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Effects of polyphenol compounds on influenza A virus replication and definition of their mechanism of action

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ABSTRACT

A set of polyphenol compounds was synthesized and assayed for their ability in inhibiting influenza A virus replication. A sub-set of them showed low toxicity. The best compounds within this sub-set were **4** and **6g**, which inhibited the viral replication in a dose-dependent manner. The antiviral activity of these molecules was demonstrated to be caused by their interference with intracellular pathways exploited for viral replication: (1) MAP kinases controlling nuclear-cytoplasmic traffic of viral ribonucleoprotein complex; (2) redox-sensitive pathways, involved in maturation of viral hemagglutinin protein.

1. Introduction

Each year influenza A viruses (IAV) cause thousands of deaths and hospitalizations. The pandemic caused by the 2009 influenza A (H1N1) virus—the first of the 21st century—was characterized mainly by mild-moderate infections similar to those seen with seasonal influenza,[1] but several cases of acute respiratory distress syndrome and pneumonia in previously healthy persons were also reported.[2] Influenza A viruses are enveloped, negative strand RNA viruses belonging to the *Orthomyxoviridae* family. Their genome consists of eight single-stranded RNA segments encoding 11 proteins including hemagglutinin (HA), one of the main surface glycoproteins.[3] The receptor binding site of HA is necessary for the virus to bind galactose-bound sialic acid on the surface of host-cells, and 16 different subtypes have been isolated thus far from different hosts.[4] Influenza virus replication has been studied in depth, and several antiviral agents all targeting viral structures, have been developed.[5] Among them, amantadine and rimantadine have a specific inhibitory effect on type A but not on type B influenza viruses.[6] These agents block the ion-channel activity of the viral matrix (M2) protein, which is mainly required for virus uncoating.[6] Currently, the viral neuraminidase (NA) inhibitors such as oseltamivir, zanamivir, and peramivir are the mainstay of pharmacological protocols to fight global influenza pandemics.[7] However, the long-term efficacy of these inhibitors is often limited by their toxicity and by the high incidence in the selection of drug-resistant viral mutants that is almost certain.[8–10] Actually, the main effort of the preclinical research is addressed to block the viral replication by interfering with pathogen-exploited host-cell machinery.[11] This approach could give important advantages, including the broad-spectrum efficacy, the antigenic properties, and the reduced probability to select drug-resistant viral strains.[11] In particular, a very promising and innovative strategy to develop new antiviral agents could be based on the inhibition of intracellular pathways that are specifically activated by influenza virus to ensure its replication.[12,13] Interestingly, many of these pathways are highly sensitive to changes in the intracellular redox state,[14,15] and as a matter of fact their activation is induced by the oxidative stress caused by infections by DNA or RNA virus, including influenza.[11,15]

Dietary polyphenols, such as resveratrol (RV) [5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol (found in red wine) and curcumin [diferuloyl methane; 1,7-bis-(4-hydroxy-3-methoxy-

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phenyl)-1,6-heptadiene-3,5-dione] (found in curry powder), were reported to posses anticancer,[16,17] anti-inflammatory,[18,19] and antiviral properties.[20–22]

Curcumin is a colouring compound present in the rhizome of *Curcuma longa L.* It has been used for thousands of years in Southeast Asia and Indian folk medicine to treat various diseases and eradicate health problems.[23] It shows many pharmacological properties including anti-inflammatory,[24] antioxidant, iron-chelating, and some other activities.[25] For example, the antioxidant activity of curcumin is responsible for its ability to decrease the incidence of colon cancer, and for its anti-atherogenic property.[26] It has cytoprotective effect on PC12 cells against 1-methyl-4-phenylpridinium ions-induced neurotoxicity through the anti-apoptotic and anti-oxidative properties of the Bcl-2-mitochondria-ROS-iNOS pathway.[27] Recently, curcumin has been proven to exert anti-influenza activity by inhibition of the virus-cell attachment. These studies demonstrated that treatment of cells with curcumin greatly reduced the yield of IAV at sub-cytotoxic doses. Pre-incubation of virus with curcumin pronouncedly inhibited influenza virus plaque formation.[28]

