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Differential antiviral and anti-inflammatory mechanisms of the flavonoids biochanin A and baicalein in H5N1 influenza A virus-infected cells

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ABSTRACT

From a panel of 22 flavonoids, we identified six compounds (apigenin, baicalein, biochanin A, kaempferol, luteolin, naringenin) that inhibited influenza A nucleoprotein production in human lung epithelial (A549) cells infected with the highly pathogenic avian influenza H5N1 virus strain A/Thailand/Kan-1/04 in non-toxic concentrations. Baicalein (IC_{50}: 18.79 ± 1.17 μM, selectivity index 5.82) and biochanin A (IC_{50} 8.92 ± 1.87 μM, selectivity index 5.60) were selected for further experiments. Both compounds reduced H5N1 infectious titres (baicalein 40 μM: 29-fold reduction, biochanin A 40 μM: 55-fold reduction after infection at MOI 0.01), virus-induced caspase 3 cleavage, nuclear export of viral RNP complexes, and enhanced the effects of the neuraminidase inhibitor zanamivir. Biochanin A and baicalein also inhibited the replication of the H5N1 strain A/Vietnam/1203/04. Time of addition experiments indicated that both compounds interfere with H5N1 replication after the adsorption period. Further mechanistic investigations revealed clear differences between these two flavonoids. Only baicalein interfered with the viral neuraminidase activity (39 ± 7% inhibition at 100 μM, the maximum concentration tested). In contrast to baicalein, biochanin A affected cellular signalling pathways resulting in reduced virus-induced activation of AKT, ERK 1/2, and NF-kB. Moreover, biochanin A inhibited the virus-induced production of IL-6, IL-8, and IP-10 while baicalein inhibited IL-6 and IL-8 production without affecting IP-10 levels. In primary human monocyte-derived macrophages, only baicalein but not biochanin A impaired H5N1 virus replication. Both flavonoids interfered with the H5N1-induced production of IL-6, IP-10, and TNF-α but not of IL-8 in macrophages. These findings indicate that closely related flavonoids can exert anti-H5N1 effects by different molecular mechanisms.

1. Introduction

Highly pathogenic H5N1 influenza A viruses represent a major pandemic threat. Complication rates are much higher in H5N1 patients than in seasonal influenza or pandemic H1N1/09 patients (Cheung et al., 2006; de Jong, 2008; Hien et al., 2009; McKimm-Breschkin et al., 2007). As of 10th August 2012, 608 confirmed human H5N1 cases had resulted in 359 deaths (www.WHO.int).

During an initial pandemic phase, matched vaccines will be restricted and antiviral drugs will be critical. The efficacy of the approved anti-influenza drugs (adamantanes, neuraminidase inhibitors) is limited, resistant strains emerge, and H5N1 strains appear to be less sensitive to the established anti-influenza drugs than seasonal influenza strains (Bavagnoli and Maga, 2011; Cheung et al., 2006; Cinatl et al., 2007a,b; Deyde et al., 2007; Hampson, 2008; Kieny and Fukuda, 2008; Mckimm-Breschkin et al., 2007; Michaelis et al., 2009; Moscona, 2009; Salter et al., 2011; Sugrue et al., 2008; van der Vries et al., 2008). Hence, additional anti-influenza therapies are needed.

High replication rates and a dysregulation of the host immune response resulting in hypercytokinaemia (“cytokine storm”) are thought to contribute to H5N1 disease severity in humans (Cheung et al., 2002; de Jong et al., 2006; Maines et al., 2008). The control of viral replication and disproportionate immune responses may thus be critical for the successful anti-H5N1 therapies (Cheung et al., 2002; de Jong et al., 2006; Hatta et al., 2010; Lee et al., 2007; Li et al., 2011; Maines et al., 2008; Michaelis et al., 2009; Zheng et al., 2008).

In 2009, the “WHO public health research agenda for Influenza” expressed a need for additional drugs including those that exert immunomodulatory effects and recommended to investigate natural products for anti-influenza activity (www.who.int). Flavonoids

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may exert multiple pharmacological effects including anti-oxidative, anti-inflammatory, and antiviral activities including inhibition of seasonal influenza A (H1N1) viruses (Chen et al., 2011; Grienke et al., 2012; Harborne and Williams, 2000; Kim et al., 2010; Nijveldt et al., 2001; Xu et al., 2010). They may interfere with the influenza virus neuraminidase (Grienke et al., 2012; Wang et al., 2006; Liu et al., 2008), the virus host cell uptake (Song et al., 2007; Wang et al., 2006), or cellular signalling events like the activation of nuclear factor kB (NFkB), AKT, ERK 1/2, p38, and/or JNK (Biswas et al., 2005; Cavet et al., 2011; Hecht et al., 2006; Kole et al., 2011; Sen et al., 2006) involved in influenza virus replication and virus-induced pro-inflammatory gene expression (Hayashi et al., 2008; Ludwig, 2011; Michaelis et al., 2011; Pinto et al., 2011; Shin et al., 2007). Studies that investigate flavonoids in H5N1-infected cells are missing.

