

In vitro Broad Antiviral Function against HBV, HSV, H3N2 Replication by Baicalin and Oroxylin A-7-O-B-D-Glucoside

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Abstract

Flavonoids have been previously shown to possess anti-viral activities *In vitro*. Oroxylin A-7-O-β-D-glucoside (OAG), a flavonoids produced by microbial conversion, and its substrate baicalin, were assayed for antiviral function against hepatitis B virus (HBV), herpes simplex virus type 2 (HSV-2) and influenza A virus (H3N2). Incubation with 100 µg/ml OAG or baicalin for 9 days reduced human HBV-transfected liver cell line HepG2 2.2.15 secretion of Hepatitis B surface antigen (HBsAg) by 83.17%, and 47.175%, respectively, and Hepatitis B e antigen (HBeAg) by 27.35%, 25.56% respectively.

OAG and baicalin inhibited HSV-II-induced cell death in a concentration dependent manner (ranging from by 75% and 62.5%, respectively at 12.5 µg/ml and 50%, 37.5%, respectively at 6.25 µg/ml).

OAG (100 µg/ml) and baicalin (50 µg/ml) also effectively inhibited H3N2-induced toxicity in MDCK by 62.5% and 50%, respectively.

In summary, OAG and baicalin could inhibit several viruses *In vitro* and OAG was more potent than baicalin. OAG may represent a candidate antiviral with broad activity against HBV, HSV-2 and H3N2 infection.

Keywords: Oroxylin A-7-O-B-D-Glucoside; Baicalin; Hepatitis B Virus; Herpes Simplex Virus Type 2; H3N2

Introduction

Worldwide, 240 million people are chronically infected with Hepatitis B virus (HBV) [1]. Currently only interferon- α and nucleotide analogues (NAs) are approved for the treatment of HBV. However, they rarely clear chronic HBV infection. These therapies also induce dose-dependent side effects and can drive drug resistance during long-term treatment [2].

Herpes simplex virus type 2 (HSV-2) is a large double-stranded DNA and enveloped virus causing a broad range of symptoms including genital disease and encephalitis in humans, particularly immunocompromised patients [3-5] HSV-2 is mainly transmitted sexually, but also can be vertically transmit from mother to child during birth. HSV-2 infection can be treated with NAs such as acyclovir, but frequent NA use can cause relatively high toxicity and emergence of drug-resistant HSV strains especially in immunocompromised patients [6]. Influenza A virus (IAV) is a common human pathogen, and can cause epidemics of severe respiratory illness, causing significant mortality and economic loss [7,8]. Three drugs are currently available for the treatment of IAV, including RNA polymerase inhibitors (ribavirin), neuraminidase inhibitors (zanamivir), and M2 channel blockers (amantadine) [9,10].

Alarmingly, viral resistance to these therapies is increasingly reported [11,12], and safer and more effective antiviral are required to treat HBV, HSV and H3N2 infections.

Baicalin is an extract of the root of *Scutellaria baicalensis* Georgi, and has been shown to provide multiple beneficial effects including anti-oxidative, anti-tumor and anti-bacterial functions [13-15]. In addition, baicalin has exhibited antiviral activity against HBV, HSV and H3N2 [16-18]. However, clinical application of baicalin is restricted by its low solubility and poor bioavailability. Microbial transformation is increasingly used to structurally modify natural compounds due to its high specificity and environmental compatibility, for example for ginsenosides [19,20]. To resolve this bottleneck in the clinical use of baicalin, we previously established a microbial transformation method which *Rhizopus delemar* As3.646 efficiently transforms baicalin to oroxylin A-7-O-β-D-glucoside (OAG). In this study, we compared the antiviral activity of baicalin and OAG against HBV, HSV and IAV. This is the first report to show the broad antiviral potential of OAG.

