Chlorogenic compounds from coffee beans exert activity against respiratory viruses

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Abstract

Chlorogenic acids (CGAs) are secondary metabolites in diverse plants. Some CGAs extracted from traditional medicinal plants are known for their healing properties, e.g. against viral infections. Also green coffee beans are a rich source of CGAs, with 5-*O*-caffeoylquinic acid being the most abundant CGA in coffee. We previously reported the synthesis of regioisomers of lactones, bearing different substituents on the quinidic core. Here, 3,4-*O*-dicaffeoyl-1,5-γ-quinide and three dimethoxycinnamoyl-γ-quinides were investigated for *in vitro* antiviral activities against a panel of 14 human viruses. Whereas the dimethoxycinnamoyl-γ-quinides did not show any antiviral potency in cytopathogenic effect (CPE) reduction assays, 3,4-*O*-dicaffeoyl-1,5-γ-quinide exerted mild antiviral activity against herpes simplex viruses, adenovirus and influenza virus. Interestingly, when the compounds were evaluated against respiratory syncytial virus (RSV), a potent antiviral effect of 3,4-*O*-dicaffeoyl-1,5-γ-quinide was observed against both subtypes of RSV, with EC_{50} values in the submicromolar range. Time-of-addition experiments revealed that this compound acts on an intracellular post-entry replication step. Our data show that 3,4-*O*-dicaffeoyl-1,5-γ-quinide is a relevant candidate for lead optimization and further mechanistic studies, and warrants clinical development as a potential anti-RSV drug.

Key words:

Chlorogenic acids, quinides, coffee, antiviral activity, influenza virus, respiratory syncytial virus

Abbreviations:

5-CQA, 5-*O*-caffeoylquinic acid; **ACV^{r}**, resistant to acyclovir; **AV-2**, adenovirus type 2; **CC_{50}**, 50% cytotoxic concentration; **$CCID_{50}$**, 50% cell culture infectious dose; **CGAs**, chlorogenic acids; **CGLs**, chlorogenic acid lactones; **CPE**, cytopathogenic effect; **CQAs**, caffeoylquinic acids; **DPBA**, 2,4-dioxo-4-phenylbutanoic acid; **DS-10000**, dextran sulfate 10 kDa; **EC_{90}**, 90% effective concentration; **EC_{99}**, 99% effective concentration; **GFP**, green fluorescent protein; **HEK293T**, human embryonic kidney 293T; **HEL**, human embryonic lung; **HIV**, human immunodeficiency virus; **MCC**, minimal cytotoxic concentration; **MDCK**, Madin-Darby canine kidney; **MFI**, mean fluorescence intensity; **ND**, not determined; **PA-Nter**, N-terminal domain of the PA subunit; **PFU**, plaque forming units; **rgRSV**, recombinant green RSV; **RSV**, respiratory syncytial virus; **TK^{-}**, thymidine kinase-deficient; **vRNP**, viral ribonucleoprotein.

Introduction

Chlorogenic acids (CGAs) are secondary metabolites in leaves, roots and fruits of many plants [1]. Especially green coffee beans have a high content of CGAs, ranging from 4 to 8% (of dry mass) for *Coffea arabica* (L., Rubiaceae) and from 7 to 14% for *Coffea canephora* (Pierre ex A.Froehner, Rubiaceae) [2]. The chlorogenic acids in coffee are usually esters of D-(–)-quinic acid (**Fig. 1**, compound **1**) with certain cinnamic acids, such as caffeic acid and ferulic acid (**Fig. 1**, compounds **2** and **3**, respectively). A large family of CGAs can be generated as mono- or multi-esters and more than 60 different CGAs and their derivatives have been identified in coffee, although with limited characterization for each of them [3,4]. 5-*O*-caffeoylquinic acid (5-CQA, **Fig. 1**, compound **5**) is the most abundant CGA in coffee, hence its common name *chlorogenic acid* [1].

During the roasting process of green coffee beans, chlorogenic acids undergo transesterification, epimerization and lactonization reactions that give rise to the formation of various derivatives including chlorogenic acid lactones (CGLs), also known as quinides, that contribute to the bitterness and the final taste of the coffee beverage [1,5]. A key point for the coffee industry is to identify and evaluate the content of chlorogenic acids and quinides in coffee, in order to characterize its composition and optimize the roasting process to obtain the best taste profile.