Here, we investigated 22 flavonoids for their effect on H5N1 nucleoprotein expression in A549 lung epithelial cells. Baicalein and biochanin A were further investigated for their effects on H5N1 replication and H5N1-induced cytokine expression in A549 cells and in primary human monocyte-derived macrophages (MDMs).

2. Materials and methods

2.1. Drugs

All investigated flavonoids (Suppl. Fig. S1; Suppl. Table S1) were purchased from Indofine Chemical Company (Hillsborough, NJ, USA), ribavirin from Sigma–Aldrich Chemie GmbH (Munich, Germany), and zanamivir from GlaxoSmithKline (Munich, Germany).

2.2. Cells and viruses

A549 cells (human lung carcinoma; ATCC, Manassass, VA, USA: CCL-185) and Vero cells (African green monkey kidney; ATCC: CCL81) were grown at 37 °C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin, and 100 μg/ml streptomycin. Human monocytes were isolated from buffy coats of healthy donors (Institute of Transfusion Medicine and Immune Haematology, German Red Cross Blood Donor Centre, Goethe-University, Frankfurt/Main, Germany) and $CD14^+$ monocytes were differentiated into MDMs as described previously (Geiler et al., 2010).

All experiments with H5N1 were performed in the safety laboratories of the Institute of Medical Virology, Clinics of the Goethe-University, Frankfurt/Main, Germany that are approved for the work with highly pathogenic avian influenza viruses (biosafety level 3+). The H5N1 influenza strain A/Vietnam/1203/04 was received from the World Health organisation (WHO) Influenza Centre (National Institute for Medical Research, London, UK), the H5N1 strain A/Thailand/1(Kan-1)/04 from Dr. Puthavathana (Mahidol University, Bangkok, Thailand). Virus stocks were prepared by infecting Vero cells, and aliquots were stored at −80 °C. Virus titres were determined as 50% tissue culture infectious dose ($TCID_{50}$/mL) in confluent Vero cells. Twenty-four hours of post infection, aliquots of the supernatants were taken. Infectivity was determined by endpoint dilution titration on Vero cells and detection of the cytopathogenic effects after 3–4 days. Virus titres were calculated by the method of Reed and Muench (1938).

2.3. Cell viability assay

Cell viability was investigated using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega GmbH, Mannheim, Germany) following the manufacturer's instruction. This assay quantifies the amount of ATP present which is representative of the number of metabolically active cells. Cell viability was determined after 48 h of treatment if not indicated otherwise.

2.4. Immune staining

Cells were fixed with 40:60 acetone/methanol for 15 min. For the detection of influenza A nucleoprotein, the monoclonal antibody MsX Influenza A (Millipore, Molsheim, France) was used as primary antibody in combination with a biotin-conjugated secondary antibody. Visualisation was performed using a streptavidin-peroxidase complex with 3-amino-9-ethylcarbazole as substrate. For fluorescence staining, the Alexa Fluor 488 goat anti-mouse IgG (H&L) (Invitrogen, Eugene, Oregon, USA) was used as secondary antibody. To detect NF-kB p65 nuclear localization, a rabbit monoclonal anti-NF-kB p65 antibody (New England Biolabs GmbH, Frankfurt/Main, Germany) was used as first antibody and Alexa Fluor 488 goat anti-rabbit IgG (H&L) as secondary antibody. Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich Chemie GmbH, Munich, Germany). An Olympus IX 1 fluorescence microscope (Olympus, Planegg, Germany) was used for visualisation.

2.5. Virus yield reduction assay

Infectious virus titres were determined as described previously (Geiler et al., 2010). Briefly, A549 cells or MDMs were infected with H5N1 viruses in MEM supplemented with 2% FBS, 100 IU/mL penicillin, and 100 mg/mL streptomycin. After 24 h, aliquots of the supernatants were taken and virus titres determined as described under Section 2.2. Flavonoids, zanamivir, or their combinations were present starting from a 1 h pre-incubation period prior to infection if not stated otherwise. For time of addition experiments, flavonoids were added exclusively for a 1 h pre-incubation period prior to infection (pre-treatment), added together with virus exclusively for the 1 h adsorption period (during adsorption), or added exclusively after the virus adsorption period (post infection).

2.6. Neuraminidase inhibition assay

The neuraminidase enzyme activity was determined using the NA-Star Neuraminidase Inhibition resistant assay Kit (AB Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol. This assay uses a chemiluminescent substrate for neuraminidase, sodium (2-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2′-(5-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-*yl*-phenyl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranoside)onate, to measure the activity of neuraminidases derived from influenza A viruses of interest. Briefly, influenza viruses were incubated with flavonoids (zanamivir served as positive control) for 10 min (37 °C), the neuraminidase substrate was added, and the luminescence signal indicating neuraminidase activity was determined.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected and stored at −80 °C. The concentrations of IL6, IL8, CXCL10, and TNFα were detected by ELISA using the Duo Set kit (R&D systems GmbH, Wiesbaden, Germany) according to the manufacturer's protocol. These kits provide calibrated immunoassay standards, predesigned capture antibodies, biotinylated detection antibodies, and streptavidin-coupled horse radish peroxidase for the measurement of detection antibody binding by tetramethylbenzidine.