Unfortunately, curcumin is water insoluble, poorly dissolves in organic phase, and shows low bioavailability in vivo after oral administration.[29,30] It is unstable at neutral-basic pH values and in serum-free medium degrading to vanillin, ferulic acid, feruloyl methane and trans-6-(40-hydroxy-30-methoxy-phenyl)-2,4-dioxo-5-hexenal.[31]

RV is a very interesting polyphenol belonging to the stilbene family. It is found in several fruits, vegetables and beverages including red wine. It is one of the most important plant polyphenols with proven salutary activity on animal health. The most important source of polyphenols and in particular RV for human diet is grape (*Vitis vinifera*).[32]

In the last two decades the potential protective effects of RV against cardiovascular[33] and neurodegenerative diseases,[34] as well as the chemo-preventive properties against cancer,[35] have been largely investigated. RV appears to be capable of interfering with several intracellular signaling pathways, including those activated by protein kinase C (PKC) and by mitogen-activated protein kinases (MAPKs).[36]

RV has been also reported to exert an antiviral activity in different experimental systems.[37–39] A few years ago, we showed that RV inhibits influenza virus replication in vitro and it is also effective in increasing the survival of influenza virus-infected mice.[22] Surprisingly, these effects are not related to its well-known antioxidant activity. They are rather related to the inhibition of intracellular pathways JNK (c-Jun N-terminal kinase) and p38MAPK involved in regulation of viral ribonucleoprotein (vRNP) complex traffic across the nuclear membrane, a key step of viral replication cycle that precedes viral assembly and release.[12]

The biological activity of RV is limited by its low bioavailability and metabolic instability. In fact, stilbene double bond is readily oxidized by cytochrome P450 monooxygenases into highly reactive epoxides, that may act as carcinogenic metabolites.[40,41] Further, under UV irradiation, RV converts to the (*Z*)-isomer[42,43] and this was claimed as one of the reason for the presence of small amounts of (*Z*)-resveratrol in wines. The conversion from (*E*)- to (*Z*)-configuration makes resveratrol less stable[44] and consequently decreases its biological activity.

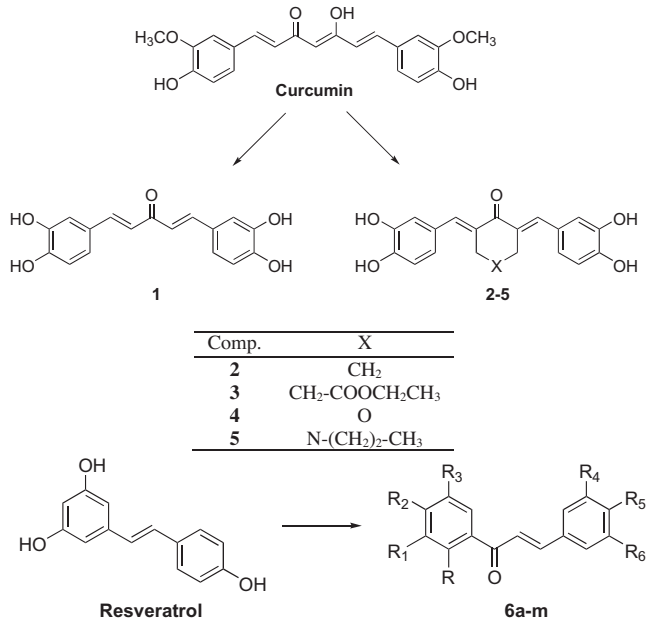
Therefore, pursuing our studies on compounds active against influenza and taking advantage of our experience in the synthesis of polyhydroxylated analogues,[45] we decided to synthesize a number of curcumin and resveratrol analogues with the aim of obtaining compounds with improved ability in interfering with the cell pathways exploited by the virus for its replication, such as kinase pathways and intracellular redox state. These compounds were evaluated for their antiviral activity and their structures are reported in Figure 1.

2. Chemistry

All derivatives tested except **6b**, were prepared as reported in literature.[45a–f] Method of synthesis of compound **6b**, chemical and physical data are reported in Supplementary data.