Many studies demonstrated that polyphenols are capable of crossing the gastrointestinal barrier of humans to circulate in plasma as intact molecules or hydrolysis metabolites, such as caffeic acid [6-8]. Furthermore, several reports suggested a positive impact of CGAs and their derivatives on human health, via dietary uptake: they are anti-oxidant, anti-inflammatory, and anti-spasmodic [9,10]; they may reduce the risk for cardiovascular diseases, cancer, type 2 diabetes, and Alzheimer's disease [11-14], and they exert antiviral activity, such as against

respiratory syncytial virus (RSV) [15-17], human immunodeficiency virus (HIV) by inhibiting the viral enzyme integrase [18,19], and hepatitis B virus [20].

Starting from D-(–)-quinic acid, we designed a simple synthesis for regioisomers of lactones, bearing different substituents on the quinidic core. We previously reported the synthesis of 3,4-*O*-dicaffeoyl-1,5-γ-quinide (**Fig. 1**, compound **6**) [21], and three dimethoxycinnamoyl-γ-quinides (**Fig. 1**, compounds **7**, **8** and **9**) through coupling of 3,4-dimethoxycinnamic acid (**Fig. 1**, compound **4**) [22]. In our current study, we evaluated the *in vitro* antiviral properties of these quinides for a panel of human viruses, such as herpes simplex virus, adenovirus, influenza virus and RSV. In this paper we demonstrate that 3,4-*O*-dicaffeoyl-1,5-γ-quinide exerts potent antiviral activity against RSV in the submicromolar range, and acts as an intracellular post-entry inhibitor of viral replication.

Results and discussion

In order to determine the biological activity of our chlorogenic quinides, compounds **6**, **7**, **8** and **9** were evaluated against a wide range of human RNA and DNA viruses in different cell types. Since cinnamic and chlorogenic acids are largely present in coffee and can additionally be generated as metabolites of chlorogenic compounds after coffee consumption, caffeic acid, ferulic acid, 3,4-dimethoxycinnamic acid, and 5-CQA (compounds **2**, **3**, **4** and **5**, respectively) were also included in the antiviral screen. The HEL cell culture model was used to evaluate the compounds against herpes simplex virus type I (KOS), herpes simplex virus type 2 (G), vaccinia virus, vesicular stomatitis virus, thymidine kinase-deficient herpes simplex virus type 1 (KOS) resistant to acyclovir (ACV^r) and adenovirus type 2 (AV-2). Based on the microscopical analysis of the virus-induced cytopathogenic effect (CPE), the antiviral potency of the compounds was determined and compared to different reference antiviral drugs (i.e., brivudin, cidofovir, acyclovir, ganciclovir, zalcitabine and alovudine). As summarized in **Table 1**, compounds **2** and **6** showed very weak activity against the tested herpes simplex viruses. However, in a repeat experiment this antiviral effect was lost, presumably because of a slightly more efficient viral infection of the cells as evidenced by the lower potency of the reference compounds in this particular assay. No antiviral effect of the 8 compounds was observed against vaccinia virus. Likewise, the replication of vesicular stomatitis virus was not inhibited by the compounds both in HEL (**Table 1**) and in HeLa cell cultures (not shown). However, in the antiviral test against adenovirus type 2, a clear effect of compounds **5** and **6** was noted. Whereas for the single caffeic acid (compound **2**) a borderline activity against AV-2 was detected, the mono- and di-ester (compounds **5** and **6**, respectively) had improved activity. In fact, the latter exerted anti-AV-2 activity in the lower micromolar range (mean

EC_{50} of 15 µM), which is comparable to the activity of the included reference compounds cidofovir, zalcitabine and alovudine [23].

In addition, Vero cells were utilized to evaluate the compounds against para-influenza type 3 virus, reovirus type 1, Sindbis virus, Coxsackie virus B4 and Punto Toro virus, together with the reference compounds DS-10000 (dextran sulfate 10 kDa) and ribavirin. Unfortunately, the compounds did not produce antiviral effects on this panel of viruses (data not shown).