Table 1
Concentrations of flavonoids that reduce A549 cell viability (CC_{50} determined after 48 h of incubation) and influenza A nucleoprotein expression in H5N1 strain A/Thailand/1(Kan-1)/04 (MOI 0.01)-infected A549 cells (IC_{50} determined 24 h post infection) by 50%.

Flavonoid	CC_{50} (μM)	IC_{50} (μM)	Selectivity index ($CC_{50}/_{50}$)
Apigenin	40.55 ± 3.27	16.01 ± 4.33	2.53
Baicalein	109.41 ± 4.35	18.8 ± 1.17	5.82
Biochanin A	49.91 ± 1.14	8.92 ± 1.87	5.60
Kaempferol	>160[a]	72.41 ± 7.42	>2.21
Luteolin	63.57 ± 3.12	20.07 ± 11.83	3.17
Naringenin	>160	70.68 ± 8.08	>2.26

[a] Maximum concentration tested.

2.8. Western blot

Cells were lysed in Triton X-sample buffer and separated by SDS–PAGE. Proteins were detected using antibodies directed against AKT, phosporylated AKT, ERK 1/2, phosphorylated ERK 1/2, p38, phosphorylated p38, JNK, phosphorylated JNK, nuclear factor-kappa B subunit p65 (NF-kB p65), inhibitor of kappa-B (IkB), caspase-3 (New England Biolabs GmbH, Frankfurt am Main, Germany), or β-actin (Sigma–Aldrich Chemie GmbH, Munich, Germany). Proteins were visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

2.9. Caspase-3 inhibition assay

The caspase-3 inhibitory effects of flavonoids were measured using the cell free caspase-3 enzyme inhibition assay kit (EMD Chemical Inc., Darmstadt, Germany) according to the manufacturer's protocol. This assay measures caspase-3 activity using the fluorimetric caspase-3 substrate Ac-DEVD-AMC.

2.10. Statistics

Two groups were compared by student's *t*-test, more groups were compared by ANOVA. Pairwise Multiple Comparison was per-