3. Result

In a first set of experiments the cytotoxicity of the compounds has been evaluated in a test on confluent monolayers of MDCK cells. Cells were plated at concentration of 2×10^5/ml, treated after 24 h with various concentrations (range 5–40 μg/ml) of the synthesized compounds (**1, 2, 3, 4, 5, 6a–m**), and incubated for the following 24 h. Microscopical examination, Trypan blue exclusion and cells counts demonstrated that the compounds **1, 2, 3, 5, 6a, 6b, 6d, 6e, 6h, 6i, 6l** and **6m**, produced a dose-dependent toxic effect. Indeed, morphological alterations, loss of cells viability and modification of cell multiplication rate in treated cells were observed (data not shown). These substances have been excluded for the following experiments. On the contrary, the treatment with the other compounds did not induce any toxicity on cell monolayer, even if the compound **6g** caused a slight alteration of cell morphology starting from 20 μg/ml and the compounds **4, 6c**, and **6f** starting from 40 μg/ml. These results were further confirmed using the



Comp.	X
2	CH_2
3	CH_2-$COOCH_2CH_3$
4	O
5	N-$(CH_2)_2$-CH_3

Comp.	R	R_1	R_2	R_3	R_4	R_5	R_6
6a	H	H	H	H	OH	OH	H
6b	H	H	OH	H	OH	H	OH
6c	H	OH	H	OH	H	OH	H
6d	H	H	OH	H	OH	OH	H
6e	H	NH_2	H	H	OH	OH	H
6f	OH	H	H	H	H	Cl	H
6g	OH	H	OH	H	H	OH	H
6h	OH	H	OH	H	H	OCH_3	H
6i	OH	H	OH	H	H	CH_3	H
6l	OH	H	OH	H	OH	OH	H
6m	OH	H	OCH_3	H	H	F	H

Figure 1. Structures of curcumin and resveratrol analogues.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) proliferation assay (data not shown).

3.1. Compounds 4 and 6g inhibit late phases of influenza virus life-cycle

First, to assess whether the compounds that did not cause toxic effect on cells, could exert an antiviral effect on influenza virus replication, confluent monolayers of MDCK cells highly permissive to influenza virus replication, were infected with influenza A/Puerto Rico/8/34 H1N1 (PR8) virus at low multiplicity of infection (0.01 M.O.I.) to allow multi-cycle replication. One hr after adsorption the cells were washed and different concentrations of compounds or DMSO (control infected CI cells) were added to MDCK and maintained for the duration of the experiment. As shown in Figure 2A, 24 h post infection (p.i.) virus titer, measured as the amount of virus released into the cell supernatant by means of hemagglutinating unit (HAU) assay, was dose-dependently (range 5–20 μg/ml) inhibited by compounds **4** and **6g**. In particular, viral replication was markedly reduced by 10 μg/ml of **6g** (75% vs CI cells) and by 20 μg/ml of **4** (75% vs CI cells) while the concentration of 20 μg/ml of **6g** was able to completely inhibit viral replication. However, since it induced some alteration of the monolayer, we chose to not use it for the following experiments. The compounds **6c** and **6f** were able to inhibit influenza virus in a dose-dependent manner (data not shown), however the molecular mechanisms underlying their anti-influenza activity are still under investigation.

Next, to identify the step(s) of the influenza virus life-cycle that were affected by compounds **4** and **6g**, we first evaluated the effect of these compounds on the PR8 virus alone. No significant antiviral effect was detected when a stock solution of PR8 virus was incubated for 1 h with **4** or **6g** and then used for infection. Similarly, virus production was not affected when compounds were present in cell culture medium only during the 1 h phase of viral adsorption or when MDCK cells underwent a overnight pre-infection treatment with two compounds, with drugs washout right before viral challenge (data not shown). Then, **4** and **6g** were added to MDCK cells immediately after virus challenge and afterwards removed at different time points (Fig. 2B). Post-infection treatment with both compounds for 2 or 4 h produced no significant antiviral effect, which confirmed that they did not act by preventing virus entry into the cells. In MDCK exposed to **4** or **6g** for the first 6 h after infection, slight decreases (about 30%) in viral replication were noted 24 h p.i. More substantial inhibition was observed when exposure was extended to the first 8 h (55%) or 24 h (75%) p.i. In other experiments, the treatment was started at different times after PR8 infection, and the compounds remained in the cell culture medium through 24 h after infection (Fig. 2C).

