetin 1 μM	1.8 ± 0.1 (3)
Quercetin	None	4.8 ± 1,2 (3)
	Baicalein 0,5 μM	4.2 ± 0.2 (3)
	Baicalein 1 μM	2.1 ± 0.6 (3)

activity of the EGF receptor, Quercetin partially inhibited production of immediate early proteins and strongly inhibited early protein production, suggesting that quercetin operates at a time point between immediate early and early protein expression, possibly after IE expression. This had been shown by Evers for genistein, an isoflavone, inhibiting HCMV induction of NFκB by IE proteins (Evers et al., 2005). Combinations of quercetin with baicalein showed additive effects, especially when baicalein was added to fixed concentrations of quercetin, which probably reflects the higher efficiency of baicalein. Their possible synergistic effect with polymerase inhibitors is of interest for further testing.

Structure activity relationship is difficult to predict because of the complexity of these molecules. The size and the numerous possible chemical substitutions make this family steric inhibitors as well as chemical reagents. Compounds structurally similar may have very different antiviral or cytotoxic activities and different targets. The complete lack of antiviral activity of gallic acid and the toxicity of the chalcone derivative from baicalein are consistent with this, indicating how each molecule must be assessed independently, as there is no predictable effect of the arrangement of hydroxyl groups' positions.

Our results show that some flavonoids have a specific *in vitro* inhibitory potential against HCMV, some with low toxicity and variable molecular targets from the viral replication cycle. It is difficult to be definitive regarding structure–activity relationships in flavonoid compounds, due to the differing biological properties of compounds, which have similar structures and chemical group substitutions. Nevertheless baicalein and quercetin are very interesting compounds for further study, as they have additive effects without increased toxicity, consistent with the fact that they target two distinct stages of the viral cycle. If similar results are obtained with *in vivo* studies, these molecules may provide good alternative or complements to antivirals currently used in clinical practice.

References

Amoros, M., Simoes, C.M., Girre, L., Sauvager, F., Cormier, M., 1992. Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. J. Nat. Prod. 55, 1732–1740.

Baldanti, F., Lurain, N., Gerna, G., 2004. Clinical and biologic aspects of human cytomegalovirus resistance to antiviral drugs. Hum. Immunol. 65, 403–409.

Balfour Jr., H.H., 1999. Antiviral drugs. N. Engl. J. Med. 340, 1255–1268.

Beecher, G.R., 2003. Overview of dietary flavonoids: nomenclature, occurrence and intake. J. Nutr. 133, 3248S–3254S.

Cazarolli, L.H., Zanatta, L., Alberton, E.H., Figueiredo, M.S., Folador, P., Damazio, R.G., Pizzolatti, M.G., Silva, F.R., 2008. Flavonoids: prospective drug candidates. Mini Rev. Med. Chem. 8, 1429–1440.

Danve, C., Morfin, F., Thouvenot, D., Aymard, M., 2002. A screening dye-uptake assay to evaluate in vitro susceptibility of herpes simplex virus isolates to acyclovir. J. Virol. Methods 105, 207–217.

Ducancelle, A., Alain, S., Petit, F., Sanson Le Pors, M.J., Mazeron, M.C., 2007. Development and validation of a non-radioactive DNA polymerase assay for studying cytomegalovirus resistance to foscarnet. J. Virol. Methods 141, 212–215.

Evers, D.L., Chao, C.F., Wang, X., Zhang, Z., Huong, S.M., Huang, E.S., 2005. Human cytomegalovirus-inhibitory flavonoids: studies on antiviral activity and mechanism of action. Antiviral Res. 68, 124–134.

Goldstein, L.C., McDougall, J., Hackman, R., Meyers, J.D., Thomas, E.D., Nowinski, R.C., 1982. Monoclonal antibodies to cytomegalovirus: rapid identification of clinical isolates and preliminary use in diagnosis of cytomegalovirus pneumonia. Infect. Immun. 38, 273–281.

Hantz, S., Garnier-Geoffroy, F., Mazeron, M.C., Garrigue, I., Merville, P., Mengelle, C., Rostaing, L., Saint Marcoux, F., Essig, M., Rerolle, J.P., Cotin, S., Germi, R., Pillet, S., Lebranchu, Y., Turlure, P., Alain, S., 2010. Drug-resistant cytomegalovirus in transplant recipients: a French cohort study. J. Antimicrob. Chemother. 65, 2628–2640.

Haun, L., Kwan, N., Hollier, L.M., 2007. Viral infections in pregnancy. Minerva Ginecol. 59, 159–174.

Hayashi, K., Hayashi, T., Otsuka, H., Takeda, Y., 1997. Antiviral activity of 5,6,7-trimethoxyflavone and its potentiation of the antiherpes activity of acyclovir. J. Antimicrob. Chemother. 39, 821–824.

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

Lin, Y.M., Flavin, M.T., Schure, R., Chen, F.C., Sidwell, R., Barnard, D.L., Huffman, J.H., Kern, E.R., 1999. Antiviral activities of biflavonoids. Planta Med. 65, 120–125.

Lyu, S.Y., Rhim, J.Y., Park, W.B., 2005. Antiherpetic activities of flavonoids against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in vitro. Arch. Pharm. Res. 28, 1293–1301.

Mitrocotsa, D., Mitaku, S., Axarlis, S., Harvala, C., Malamas, M., 2000. Evaluation of the antiviral activity of kaempferol and its glycosides against human cytomegalovirus. Planta Med. 66, 377–379.

Mocarski, E.S., Shenk, T., Pass, R.F., 2007. Cytomegaloviruses. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia, pp. 2701–2772.

Nassetta, L., Kimberlin, D., Whitley, R., 2009. Treatment of congenital cytomegalovirus infection: implications for future therapeutic strategies. J. Antimicrob. Chemother. 63, 862–867.

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

Weislow, O.S., Kiser, R., Fine, D.L., Bader, J., Shoemaker, R.H., Boyd, M.R., 1989. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. 81, 577–586.