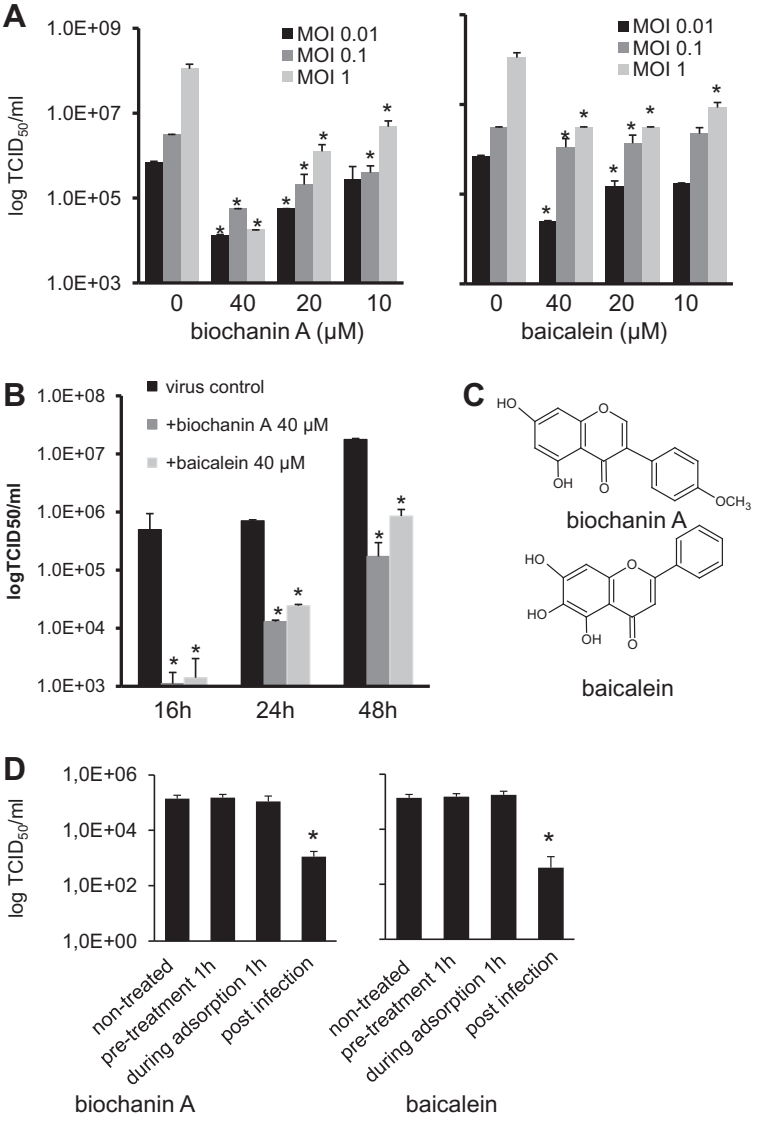

Fig. 1. Effects of biochanin A and baicalein on viral titres in H5N1 strain A/Thailand/1(Kan-1)/04-infected A549 cells. (A) Concentration-dependent effects of biochanin A or baicalein on viral titres in A549 cells infected at different MOIs 24 h post infection. *$p < 0.05$ compared to virus control. (B) Time-dependent effects of biochanin A (40 μM) or baicalein (40 μM) on viral titres in H5N1 (MOI 0.01)-infected A549 cells at different time points post infection. *$p < 0.05$ compared to virus control. (C) Chemical structures of baicalein and biochanin A. (D) Time of addition experiments in A549 cells infected at MOI 0.01. Cells were treated with biochanin A (40 μM) or baicalein (40 μM) for 1 h prior to infection (pretreatment 1 h), during the 1 h viral adsorption period (during adsorption 1 h), or after the 1 h adsorption period and virus removal (post infection). Virus titres were determined 24 h post infection. *$p < 0.05$ compared to virus control.

formed by Student–Newman–Keuls-test. All statistical analyses were performed using SigmaStat (Systat, Erkrath, Germany).

3. Results

3.1. Effects of flavonoids on H5N1 nucleoprotein production in A549 cells

Six out of 22 flavonoids (apigenin, baicalein, biochanin A, kaempferol, luteolin, naringenin) reduced H5N1 nucleoprotein expression in strain A/Thailand/1(Kan-1)/04 (MOI 0.01)-infected A549 cells by 50% (IC_{50}) at non-toxic concentrations (Table 1, Suppl. Table S1). The IC_{50} values were determined 24 h post infection, the CC_{50} values (concentrations that decrease cell viability by 50%) were determined after 48 h of incubation. Due to their molar activities and their selectivity indices, baicalein (IC_{50}: 18.79 ± 1.17 μM, CC_{50}: 109.41 ± 4.35, selectivity index 5.82) and biochanin A (IC_{50} 8.92 ± 1.87 μM, CC_{50}: 49.91 ± 1.14, selectivity index 5.60) were selected for further experiments. Their effects on A549 cell viability were additionally investigated after 24 and 72 h incubation periods. After 24 h, both compounds showed higher CC_{50} values than after 48 h (baicalein: >160 μM; biochanin A: 111.44 ± 10.22 μM). While the CC_{50} value of baicalein further decreased after 72 h (69.32 ± 9.90 μM) compared to the 48 h time point, treatment of A549 cells with biochanin A for 72 h (50.11 ± 10.55 μM) did not result in a decreased CC_{50} value compared to 48 h treatment.

3.2. Effects of biochanin A and baicalein on H5N1 virus titres

Biochanin A and baicalein reduced the virus titres in A549 cells infected with two different H5N1 strains (A/Thailand/1(Kan-1)/04, A/Vietnam/1203/04) at MOIs between 0.01 and 1 in a concentration-dependent manner (Fig. 1A; Suppl. Fig. S2). To investigate the antiviral effects over time, virus titres were determined in A/Thailand/1(Kan-1)/04 (MOI 0.01)-infected A549 cells after 16, 24, and 48 h in the absence or presence of biochanin A (40 μM) or baicalein (40 μM) (Fig. 1B). Both substances exerted sustained antiviral effects. The chemical structures of baicalein and biochanin A are presented in Fig. 1C.

Time of addition experiments revealed that both compounds affect viral replication after the viral adsorption period (Fig. 1D). Addition of both substances after the viral adsorption period was sufficient to exert maximal antiviral effects.

Novel anti-influenza drugs may be used in combination with established anti-influenza drugs, especially neuraminidase inhibitors. Therefore, it is important to know whether novel drugs may affect the efficacy of neuraminidase inhibitors. To investigate this,