The highest inhibition (about 79%) of viral replication was achieved when treatment began 2 and 4 h after viral challenge. When treatment was delayed until 6 h after infection, effects were more limited but still observable and finally, when two compounds were added 8 h p.i., no significant inhibition was noted. These data indicate that the antiviral activity of the compounds is largely related to their inhibition of virus life-cycle steps occurring 2-8 h p.i., and possibly related to post-transcriptional events.

3.2. Compound 4 inhibits expression of late viral proteins

Nucleoprotein (NP) is the most abundant influenza A viral protein that is synthesized immediately after infection, whereas the major external glycoprotein hemagglutinin (HA), neuraminidase (NA) and matrix protein 1 (M1) are late gene products.[3] In order to determine whether the inhibition of viral replication was related to the modulation of viral protein synthesis, the cells were infected with PR8, and compounds **6g** (10 μg/ml) or **4** (20 μg/ml) were immediately added after PR8 adsorption. Twenty-four hours after infection, protein from cell lysates were separated by sodium dodecyl sulfate-polyacrylammide gel electrophoresis in reducing conditions and immunoblotted with anti-influenza Abs. As shown in Figure 3 (left panel), while the treatment with **6g** did not cause any effect on the expression on viral proteins, except for NA

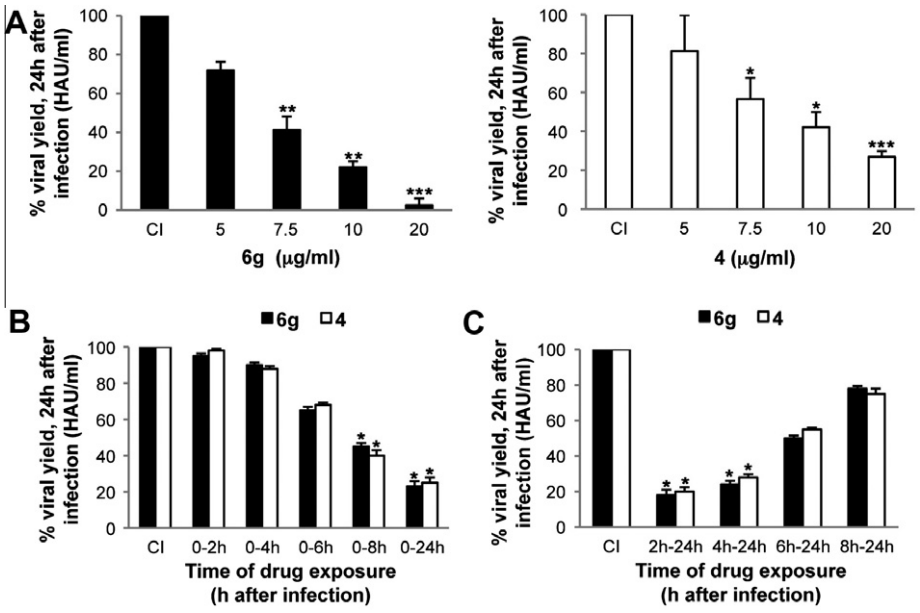


Figure 2. Compounds **4** and **6g** inhibit specific steps of influenza virus life-cycle. (A) Different concentrations of each compound were added to PR8-infected (M.O.I = 0.01) MDCK cells. Viral yields 24 h p.i. are expressed as percentages of those recorded for control infected (CI) cells treated with 0.02% dimethyl sulfoxide (DMSO; the concentration present in culture medium containing the highest dose of compounds). Values shown are means of four experiments, each run in duplicate. $^{*}P$ <0.01; $^{**}P$ <0.001; $^{***}P$ <0.0001 vs CI. Viral yields 24 h p.i. for CI cells and cells infected/treated as follows: (B) **4** and **6g** (20 and 10 μg/ml, respectively) were added to cell cultures immediately after PR8 challenge and removed at different time points (2, 4, 6, 8 or 24 h) after infection; (C) **4** and **6g** were added 2, 4, 6, or 8 h p.i., and maintained in the culture medium for 24 h after infection. Values shown are means of two experiments, each run in duplicate. $^{*}P$ <0.01 vs CI.