Material and Methods

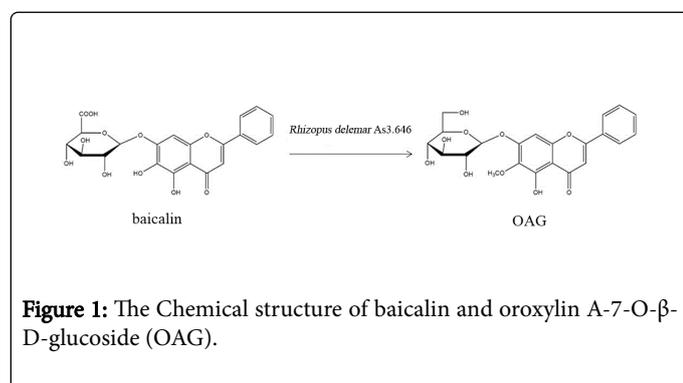
Chemicals and strain

Baicalin was purchased from Ningbo traditional Chinese Pharmaceutical Co. Ltd. Ribavirin, Dimethylsulfoxide (DMSO) and 3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Ethanol, methanol, ethyl acetate, petroleum ether, chloroform were obtained from Sinopharm Chemical Reagent Co. Ltd. *Rhizopus delemar* As3.646 was received from the department of biosynthesis and medical chemistry at the Fudan University School of Pharmacy.

Biotransformation, extraction, isolation and identification of OAG

Rhizopus delemar As3.646 seeds were used to inoculate 20 L of potato dextrose culture medium (Potato Starch 5.0 g/L Glucose 20 g/L pH 6.0 ± 0.2) which was cultured at 28°C 180 r/min for 2 days, then pretreated with the substrate, and cultured for a further 3 days. Mycelium was then filtered from the fermentation liquid and the remaining fluid was extracted with ethyl acetate for three times. The extract was then concentrated under the reduced pressure. The mycelium was soaked in ethanol for 1 hour, suction filtered, and the soaking solution was concentrated without ethanol, extracted with ethyl acetate three times, and concentrated under the reduced pressure. The two ethyl acetate extracts were pooled. Five grams of ethyl acetate extract was first fractionated with silica gel column chromatography (mesh 200-300, 300 g, Qingdao Marine Chemical Factory, China), and 6 fractions were collected with a gradient mobile phase of chloroform and ethanol. Fraction 3 was subjected to high performance liquid chromatography (ODS column: 5 μm, 250 × 10 mm, SunFire) with methanol/H₂O (50:50) as the mobile phase to generate compound 1 (20 mg; (Figure 1)).



The purified compound 1 was a yellow powder with a melting point of 234–235°C. ESI-MS showed peaks at *m/z* 447 [M+Na] and 469 [2M+Na]. This compound was later identified as OAG (Figure 1) based on its ¹H-NMR (DMSO-d₆) and ¹³C-NMR (DMSO-d₆) data that were consistent with the literature [17,21].

Anti-HBV test

Cell culture: The HBV stably transfected cell line HepG2 2.2.15 was provided by the Chinese Academy of Medical Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) at 37°C in 5% CO₂ and in a humidified incubator. Test reagents in this study were dissolved in DMSO. Cells were seeded in 24-well tissue culture plates at approximately 1 × 10⁶/ml, and cultured in medium containing 0.2% DMSO and maintained for 48 h prior to the OAG and baicalin treatment to stabilize the HBV DNA level. The cell culture supernatants and HepG₂ 2.2.15 cells were collected immediately before

the first dose (day 0) and after 9 days of treatment, and were then stored at -70°C until analysis.

Toxicity assay: Cells were cultured in 96-well plates at 2 × 10⁵ cells/well. After 4 days, the culture media were removed and media containing five concentrations of OAG and baicalin, ranging from 12.5 to 200 μg/ml, were added for another 4 d. MTT solution (10 ml in 100 ml medium) was then added to all wells, and incubated at 37°C for 4 h, then the media was removed and 100 μl DMSO added. The appearance of dark blue crystals was directly quantified by microplate reader at a wavelength of 490 nm [17].