We next investigated the inhibitory potency of the selected CQAs and quinides towards influenza virus replication. All compounds were assessed in an enzymatic assay with recombinant influenza PA endonuclease, as well as in the cell-based influenza vRNP (viral ribonucleoprotein) reconstitution assay and influenza virus yield assay (**Table 2**). The influenza RNA polymerase complex is gaining much interest as a target to halt influenza virus replication, since major progress has been made in recent years in unravelling the structure and functions of this protein complex [24]. Its endonuclease activity, which resides in the N-terminal part of the PA-subunit (PA-Nter) [25,26], is essential to provide capped primers for viral mRNA synthesis [27]. In the enzymatic assay with influenza PA-Nter endonuclease, compounds **2**, **5** and **6** showed promising inhibitory activity reaching IC_{50} values in the same range as the reference compound 2,4-dioxo-4-phenylbutanoic acid (DPBA) [28]. The PA inhibitory activity of the compounds depends on the presence of two hydroxyl groups. The strong activity of compound **2** (IC_{50} = 16 µM) disappeared when one (compound **3**) or both (compound **4**) hydroxyl functions on the phenyl substituent were replaced by a methoxy group. Consistent with the crucial role of the hydroxyl groups, the activity of 3,4-dihydroxylphenyl bearing compounds **5** and **6 (**IC_{50} = 18 and 34 µM, respectively) was completely lost in their 3,4-dimethoxyphenyl analogues **7** and **8**. Also compound **9**, which does not carry a dihydroxy function, was devoid of inhibitory activity. All influenza PA endonuclease inhibitors reported thus far inhibit the PA enzyme through chelation of its metal

cofactor(s) within the catalytic core [24]. In this work, only compounds bearing a 3,4-dihydroxyphenylgroup were able to inhibit the influenza PA endonucleolytic activity (i.e. compounds **2**, **5** and **6**), while activity was abolished in their analogues carrying only one or no hydroxyl groups. This observation underscores the relevance of the catechol pharmacophore to chelate the metal ions in the PA active site, as previously demonstrated by us and others [29-31].

To determine the anti-influenza activity of the test compounds in cell culture, we first performed a luciferase reporter-based influenza vRNP reconstitution assay in HEK293T cells (**Table 2**). None of the compounds showed toxicity at concentrations up to 200 µM. For two compounds which were active in the enzymatic assay, i.e., **2** and **6**, we also observed selective activity in the vRNP reconstitution assay, with selectivity indices (defined as the ratio between the CC_{50} and EC_{50}, i.e. the 50% cytotoxic and 50% effective concentration) >6 and >10, respectively. This indicates that both compounds are able to inhibit influenza RNA synthesis, and suggests that they could be classified as PA inhibitors. Compound **8** on the other hand, was devoid of activity in the PA enzymatic assay, yet displayed weak activity (EC_{50} = 135 µM) in the vRNP reconstitution assay. This observation suggests that **8** might inhibit the influenza polymerase in an endonuclease-independent manner. For compounds **2** and **6**, the dose-response curves in either the PA-enzyme or vRNP reconstitution assay are shown in **Fig. 2**.

In a second stage, the active compounds from the vRNP assay were subjected to a virus yield assay in MDCK cells. In this assay, only compound **6** was able to inhibit viral replication (EC_{90} = 77 µM; i.e. the compound concentration causing a 1-$\log_{10}$ reduction in viral RNA copy number), albeit a lot less potently than reference compound ribavirin, and compounds **2** and **8** were devoid of activity. Thus, whereas three compounds demonstrated potent inhibition in the enzymatic assay, only one compound (i.e., compound **6**) was proven to have activity in

influenza virus-infected cell culture. This might indicate that the compounds do not enter the cells efficiently, a problem commonly faced with potential influenza PA endonuclease inhibitors [32].

Finally, we determined the antiviral activity of the different compounds against another airway pathogen, respiratory syncytial virus. Alike influenza, human RSV is a major etiological agent of serious and acute lower respiratory tract infections in vulnerable populations such as infants, elderly and immunocompromised persons. Despite the huge clinical impact of RSV, there are currently no approved vaccines or small molecule antiviral drugs against RSV, and the standard of care for the management of RSV disease is limited to supportive care [33-35]. Therefore, novel therapeutics are needed to reduce the severity of RSV infection and limit hospital admissions.

The compounds were tested against RSV in the HeLa cell line. As summarized in **Table 3**, compound **5** exerted some weak activity against RSV in a CPE reduction assay (mean EC_{50} of 24 µM). Interestingly, for compound **6** an anti-RSV efficacy in the submicromolar range was observed (mean EC_{50} of 0.19 µM), which surpassed the activity of the only approved anti-RSV drug ribavirin (**Table 3**). Both active quinide compounds did not display cellular cytotoxicity at 100 µM (the highest concentration tested), resulting in selectivity indices of >4 and >500, respectively. Since compound **6** was the most promising anti-RSV candidate from our antiviral screen, we focused our further RSV research on this quinide.

To assess whether or not this antiviral effect of compound **6** may be cell type-dependent, a comparable CPE reduction assay was performed in the human larynx epidermoid carcinoma cell line HEp-2, for which a similar EC_{50} (i.e., 0.15 µM) was recorded as obtained in HeLa cells (data not shown).