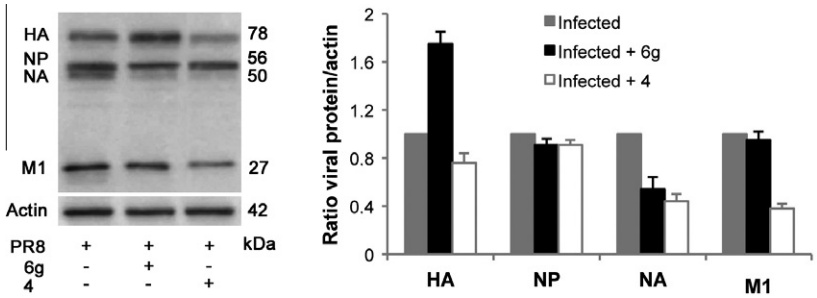


Figure 3. Compound **4** inhibits expression of late viral proteins. (Left panel) expression of viral proteins in influenza A/PR8/34 H1N1 infected cells untreated or treated with **6g** or **4**, respectively. After 24 h, cells were lysed and cell homogenates were separated in reducing conditions by 12% gel, transferred to nitrocellulose membrane and immunostained with goat polyclonal anti-influenza Abs. PR8 virus protein molecular weights are indicated to the right of the figure. HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M1, matrix protein 1. Results are shown for one representative experiment of three performed. (Right panel) densitometric analysis of viral proteins expression shown in (left panel). Results are expressed as ratio of each viral protein to actin.

protein (by 42% vs untreated cells), a significant inhibition of some viral proteins was observable after treatment with **4**.

In particular, densitometric analysis (Fig. 3, right panel) revealed decreased expression of HA (by 25% vs untreated), NA (by 56% vs untreated) and M1 (by 62% vs untreated), even if the expression of NP protein was slight inhibited. These results suggest that the antiviral activity exerted by **4** could be due to an interference with viral protein synthesis, while the effect of **6g** to the inhibition of other steps of virus life-cycle.

3.3. Compounds 6g and 4 retain viral NP in the nucleus of infected cells

During influenza virus replication, viral RNAs are packaged into helical ribonucleoprotein (RNP) complexes with polymerase and NP in the host-cell nucleus and are subsequently exported into the cytosol to be assembled with the other structural proteins.[3] Our previous studies demonstrated that RV blocks the nuclear-cytoplasmic translocation of RNP complexes.[22] To determine whether the same mechanism was involved in **4** and **6g** inhibition of influenza virus replication, immunofluorescence analysis of vRNP trafficking was performed. MDCK cells were infected with PR8 at a high M.O.I. to allow single-cycle replication. Eight hours after infection, cells were fixed, permeabilized and stained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI), and then incubated in turn with primary anti-NP antibody and with fluorescein isothiocyanate-conjugated secondary antibody. As shown in Figure 4 in the untreated cells, NP was located predominantly in the cytosol, and addition of compounds **4** and **6g** led to the inhibition of viral RNP export from nucleus to cytoplasm. In particular, the treatment with **6g** caused a strong inhibition of nuclear-cytoplasmic traffic of NP, suggesting that this compound could interfere with some pathways that regulate this important step of influenza virus replication. These results were confirmed by western blot analysis of NP localization in the nuclear and cytoplasmic extracts after 8 h p.i. As shown in Figure 5 the treatment with the compounds, especially with **6g**, caused an inhibition of NP levels in the cytosol, while the protein was mainly expressed in the nucleus of the cells. Several kinase cascades, such as PKC and MAPKs are activated during influenza virus infection.[11] Pleschka et al.[46] demonstrated that ERK (extracellular signal-regulated kinase) phosphorylation promotes vRNP traffic and virus production. Our previous studies demonstrated the role of p38MAPK activity in nuclear export of RNP complexes of influenza virus. In particular, the inhibition of this kinase decreased vRNP traffic, phosphorylation of viral NP (a key event for the export in the cytoplasm), and viral titers in cells supernatants.[12] Moreover, it is known that RV interferes with several intracellular pathways including those activated by PKC and MAPKs.[36]