Measurement of HBsAg and HBeAg: Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the supernatant was quantified after 9 days of culture in the presence or absence of OAG and baicalin [16]. The concentration of HBsAg or HBeAg was measured by an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's protocol. Each measurement was performed in triplicate. The inhibition rates (%) of the tested compounds on HBsAg or HBeAg concentration were calculated as follows: inhibition ratio = [OD(control) - OD(sample)] / OD(control) × 100% [17].

Analysis of HBV DNA, HBV DNA copy numbers by quantitative PCR: HepG2 2.2.15 cells were grown in 24-well culture plates at a density of 5 × 10⁵ cells/ml. The medium with the compounds was replaced every 4 days. On the eighth day, HBVDNA in the replaced medium was detected by qPCR in BIO-RAD iCycler (BIO-RAD, USA). The forward primer was 5'-TGTCCTGGTTATCGCTGG-3', the reverse primer was 5'-CAAACGGGCAACATACCTT-3', and the Taqman probe was FAM-5'-TGTGTCTGCGGCGTTTTATCAT-3'-TAMRA. After initial incubation at 95°C for 90 s to activate the Taq polymerase, 40 cycles of amplification were conducted using the following program: 95°C for 5 s, 60°C for 30 s and 72°C for 20 s. The standards were prepared by serial dilutions of a known amount of GAPDH amplicon. The HBVDNA copy number was normalized to GAPDH and calculated by the iQTM Real-Time System software (Version 3.0 for windows, BIORAD, USA). HBV DNA inhibition rate (%) = (copy number of the control - copy number of the study sample) / copy number of the control × 100% [22].

Anti-HSV-II Test

HSV-II cytotoxicity: Vero cells were seeded onto 96-well plates at 5 × 10⁴ cells per ml in RPMI medium (Gibco) supplemented with 10% FCS (Gibco). Then 100 μl of serial dilutions of medium containing OAG or baicalin were added to each well a confluent cell monolayer in three times. After incubation at 37°C with 5% CO₂ for 72 h, cells were observed by electron microscopy and the MTT assay was used to assess the impact of HSV infection on cell growth [22].

Anti-HSV-II activity: Cells were seeded in 96-well plates at 5 × 10⁴ cells per well in 100 μl. After incubation at 37°C with 5% CO₂ for 24 h, cells were inoculated with HSV-II (100 TCID₅₀). [23]. After 1.5 h, the cells were washed three times with PBS, then media containing the indicated concentrations of OAG or baicalin was added. After a further 72 h, virus replication was assessed by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Anti-H3N2 test

H3N2 cytotoxicity: MDCK cells were grown in DMEM (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA)

at 37°C in a humidified incubator with 5% CO₂ in 24-well plates at approximately 2 × 10⁵/ml for 2 days. Then 100 µl of serial dilutions of medium containing OAG or baicalin were added to each well a confluent cell monolayer in triplicate. The cells were cultured for a further 9 d, and the medium was replaced every 3 days. Cell viability was assessed by MTT assay, as previously described [24].

Anti-H3N2 activity: MDCK cells were cultured in 96-well plates at 2 × 10⁴ per well. After incubation at 37°C with 5% CO₂ for 24 h, cells were inoculated with H3N2 (100 TCID₅₀), then, 2 h later, washed three times with PBS. Then 100 µl of serial dilutions of medium containing OAG or baicalin were added to each well. After incubation for a further 72 h, a µl mix (including 6% bovine serum DMEM medium and an equal volume of 1.5% agarose) was added to each well. The cells were further cultured at 34°C under 5% CO₂ for 48 h, then the cover layer was removed and the cell monolayer was fixed with 10% formalin, stained with 1% crystal violet, and plaques were counted [24].

Results

In vitro anti-HBV activity of OAG

The influence of OAG on cell viability was assayed in cultured HepG2 2.2.15 cells. As shown by MTT assay cell proliferation was not inhibited in the presence of 50 µg/ml baicalin or 100 µg/ml OAG (Figure 2). The cytotoxic concentrations of baicalin and OAG 50% were 108.07 µg/ml and 177.85 µg/ml, respectively. These results were used to determine the dose range in the following experiments.