All the experiments described above were performed using the Long strain of the RSV subtype A, but RSV consists of two antigenic subtypes, A and B. To determine the activity spectrum of compound **6**, a representative lab strain of the B subtype was used in a cell protection assay in HeLa cells. As shown in **Figure 3A**, the quinide compound proved equally potent against both subtypes of RSV. Although subtype A has been often associated with the more severe symptomatic manifestations, also subtype B circulates frequently with clear clinical manifestations [36]. Thus, having a compound that inhibits both subtypes would be beneficial to treat all circulating RSV strains.

To carry out a preliminary study on the mode of action of compound **6**, we used a recombinant green RSV (rgRSV) strain [37] that carries the gene encoding for green fluorescent protein (GFP). As this gene is stably inserted in the viral genome, infection of cells with rgRSV results in intracellular expression of GFP that can be detected by fluorescence microscopy and flow cytometry. As displayed in **Fig. 3B**, infection of HeLa cells with rgRSV was highly efficient: about 91% of the cells were infected and stained positive for GFP. Importantly, treatment of the cells with compound **6** resulted in a clear dose-dependent reduction of RSV infection as evidenced by the reduction in GFP expression (**Fig. 3C**), with an EC_{50} value of 0.17 μM that fits nicely with the observed activity in the CPE assays for RSV A and B (**Fig. 3A**). At a compound concentration of 4 μM, viral infection was markedly inhibited (**Fig. 3B**): less than 20% stained GFP-positive, and the mean fluorescence intensity (MFI) was reduced by 85% as compared to the virus-infected control.

We next used rgRSV in a time-of-addition experiment to assess whether the antiviral effect of compound **6** was different when added at various time points post infection. The reference compounds DS (an attachment/entry inhibitor) and ribavirin (a transcription/replication inhibitor) were included as controls. As expected, viral infection was completely prevented when the compounds were administered to the cells prior to adding the virus (**Figure 3D**).

When the cells were first exposed to the virus and then treated with the compounds at 1.5h post virus inoculation, compound **6** markedly inhibited virus replication whereas the attachment inhibitor DS completely lost its antiviral effect. The administration of compound **6** could even be delayed until 5h after virus infection without greatly reducing its antiviral effect. In addition, compound **6** did not exert any direct virucidal activity: pre-incubation of the virus stock with the compound could not prevent viral infection of the cells, excluding a direct effect of the compound on the integrity of the viral particles (data not shown). Taken together, the time-of-addition experiment revealed that the quinide compound acts on a post-entry stage, inhibiting RSV through an intracellular antiviral mechanism of action. Whereas the current data have not pointed to a specific antiviral target yet, one can speculate that the viral replication machinery, and more particular the drugable viral RNA-dependent RNA polymerase might be a potential target [38]. Nevertheless, our data show that compound **6** is a relevant candidate for lead optimization and further anti-RSV and mechanistic studies.

Our results are in agreement with previous studies that reported antiviral activity against RSV for dicaffeoylquinic acids [15-17]. These groups independently recorded anti-RSV activities for 4,5-*O*-dicaffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid, in the lower micromolar range (Figure 1, compounds **11** and **10**, respectively). Here we show that a quinide backbone instead of the quinic acid structure even improved the anti-RSV potency (activity in the submicromolar range for compound **6**). Interestingly, whereas the di-ester structures had profound and comparable anti-RSV activity, the mono-ester variant (corresponding to our compound **5**) exerted significantly weaker antiviral effect [15]. This indicates that two caffeic acid side-arms are needed on a quinic acid or quinide central ring to obtain anti-RSV effect, but their relative positioning on the central core might be less stringent. Remarkably, luteoside (a phenylpropanoid glycoside from the medicinal plant *Markhamia lutea*) was reported to exhibit potent *in vitro* activity against RSV [39]. Although luteoside has a much more

complicated chemical structure as compared to the dicaffeoylquinic acids and our quinides, it also carries one caffeoyl and one caffeoyl-like side arm on its sugar backbone. This suggests that there might still be ample possibilities for structure improvement and analogue design, in order to improve the antiviral potential of this category of plant-derived antivirals. Despite the challenging medicinal chemistry optimization, compound **6** is an interesting chemical tool compound for further elucidation of the mode of action and to better understand how RSV replicates in cells.

In conclusion, our antiviral investigations indicated that dimethoxycinnamoyl-γ-quinides are devoid of antiviral potencies in CPE reduction assays, but pointed to 3,4-*O*-dicaffeoyl-1,5-γ-quinide as a potent and interesting RSV inhibitor with relevance for further development.