Therefore to determine the underlying mechanism for the inhibitory effect of **4** and **6g** on nuclear translocation of vRNP complexes, the possible involvement of the PKC pathway in the antiviral activity of the compounds was investigated in human NCI-H292 infected cells. As shown in Figure 6, the phosphorylation of PKD, the PKC downstream effector,[47] was observed in infected cells 6 h p.i., and this event was markedly reduced by compounds **4** and **6g**. Then, the phosphorylation of p38MAPK, JNK, and ERK pathways was analyzed. As shown in Figure 6, the compound **6g** strongly diminished p38MAPK and JNK phosphorylation, but had no effect on that of ERK 1 and 2. Similar results were obtained in cells treated with **4**, although the inhibition of p38MAPK phosphorylation was less pronounced. These results indicate that the strong inhibition of p38MAPK phosphorylation by **6g** leads to vRNP retaining in the nucleus of infected cells.

3.4. Compound 4 restores the intracellular redox balance during viral infection and affects viral HA localization

Influenza virus infection is associated with redox changes characteristic of oxidative stress, including depletion of GSH levels and a general oxidative stress in both in vivo and in vitro experimental models.[15] Our recent studies have demonstrated a key role of GSH levels in the regulation of viral HA maturation. Indeed, the addition of a GSH derivative was able to restore the reduced environment in infected cells and, as a consequence, to interfere with intracellular redox-regulated pathways responsible for the folding of viral proteins. The final effect on viral replication was an impairment of viral propagation by impeding HA plasma-membrane insertion.[13] Therefore we evaluated the potential antioxidant activity of these compounds. Twenty-four hours after infection, as expected influenza virus-induced a significant depletion of intracellular GSH content (Fig. 7A). On the contrary, in infected cells treated with **4**, GSH levels did not diminish during viral infection, and GSH content was similar to that measured in control (mock-infected) cells. The treatment with **6g** was also able to restore the intracellular GSH content, even if in less extent. A confirmation of this difference was obtained by measuring the redox potentials of **4** and **6g**. Compound **4** showed Epa1 = 0.75 V compared to Epa1 = 1,35 V found for **6g**. It is evident the higher reducing property of 4 compared to that of **6g**.

Next, we evaluated whether these compounds could interfere with HA maturation, especially with HA localization on the plasma-membrane. Immunofluorescence studies of HA localization were performed 8 h after PR8 infection with high M.O.I. Cells were fixed and stained with anti-HA antibody and with secondary antibody conjugated with phycoerythrin. As shown in Figure 7B, viral protein HA was highly expressed, diffused into the cells and localized predominantly on the plasma-membrane of infected cells.