As shown in Figure 2, treatment of HepG2 2.2.15 cells with OAG and baicalin at various concentrations for 9 d significantly reduced secretion of HBsAg and HBeAg in a dose-dependent manner. Ribavirin, as the positive control, showed 57.33% inhibition rate on HBsAg secretion and 29.09% inhibition rate on HBeAg secretion. OAG showed a similar inhibitory effect its parental baicalin on HBeAg secretion. However, OAG inhibited HBsAg secretion more potently than baicalin. OAG also showed better inhibitory activity on HBsAg secretion at the same concentration of 50 µg/ml. Non-cytotoxic concentrations of OAG (100 µg/ml) could inhibit HBsAg secretion by 83.17%.

OAG inhibition of HBV replication *In vitro*: To further investigate the anti-HBV activity of OAG and baicalin, the levels of HBV DNA in HBV infected HepG2 2.2.15 cells were evaluated. Consistent with inhibition of HBsAg and HBeAg secretion, the cells incubated with samples (OAG, baicalin or ribavirin) for 9 days, lower levels of extracellular HBV DNA were detected, and this effect was dose-dependent (Figure 2). At the same doses 25 and 50 µg/ml, similar inhibitory effect on HBV DNA between OAG and the parental baicalin was noted. OAG showed much greater inhibitory effect at 100 µg/ml at which baicalin could not be used because of the toxicity and the effect was similar to what ribavirin showed at 50 µg/ml.

OAG inhibition of HSV-II *In vitro*

The TCID₅₀ of HSV-II in Vero cells, as detected by cytopathogenic effect (CPE), was 10⁻⁴ in the virus titration assay. The cytotoxicity of the tested compounds in Vero cells was estimated by cytotoxicity assay. The TC₅₀ was 59.54 µg/ml for OAG and 82.13 µg/ml for baicalin, indicating no or low cytotoxicity of these compounds at the EC₅₀ (Figure 3).