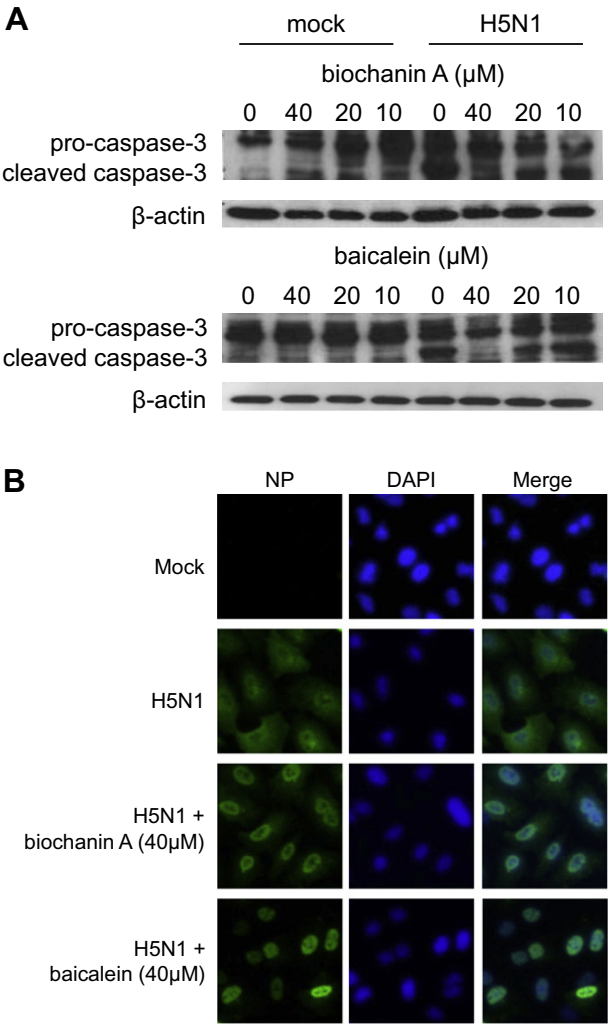

Fig. 3. Effects of biochanin A and baicalein on H5N1 influenza A virus-induced caspase-3 activation and the export of viral ribonucleoprotein (RNP) complexes. (A) Pro-caspase-3 and cleaved (activated) caspase-3 detected by Western blot in A549 cells infected with H5N1 A/Thailand/1(Kan-1)/04 (MOI 1) 24 h post infection. (B) A549 cells infected with H5N1 A/Thailand/1(Kan-1)/04 (MOI 1) and stained for influenza A virus nucleoprotein (NP) at 8 h p.i. (green). The cell nuclei were DAPI-stained (blue). NP is a constituent of the RNP complex. Therefore, the nuclear NP retention in biochanin A- and baicalein-treated H5N1-infected cells indicates the inhibition of the nuclear RNP export. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

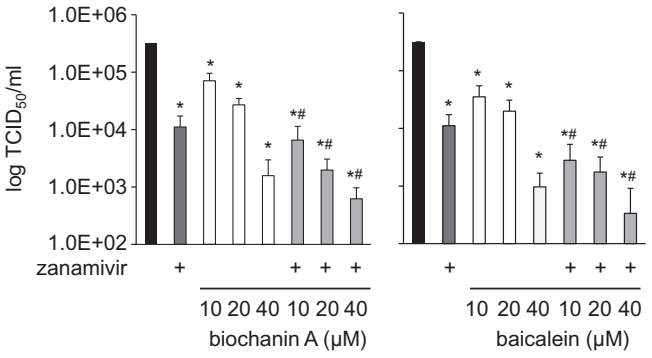

Fig. 2. Effects of biochanin A and baicalein on H5N1 titres in combination with zanamivir 10 ng/mL. A549 cells were infected with H5N1 strain A/Thailand/1(Kan-1)/04 (MOI 0.01). Virus titres were determined 24 h post infection. $^{*}p < 0.05$ compared to virus control, $^{\#}p < 0.05$ relative to either single treatment.

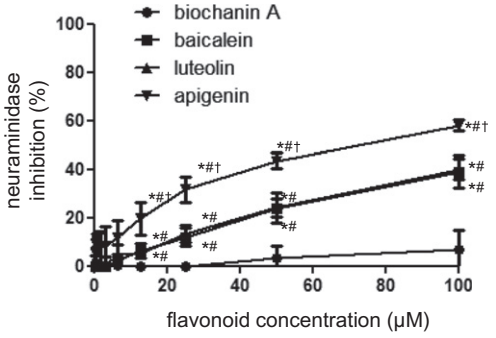

Fig. 4. Effects of selected flavonoids on influenza A virus neuraminidase activity. $^{*}P < 0.05$ relative to control; $^{\#}P < 0.05$ relative to biochanin A; $^{\dagger}P < 0.05$ relative to baicalein and luteolin.

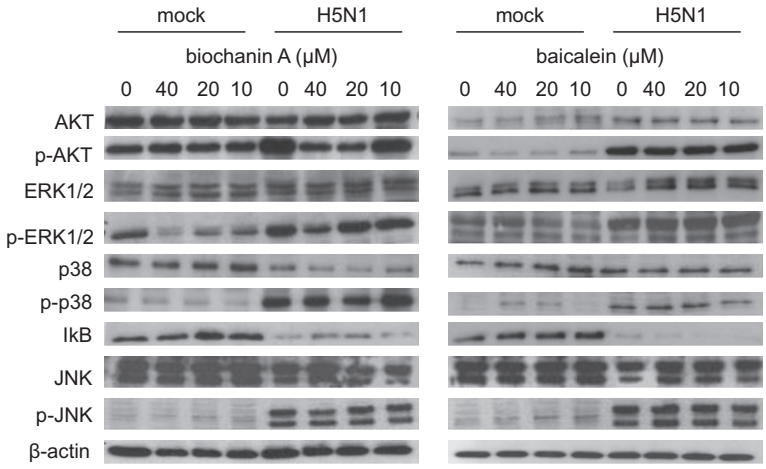

Fig. 5. Effect of biochanin A and baicalein on H5N1-induced expression and/or activation of cellular proteins. Cellular proteins were detected by Western blot in A549 cells infected with H5N1 A/Thailand/1(Kan-1)/04 (MOI 1) 24 h post infection.