Materials and methods

Chemicals

Caffeic acid (≥ 98%), ferulic acid (99%), 3,4-dimethoxy cinnamic acid (predominantly trans, 99%), chlorogenic acid hemihydrate (≥ 98%) and all reagents were purchased from Sigma-Aldrich and used without further purification. The synthesis of quinides **6**, **7**, **8** and **9** has been described in detail [21,22]. The following reference antiviral drugs were used in the antiviral assays: brivudin (G.D. Searle), cidofovir (Gilead Sciences), acyclovir (GlaxoSmithKline), ganciclovir (Roche), zalcitabine (Carbosynth), alovudine (Carbosynth), DS 10000 (Pfeifer & Langen), 2,4-dioxo-4-phenylbutanoic acid (DPBA; Interchim) and ribavirin (ICN Pharmaceuticals). All these reference compounds were at least 97% pure.

Antiviral activity evaluation by CPE reduction assays

Compounds were dissolved in DMSO to obtain a stock concentration of 50 mM that was stored in the freezer at -20° C. The antiviral activity of the compounds was determined in different cell types by diluting the stock solution in medium to a final concentration of 100 µM of compound (and 0.2% of DMSO). Further serial dilutions of the test compounds were also made in cell culture medium. (i) Human embryonic lung (HEL) cells were seeded in 96-well plates and incubated for 6 days at 37 °C until confluency was reached. Medium was aspirated and replaced by serial dilutions of the test compounds (100 µL per well). One hundred microliters of the virus (*herpes simplex virus type 1, herpes simplex virus type 2, thymidine kinase-deficient (TK⁻) herpes simplex virus type 1 resistant to acyclovir (ACV^r), vaccinia virus, adenovirus-2* or *vesicular stomatitis virus*), diluted in medium to obtain a virus input of 100 $CCID_{50}$ (50% cell culture infectious dose; 1 $CCID_{50}$ being the virus dose that is able to infect 50% of the cell culture), was added to each well. Mock-treated cell cultures

receiving solely the test compounds were included, to determine the cytotoxicity. After 4 to 10 days of incubation at 37 °C, microscopical analysis was performed to score the virus-induced CPE. (ii) African green monkey kidney (Vero) cells were seeded in 96-well plates at 30,000 cells per well and incubated for 1 day at 37 °C until confluency was reached. Medium was aspirated and replaced by serial dilutions of the test compounds (100 µL per well). One hundred microliters of the virus (*Coxsackie virus B4, Sindbis virus, parainfluenza virus type 3, Punta Toro virus* or *reovirus type 1*), diluted in medium to obtain a virus input of 100 $CCID_{50}$, was added to each well. Mock-treated cell cultures receiving solely the test compounds were included, to determine the cytotoxicity. After 3 days (for Coxsackie virus B4 and Sindbis virus) or 6 days (for para-influenza-3 virus, Punta Toro virus and reovirus-1) of incubation at 37 °C, microscopical analysis was performed to score the virus-induced CPE.

Influenza PA-Nter endonuclease assay

The plasmid-based influenza PA-Nter endonuclease assay was performed according to a previously published method with minor modifications [31]. Two micrograms of recombinant PA-Nter enzyme (residues 1-217 from the PA protein of influenza virus strain A/X-31) was incubated with 1 µg M13mp18 single-stranded plasmid (Bayou Biolabs) as a substrate; in the presence of serial dilutions of the test compounds and at a final reaction volume of 25 µL. The assay buffer was composed of 50 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM β-mercaptoethanol and 1 mM $MnCl_2$. After 2 h incubation at 37 °C, the reaction was stopped by heat inactivation (80 °C, 20 min) and the endonucleolytic digestion of the substrate was visualized by gel electrophoresis with ethidium bromide staining. The amount of remaining intact plasmid substrate was quantified by ImageQuant TL software (GE Healthcare).