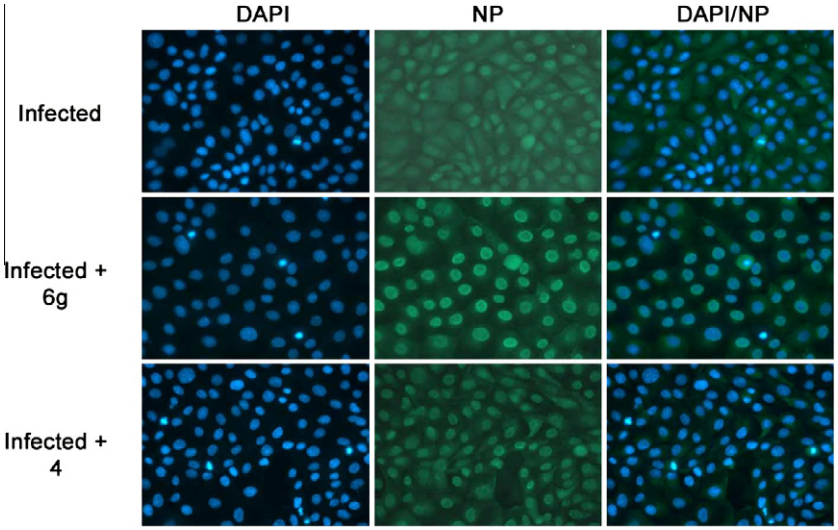


Figure 4. Compounds **6g** and **4** block the nuclear-cytoplasmic vRNP traffic. MDCK cells were infected with 1 M.O.I. to allow single-cycle replication. Cells were fixed, permeabilized and stained with monoclonal anti-NP Ab and analyzed by fluorescence microscopy. Nuclei were stained with DAPI. Merged images are shown in the third column.

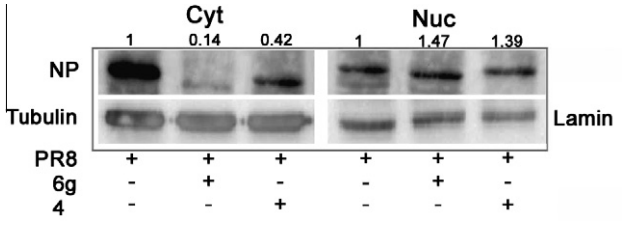


Figure 5. Compounds **6g** and **4** retain the NP in the nucleus of infected cells. Cytosolic and nuclear extracts from **4** or **6g** treated and untreated cells were subjected to SDS-PAGE and immunoblotted with anti-NP Abs. The same nitrocellulose filters were then stripped and restained with anti-α-tubulin or anti-lamin A/C. Above each blot, densitometric analysis expressed as the ratio of viral NP/tubulin or lamin is reported. Results are shown for one representative experiment of three performed.

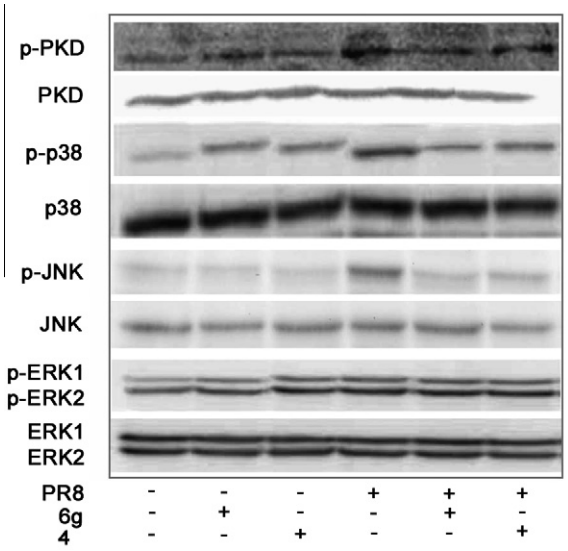


Figure 6. Compounds **4** and **6g** interfere with PKC pathway. NCI-H292 cells were mock- or PR8-infected (1 M.O.I.), treated with **4** or **6g** and lysed 6 h p.i. Proteins were separated by 10% SDS–PAGE and gels were blotted onto nitrocellulose membranes and immunostained with rabbit anti-phospho-PKD, anti-phospho-ERK 1/2, anti-phospho-p38MAPK or anti-phospho-JNK Abs. The same nitrocellulose filters were then stripped and restained with anti-PKD, anti-ERK1/2, anti-p38MAPK, or anti-JNK Abs, respectively. Results are shown for one representative experiment of three performed.

On the contrary, the treatment with the compounds, especially with **4**, inhibited HA expression, and viral protein localization on the plasma-membrane was impaired. In particular, in these latter cells HA was localized on the peri-nuclear zone (see white arrows, Fig. 7B). These results suggest that **4** could exert its antiviral activity through regulation of some redox-sensitive pathways involved in HA maturation. Further studies are in progress to better characterize the molecular mechanism involved in this inhibition.

4. Discussion

In the present study we have demonstrated the antiviral activity of compounds **4** and **6g**. These compounds inhibited the influenza A virus replication in a dose-dependent manner without inducing any cytotoxic effect. The antiviral activity of these molecules is due to inhibition of two key steps of influenza virus life-cycle: (1) nuclear-cytoplasmic traffic of vRNP; (2) maturation of viral HA protein.