A/Thailand/1(Kan-1)/04 (MOI 0.01)-infected A549 cells were treated with combinations of the neuraminidase inhibitor zanamivir (10 ng/mL) and varying biochanin A or baicalein concentrations (Fig. 2). Results indicated that both substances significantly enhanced the effects of zanamivir.

3.3. Effects of biochanin A and baicalein on H5N1-induced caspase-3 activation and on the nuclear export of viral RNP complexes

Caspase-3 activation was shown to be involved in influenza A virus replication (Wurzer et al., 2003; Geiler et al., 2010). A/Thailand/1(Kan-1)/04 (MOI 1) infection of A549 cells resulted in increased levels of cleaved caspase-3 24 h p.i. that was inhibited by biochanin A (40 μM) and baicalein (40 μM) (Fig. 3A) although neither biochanin A nor baicalein interacted directly with caspase-3 (Suppl. Table S2). Thus, both compounds inhibited virus-induced cellular signalling up-stream of caspase-3 activation.

The inhibition of virus-induced caspase-3 activation was shown to be associated with the nuclear retention of the viral ribonucleoprotein (RNP) complex (Wurzer et al., 2003; Geiler et al., 2010). Accordantly, both compounds caused nuclear retention of the viral RNP complexes (Fig. 3B).

3.4. Baicalein but not biochanin A inhibits the H5N1 neuraminidase

Flavonoids like apigenin and luteolin were previously shown to inhibit seasonal influenza A virus neuraminidases (Grienke et al., 2012; Liu et al., 2008). Among the flavonoids with the highest anti-H5N1 activity investigated here, only baicalein, apigenin, and luteolin but not biochanin A reduced H5N1 neuraminidase activity (Fig. 4). Baicalein 100 μM (the maximum concentration tested) inhibited the H5N1 neuraminidase activity by 39 ± 7%.

3.5. Differential effects of biochanin A and baicalein on H5N1-induced host cell signalling

Host cell proteins including the kinases PI3K/AKT, ERK, p38, JNK, and the transcription factor NFκB may contribute to influenza virus replication and virus-induced pro-inflammatory gene expression (Geiler et al., 2011; Hayashi et al., 2008; Ludwig, 2011; Michaelis et al., 2009; Pinto et al., 2011; Shin et al., 2007). Accordingly, infection of A549 cells with H5N1 strain A/Thailand/1(Kan-1)/04 (MOI 1) resulted in enhanced phosphorylation of AKT, ERK1/2, JNK, and p38 (Fig. 5; Suppl. Fig. S3). Biochanin A interfered with the H5N1-induced phosphorylation of AKT and ERK 1/2 but not of JNK or p38 phosphorylation (Fig. 5; Suppl. Fig. S3). Baicalein did not affect the phosphorylation of these proteins (Fig. 5; Suppl. Fig. S3).

Activation of NFκB causes degradation of its negative regulator IκB. Infection of A549 cells with A/Thailand/1(Kan-1)/04 (MOI 1)

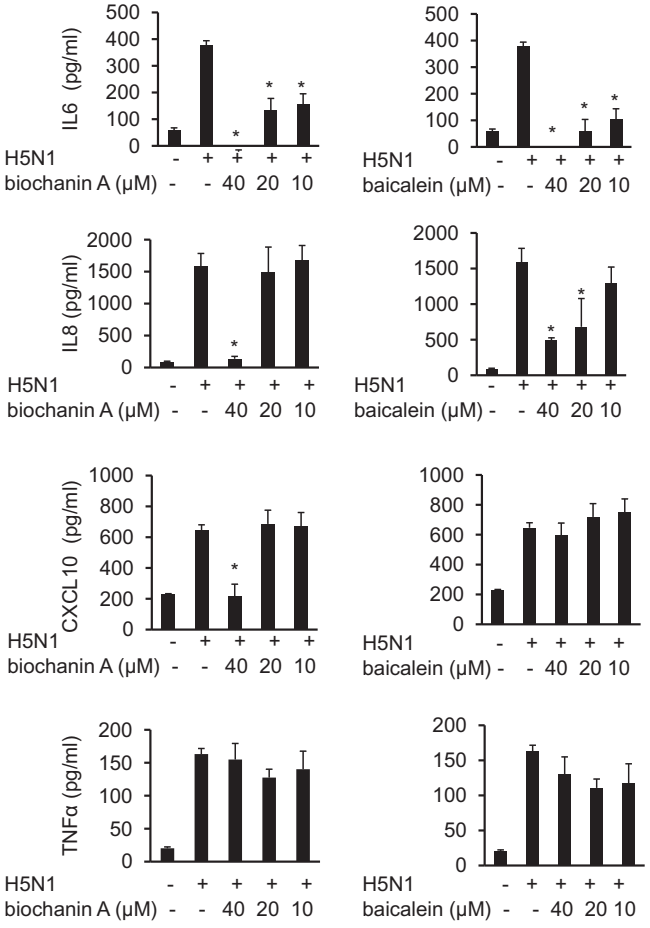

Fig. 6. Effect of biochanin A and baicalein on expression of pro-inflammatory cytokines in H5N1 strain A/Thailand/1(Kan-1)/04 (MOI 0.01)-infected A549 cells. Cell supernatants were collected at 24 h post infection and the cytokine content was analysed by ELISA. $p < 0.05$ relative to virus control.