Influenza vRNP reconstitution assay

The procedure to determine the inhibitory effect of the compounds on reconstituted influenza virus vRNPs is described in more detail elsewhere [40,41]. Briefly, four expression plasmids derived from influenza A/PR/8/34 (encoded pVP-PB1, pVP-PB2, pVP-PA and pVP-NP; generously donated by M. Kim [42], Korea Research Institute of Chemical Technology, Daejeon, South Korea) were combined with a firefly luciferase reporter plasmid (also a kind gift from M. Kim) and cotransfected into human embryonic kidney 293T (HEK293T) cells (purchased from Thermo Fisher Scientific) using Lipofectamine 2000 (Invitrogen, Life Technologies). After incubation at 37 °C for 24 h in the presence of the test compounds, the luciferase activity was determined using the ONE-Glo luciferase assay system (Promega). The 50% effective concentration (EC_{50}) was defined as the compound concentration causing 50% reduction in the vRNP-driven firefly luciferase signal, as compared to cells receiving medium instead of compound. In parallel, compound cytotoxic activity, expressed as 50% cytotoxic concentration (CC_{50}), was determined in untransfected HEK293T cells which had been incubated with serial dilutions of the compounds for 24 h, using the MTS cell viability assay. The CC_{50} values were defined as the compound concentration reducing cell viability by 50%, as compared to wells receiving medium instead of compound.

Influenza virus yield assay

The virus yield assay to determine anti-influenza virus activity in cell culture was previously published in full detail [43]. One day prior to infection, Madin-Darby canine kidney (MDCK) cells (a kind gift from M. Matrosovich, Marburg, Germany) were seeded into 96-well plates at 25,000 cells per well. At day 0, serial dilutions of the test compound were added, immediately followed by infection with influenza A/PR/8/34 virus at 150 $CCID_{50}$ per well. After 24 h incubation at 35 °C, the virus amounts in the supernatants was estimated by determining the viral genome copy number in a one-step quantitative real-time reverse transcription (qRT)-

PCR assay (CellsDirect One-Step qRT-PCR kit; Invitrogen), using influenza virus M1-specific primers and probe.

RSV antiviral assay

The anti-RSV effect of the compounds was investigated using a CPE assay method as follows: human cervical cancer (HeLa) cells (1.25 x 10^4 per well) were seeded in 96-well plates and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The overnight medium (DMEM containing 10% FBS) was removed and replaced by serial dilutions of the test compounds in medium containing 2% FBS (100 µL per well). One hundred microliters of the virus RSV A (strain Long; ATCC VR-26) or RSV B (strain 18537; ATCC VR-1580), diluted in medium to obtain a virus input of 60 or 300 $CCID_{50}$ respectively, was added to each well. Mock-treated cell cultures receiving solely the test compounds were included, to determine the cytotoxicity. After 4 to 6 days of incubation at 37 °C in a humidified 5% CO_2 atmosphere, when the cells in the virus control wells exhibited extensive CPE and destruction, microscopical analysis was performed to score the virus-induced cytopathogenicity in all samples. Antiviral activity of the compounds is expressed as EC_{50}, which represents the concentration of the test compound required to reduce the viral CPE by 50%.

For the experiments with rgRSV, a similar protocol was used but with 2 x 10^4 cells per well and a virus input of 1 x 10^5 plaque forming units (PFU)/mL. Cells were collected after 2 days, fixed in 1% formaldehyde and analyzed by flow cytometry for GFP, using a BD Accuri C6 flow cytometer (BD Biosciences) and the accompanying BD FACSDiva software.

RSV time of addition assay

HeLa cells (4 x 10^4 per well) were seeded in 96-well plates and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The overnight medium (DMEM containing 10% FBS)

was removed and replaced by fresh medium containing 2% FBS, and cells were incubated with or without test compound for 1.5 hours (time point -1.5 h; the compounds were added to the medium at all subsequent incubation steps in order to keep the compound continuously present). Next, medium was aspirated and cells were exposed to rgRSV (6 x 10^4 PFU/mL) for 2 hours at 37 °C. Then, unbound RSV was washed out from the cell monolayers, and cells were given fresh medium for another 1.5 hours. The compounds were administered either 1.5 h or 5 h after virus wash out (time points +1.5 h and +5 h). Finally, cells were collected after 48 h, fixed in 1% formaldehyde and analysed by flow cytometry for GFP, using a BD Accuri C6 flow cytometer and the accompanying BD FACSDiva software.