These fundamental steps are finely regulated by redox-sensitive intracellular pathways that are activated during viral infection. In particular, vRNP traffic is regulated by several intracellular kinases, including PKC and MAPKs.[11] In our previous paper we have demonstrated that influenza virus-activated p38MAPK phosphorylated viral NP, an event needed for vRNP nuclear export. Inhibition of p38MAP led to retention of NP in the nucleus of infected cells.[12] Here, we demonstrate that the two compounds were able to inhibit p38MAPK and, as consequence, to impede the export of vRNP from the nucleus to the cytoplasm, confirming the role of p38MAPK in the regulation of this step of viral replication.

Viral infection is often associated with redox changes characteristic of oxidative stress, and these alterations are mainly due to virus-induced depletion of intracellular GSH levels.[48] A decrease in GSH content and general oxidative stress have been also demonstrated during influenza virus infection in both in vivo and in vitro experimental models.[49–52] We have recently demonstrated a key role of GSH levels in the regulation of viral HA maturation. Indeed the addition of a GSH derivative impairs viral propagation by

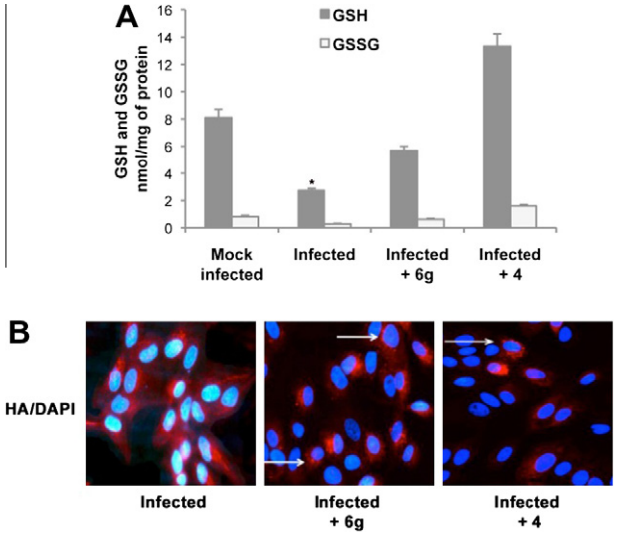


Figure 7. Compound **4** restores the intracellular redox balance and affects viral HA localization. (A) MDCK infected or mock-infected cells were treated with **4** or **6g**, and intracellular GSH and GSSG levels were measured by Glutathione assay kit 24 h p.i. Results are expressed as nanomoles per milligram of protein. Each value represents the mean of two different experiments, each run in duplicate. $^{*}P$ <0.05 versus mock-infected cells. (B) Cells were infected with PR8 (1 M.O.I.) to allow single-cycle replication. Cells were fixed, permeabilized and stained with anti-HA Ab (red fluorescence) and analyzed by fluorescence microscopy. Nuclei were stained with DAPI. Results are shown for one representative experiment of two performed.

impeding HA plasma-membrane insertion.[13] The antioxidant activity of RV has been well demonstrated in numerous studies.[53] However, in our previous paper, RV was not able to restore intracellular levels of GSH in influenza virus-infected cells.[22] Here, the addition of the new compounds, in particular of **4**, to infected cells was able to restore virus-induced depletion of GSH. Moreover, we have observed that the compounds, especially **4**, were able to interfere with HA localization on plasma-membrane, an event that occurs when HA redox-regulated maturation process is completed.[13] These results suggest that the compounds could interfere with some redox-sensitive intracellular pathways involved in maturation of viral HA. Further studies are in progress to evaluate the molecular mechanisms underlying this process.

5. Conclusion

Overall the data demonstrated that the compounds **4** and **6g** exert their anti-influenza activity by inhibiting intracellular metabolic pathways rather than viral proteins. Inactivation of host-cell functions that are essential for the virus replication offers two important advantages: not only it is more difficult for the virus to adapt to, but it can also expected to affect viral replication independently from virus type or strain.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.062.

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