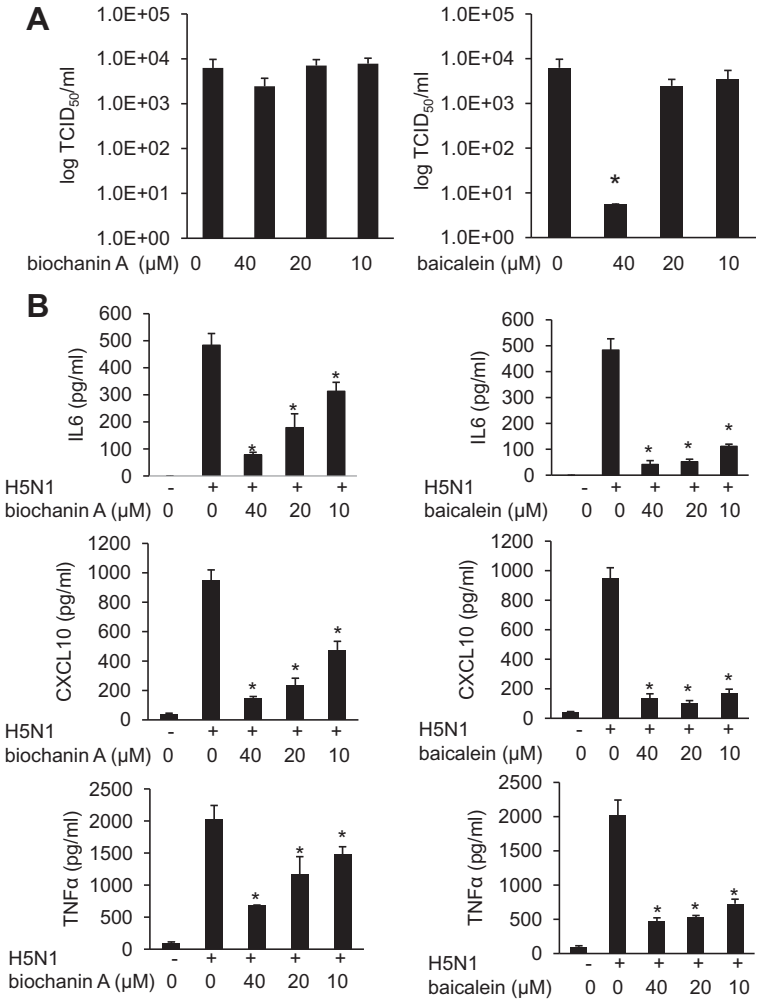

Fig. 7. Effects of biochanin A and baicalein on H5N1 replication and H5N1-induced cytokine expression in primary human monocyte-derived macrophages (MDMs). (A) MDMs were infected with H5N1 A/Thailand/1(Kan-1)/04 (MOI 1) in the absence or presence of biochanin A or baicalein and the viral titres (expressed as $TCID_{50}$/mL) were determined 24 h post infection. $^{*}p < 0.05$ compared to virus control. (B) MDMs were infected with H5N1 A/Thailand/1(Kan-1)/04. Cell culture supernatants were collected at 24 h p.i. in the absence or presence of biochanin A or baicalein treatment and investigated for cytokine levels by ELISA. $^{*}p < 0.05$ relative to virus control.

caused a decrease of cellular IκB levels that was partially prevented by biochanin A but not by baicalein (Fig. 5; Suppl. Fig. S3). NFκB then enters the nucleus where it binds to the promoters of its response genes. In accordance with H5N1-induced IκB degradation, A/Thailand/1(Kan-1)/04 (MOI 1) infection of A549 cells resulted in nuclear NFκB accumulation (Suppl. Fig. S4). Biochanin A but not baicalein inhibited this virus-induced nuclear NFκB p65 accumulation (Suppl. Fig. S4).

3.6. Differential effects of biochanin A and baicalein on H5N1-induced pro-inflammatory cytokine expression in A549 cells

The H5N1-induced host cell expression of pro-inflammatory cytokines including IL6, IL8, CXCL10, and TNFα has been correlated with the disease severity in H5N1 patients (Chan et al., 2005; de Jong et al., 2006). Biochanin A and baicalein inhibited IL6 and IL8 secretion in A/Thailand/1(Kan-1)/04 (MOI 0.01)-infected A549 cells (Fig. 6). Only biochanin A interfered with the H5N1-induced CXCL10 production. None of the substances interfered with TNF-α secretion (Fig. 6). Neither biochanin A nor baicalein influenced the secretion of the investigated cytokines in non-infected A549 cells (Suppl. Fig. S5). The inhibitory effects of biochanin A and baicalein on H5N1-induced pro-inflammatory gene expression did not correlate consistently with their effects on viral replication. This suggests that there is no direct correlation between the anti-inflammatory and virus inhibitory effects.