Statistics

The antiviral data are represented as single EC_{50} values, which is the 50% effective concentration required to reduce virus-induced cytopathogenicity by 50%. When activity was recorded against at least one virus in the first experiment, the compound was retested against the whole panel of viruses in a second experiment. The EC_{50} values were manually calculated by interpolation, assuming a semi-log dose-response effect. For the influenza PA-Nter endonuclease and vRNP reconstitution assay, the percentages of inhibition of activity from 2-3 independent experiments were plotted against the compound concentrations on a semi-logarithmic plot, using GraphPad Prism software (GraphPad Software) to obtain the IC_{50} or EC_{50} by nonlinear least-squares regression analysis. In the influenza virus yield assay, the EC_{99} and EC_{90} values were defined as the compound concentration causing respectively a 2-log_{10} and 1-log_{10} reduction in viral RNA copy number, as compared to the virus control without compound. These values were calculated by interpolation from data of two independent experiments.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends:

Figure 1. Molecular structure of D-(–)-quinic acid **1**, caffeic acid **2**, ferulic acid **3**, 3,4-dimethoxycinnamic acid **4**, 5-*O*-caffeoylquinic acid **5**, 3,4-*O*-dicaffeoyl-1,5-γ-quinide **6**, 3-*O*-[3,4-(dimethoxy)cinnamoyl]-1,5-γ-quinide **7**, 3,4-*O*-bis[3,4-(dimethoxy)cinnamoyl]-1,5-γ-quinide **8**, and 1,3,4-*O*-tris[3,4-(dimethoxy)cinnamoyl]-1,5-γ-quinide **9**, 3,5-*O*-dicaffeoylquinic acid **10**, 4,5-*O*-dicaffeoylquinic acid **11**, and luteoside A **12**. The IUPAC numbering system for chlorogenic acid is adopted [44] and, to avoid confusion, the same numbering system is used for the carbon atoms of the lactones and the acid precursors.

Figure 2. Dose-response curves for compounds **2** and **6**. Left panels: enzymatic activity with PA-Nter; right-panels: cell-based vRNP reconstitution assay. The graphs were made by non-linear regression analysis on data from 2-3 independent experiments using GraphPad Prism. The data points shown are the mean ± SD.

Figure 3. Antiviral activity of compound **6** against RSV in HeLa cells. (A) Compound **6** was tested in HeLa cells simultaneously against RSV A and B in an antiviral assay as described in the materials and methods. The mean EC_{50} values (with 95% confidence intervals) from 6 independent experiments are given. (B) Flow cytometric histogram of rgRSV infected cells. The GFP expression of virus control cells (white histogram) and infected cells treated with 4 µM compound **6** (gray histogram) is depicted. The dotted line histogram represents the background fluorescence of non-infected control cells. (C) Dose-dependent inhibition of rgRSV infection by compound **6** as determined by GFP expression with flow cytometry. For each dose of the compound, the mean fluorescence intensity (MFI) of the cells was quantified and is expressed as percentage of the MFI of the virus control cells (without compound). (D) Time-of-addition experiment of compound **6** (5 µM), dextran sulfate (DS; 1 µg/mL) and

ribavirin (50 µM) in HeLa cells infected with rgRSV. Compounds were administered either 1.5 h before virus exposure, 1.5 h or 5 h after virus wash out, and were kept in the culture medium during the whole experiment as described in detail in the materials and methods. The cells were exposed to the virus for 2 h (indicated with a grey box on the x-axis). After 48 h, cells were collected and the number of infected (GFP positive) cells was quantified by flow cytometry. The figure displays the percentage of RSV inhibition for the three starting points of compound treatment. One representative experiment out of 2 is shown.

Tables:

Table 1. Antiviral activity and cytotoxicity of compounds in HEL cell cultures.

Compound	Exp.	MCC[a]	EC_{50}[b]					
			Herpes simplex virus-1 (KOS)	Herpes simplex virus-2 (G)	Vaccinia virus	Vesicular stomatitis virus	Herpes simplex virus-1 TK^- KOS ACV^r	Adeno-virus-2
2	1[c]	>100	45	45	>100	>100	100	45
	2	>100	>100	>100	>100	>100	>100	100
3	1	>100	>100	>100	>100	>100	>100	>100
4	1	>100	>100	>100	>100	>100	>100	58
	2	>100	>100	>100	>100	>100	>100	100
5	1	>100	>100	>100	>100	>100	>100	50
	2	>100	>100	>100	>100	>100	>100	58
6	1	≥100	45	45	>100	>100	45	10
	2	>100	>20	>20	>20	>20	>20	20
7	1	>100	>100	>100	>100	>100	>100	>100
8	1	>100	>100	>100	>100	>100	>100	>100
9	1	>100	>100	>100	>100	>100	>100	>100
Brivudin	1	>250	0.02	146	17	>250	250	ND
	2	>250	0.04	250	17	>250	50	ND
Cidofovir	1	>250	4.5	3.8	29	>250	5.8	10
	2	>250	4.5	5.9	50	>250	5.8	4.0
Acyclovir	1	>250	0.2	0.2	>250	>250	10	ND
	2	>250	0.3	0.2	>250	>250	10	ND
Ganciclovir	1	>100	0.03	0.06	>100	>100	0.4	ND
	2	>100	0.07	0.2	>100	>100	0.8	ND

Zalcitabine	1	>250	ND	ND	ND	ND	ND	25
	2	>250	ND	ND	ND	ND	ND	10
Alovudine	1	>250	ND	ND	ND	ND	ND	10
	2	>250	ND	ND	ND	ND	ND	10

[a]MCC: minimum cytotoxic concentration (MCC) as determined by microscopic analysis of cell morphology, in µM.
[b]EC_{50}: 50% effective concentration, i.e. compound concentration required to reduce virus-induced cytopathogenicity by 50%, in µM.
[c]When activity was recorded against at least one virus in the first experiment, the compound was retested against the whole panel of viruses in a second experiment.
ND, not determined.

Table 2. Anti-influenza activity of the test compounds in the enzymatic assay with influenza PA-Nter endonuclease, or in cellular influenza virus assays.

Compound	**Enzyme assay with PA-Nter**[a]	**vRNP reconstitution assay in HEK293T cells**[b]		**Virus yield assay in influenza virus-infected MDCK cells**[c]		
		Cytotoxicity	Antiviral activity	Cytotoxicity	Antiviral activity	
	IC_{50} (95% CI)	CC_{50}	EC_{50} (95% CI)	CC_{50}	EC_{99}	EC_{90}
2	16 (12-22)	>200	32 (20-51)	>200	>200	>200
3	>500	>200	>200	ND	ND	ND
4	>500	>200	>200	ND	ND	ND
5	18 (15-22)	>200	>200	ND	ND	ND
6	34 (28-40)	>200	19 (12-30)	>200	≥100	77
7	>500	>200	>200	ND	ND	ND
8	>500	>200	135 (100-182)	113	>100	>100
9	>500	>200	>200	ND	ND	ND
DPBA[d]	24 (17-34)	ND	ND	ND	ND	ND
Ribavirin	ND	>200	7.1 (5.0-10)	>200	8.6	5.7

[a]Recombinant PA-Nter was incubated with the ssDNA plasmid substrate, a Mn^{2+}-containing buffer and test compounds. Cleavage of the substrate was assessed after 2 hr incubation. The IC_{50} represents the compound concentration (in µM) to obtain 50% inhibition of cleavage.

[b]HEK293T (human embryonic kidney 293T) cells were co-transfected with the four vRNP-reconstituting plasmids and the luciferase reporter plasmid in the presence of the test compounds. The EC_{50} value represents the compound concentration (in µM) producing 50% reduction in vRNP-driven firefly reporter signal, estimated at 24 h after transfection. The CC_{50} (in µM), i.e. the 50% cytotoxic concentration, was determined in untransfected HEK293T cells by MTS cell viability assay.

[c]MDCK (Madin-Darby canine kidney) cells were infected with influenza A virus (strain A/PR/8/34) and incubated with the compounds during 24 h. The virus yield in the supernatant was assessed by real-time qPCR. The EC_{99} and EC_{90} values represent the compound concentrations (in µM) producing a 2-$\log_{10}$ or 1-$\log_{10}$ reduction in virus titer, respectively. The cytotoxicity, assessed in uninfected MDCK cells, was expressed as the CC_{50} value (50% cytotoxic concentration, determined with the MTS cell viability assay, in µM).

[d]DPBA, 2,4-dioxo-4-phenylbutanoic acid. [28]

ND, not determined.

Table 3. Cytotoxicity and antiviral activity of compounds against RSV in HeLa cells.

Compound	Exp.	RSV type A strain Long	
		MCC[a]	EC_{50}[b]
2	1[c]	>100	>100
	2	>100	>100
3	1	>100	>100
	2	>100	>100
4	1	>100	>100
	2	>100	>100
5	1	>100	19
	2	>100	29
6	1	>100	0.21
	2	>100	0.16
7	1	>100	>100
	2	>100	>100
8	1	>100	>100
	2	>100	>100
9	1	50	>50
	2	50	>50
DS-10000	1	>100	0.11
	2	>100	0.09
Ribavirin	1	250	4.5
	2	250	5.6

[a]MCC: minimum cytotoxic concentration (MCC) as determined by microscopic analysis of cell morphology, in µM.
[b]EC_{50}: 50% effective concentration, i.e. compound concentration required to reduce virus-induced cytopathogenicity by 50%, in µM.
[c]The compounds were tested in two independent experiments.
All concentrations are in µM, except for DS-10000 for which the concentration is given in µg/mL.