3.7. Differential effects of biochanin A and baicalein in H5N1-infected primary human monocyte-derived macrophages (MDM)

Infection of macrophages with H5N1 resulted in higher pro-inflammatory cytokine expression than with seasonal influenza or pandemic H1N1/09 strains and this increased cytokine expression was suggested to contribute to H5N1 disease severity (Cheung et al., 2002; Deng et al., 2008; Geiler et al., 2011; Guan et al., 2004; Lee et al., 2011; Maines et al., 2008; van Riel et al., 2006; Woo et al., 2010). Baicalein but not biochanin A interfered with H5N1 strain A/Thailand/1(Kan-1)/04 replication in MDMs (Fig. 7A). Both compounds inhibited the H5N1-induced secretion of IL6, CXCL10, and TNFα (Fig. 7B) but not of IL8 (Suppl. Fig. S6). Neither biochanin A nor baicalein affected cytokine production in non-infected MDMs (Suppl. Fig. S7).

4. Discussion

Here, we identified 6 flavonoids (apigenin, baicalein, biochanin A, kaempferol, luteolin, naringenin) from a panel of 22 flavonoids that impaired H5N1 nucleoprotein expression A549 lung epithelial cells. Baicalein and biochanin A were chosen for further inves-

tigations due to their molar activities and selectivity indices. Biochanin A had not been shown before to exert anti-influenza activity, while baicalein had previously been shown to inhibit seasonal influenza A virus replication in cell culture and in mice and to increase the antiviral effects of ribavirin (Xu et al., 2010; Chen et al., 2011). Both compounds inhibited the replication of two different H5N1 strains (A/Thailand/1(Kan-1)/04, A/Vietnam/1203/04).

Although some flavonoids were described to impair the host cell uptake of influenza viruses (Wang et al., 2006; Song et al., 2007), time-of-addition experiments revealed that biochanin A and baicalein acted exclusively after virus adsorption. Both substances inhibited critical steps within the viral replication cycle including virus-induced caspase-3 activation and the nuclear export of viral RNP complexes. Also, both compounds enhanced the antiviral effects of the neuraminidase inhibitor zanamivir.

However, biochanin A and baicalein differed in their anti-influenza mechanisms. While baicalein inhibited the H5N1 neuraminidase, biochanin A did not. Biochanin A, but not baicalein, interfered with the activation of AKT, ERK 1/2, and NFκB that are constituents of cellular signalling pathways involved in influenza virus replication and virus-induced cellular pro-inflammatory cytokine production (Geiler et al., 2011; Ludwig, 2011; Michaelis et al., 2009; Pinto et al., 2011; Shin et al., 2007).

For the investigation of H5N1-induced pro-inflammatory gene expression, four cytokines were selected that had been correlated to the severity of H5N1 disease: IL6, IL8, CXCL10, and TNFα (Michaelis et al., 2009). Biochanin A and baicalein reduced the production of IL6 and IL8 in H5N1-infected A549 cells. Biochanin A but not baicalein reduced CXCL10 expression. This discrepancy may be a consequence of the different modes of anti-influenza action of these two compounds.

Finally, we compared the effects of biochanin A and baicalein on H5N1-infected primary human monocyte-derived macrophages. Alveolar macrophages are susceptible to H5N1 infection (Deng et al., 2008; van Riel et al., 2006; Maines et al., 2008) and the enhanced H5N1-induced pro-inflammatory cytokine expression in macrophages compared to macrophages infected with seasonal or pandemic H1N1/09 strains was correlated to H5N1 disease severity (Cheung et al., 2002; Guan et al., 2004; Woo et al., 2010; Geiler et al., 2011). Our results indicated that the effects of biochanin A and baicalein are cell type specific. Both compounds had interfered with H5N1 replication in A549 cells in concentrations of 10 μM. In macrophages, baicalein inhibited H5N1 replication only at a concentration of 40 μM while biochanin A did not affect H5N1 replication. There appears to be an uncoupling between the effects of biochanin A and baicalein on viral replication and virus-induced cytokine expression. In macrophages, both compounds interfered with the H5N1-induced production of IL6, CXCL10, and TNFα in concentrations of 10 μM that did not attenuate H5N1 replication. Also, there is a difference in the spectrum of H5N1-induced cytokines that were affected by biochanin A or baicalein in the different cell types. Both compounds interfered with IL6, CXCL10, and TNFα secretion in H5N1-infected macrophages. Biochanin A inhibited IL6, IL8, and CXCL10 production and baicalein IL6 and IL8 production in H5N1-infected A549 cells.

In conclusion, our data demonstrate that the flavonoids biochanin A and baicalein interfere with H5N1 replication and/or H5N1-induced pro-inflammatory gene expression in lung epithelial cells and macrophages. Biochanin A and baicalein differ in their antiviral mechanisms although the substances are structurally closely related. The decipherment of these complex pharmacological actions exerted by flavonoids in the context of H5N1 infection may result in the identification and/or design of optimised compounds for the treatment of H5N1 disease in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.10.004.

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