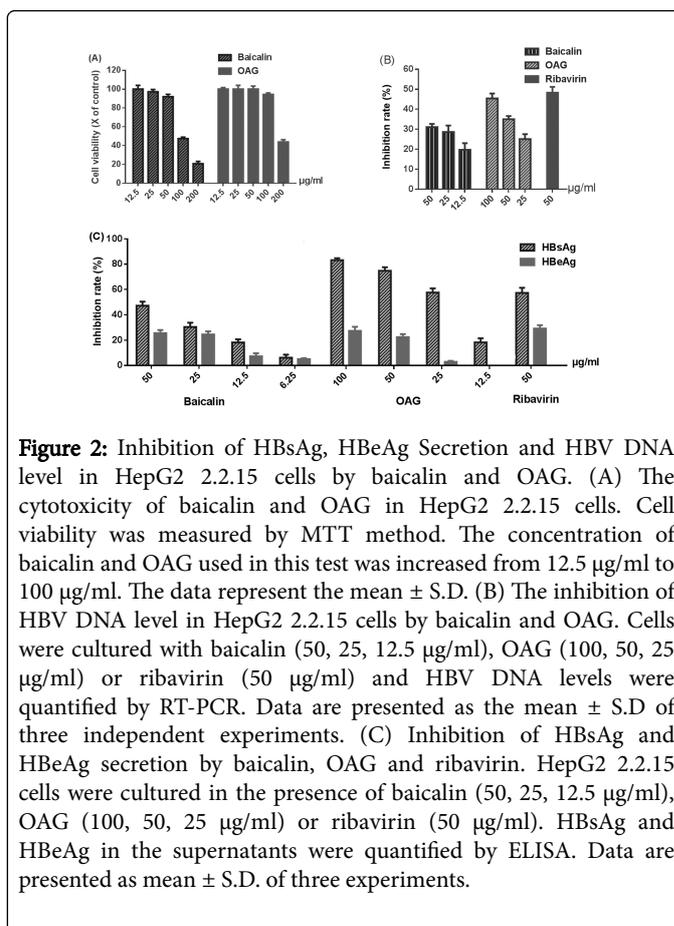


Figure 2: Inhibition of HBsAg, HBeAg Secretion and HBV DNA level in HepG2 2.2.15 cells by baicalin and OAG. (A) The cytotoxicity of baicalin and OAG in HepG2 2.2.15 cells. Cell viability was measured by MTT method. The concentration of baicalin and OAG used in this test was increased from 12.5 µg/ml to 100 µg/ml. The data represent the mean ± S.D. (B) The inhibition of HBV DNA level in HepG2 2.2.15 cells by baicalin and OAG. Cells were cultured with baicalin (50, 25, 12.5 µg/ml), OAG (100, 50, 25 µg/ml) or ribavirin (50 µg/ml) and HBV DNA levels were quantified by RT-PCR. Data are presented as the mean ± S.D. of three independent experiments. (C) Inhibition of HBsAg and HBeAg secretion by baicalin, OAG and ribavirin. HepG2 2.2.15 cells were cultured in the presence of baicalin (50, 25, 12.5 µg/ml), OAG (100, 50, 25 µg/ml) or ribavirin (50 µg/ml). HBsAg and HBeAg in the supernatants were quantified by ELISA. Data are presented as mean ± S.D. of three experiments.

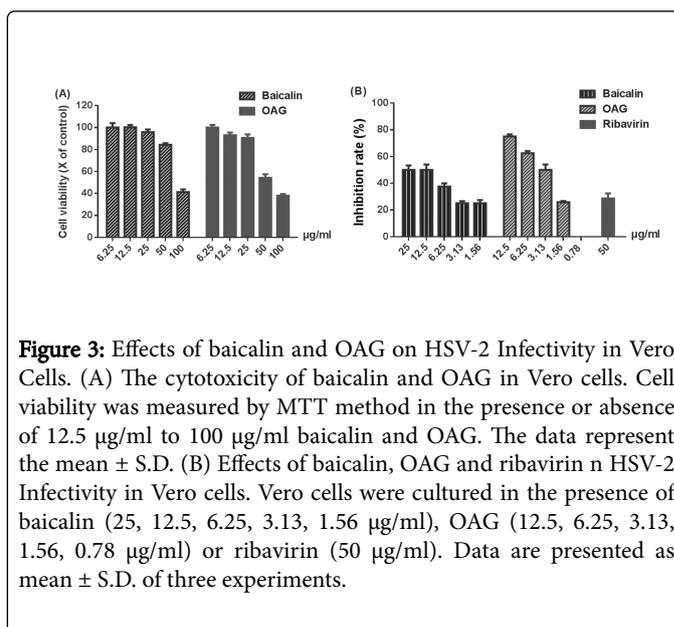


Figure 3: Effects of baicalin and OAG on HSV-2 Infectivity in Vero Cells. (A) The cytotoxicity of baicalin and OAG in Vero cells. Cell viability was measured by MTT method in the presence or absence of 12.5 µg/ml to 100 µg/ml baicalin and OAG. The data represent the mean ± S.D. (B) Effects of baicalin, OAG and ribavirin on HSV-2 Infectivity in Vero cells. Vero cells were cultured in the presence of baicalin (25, 12.5, 6.25, 3.13, 1.56 µg/ml), OAG (12.5, 6.25, 3.13, 1.56, 0.78 µg/ml) or ribavirin (50 µg/ml). Data are presented as mean ± S.D. of three experiments.

OAG inhibition of IAV H3N2 *In vitro*

The *In vitro* anti-IAV activity of OAG and baicalin was evaluated by measuring the IAV H3N2-induced CPE in MDCK cells. As shown in Figure 4, non-toxic concentrations of both compounds dose-dependently inhibited virus-induced CPE, by 50% and 25%, respectively, at OAG and baicalin concentrations of 50 µg/ml and 25 µg/ml, respectively. At 100 µg/ml, OAG inhibited CPE by 62.5%, but baicalin became toxic at 100 µg/ml. The positive control, ribavirin, inhibited CPE by 64.5% at 100 µg/ml.

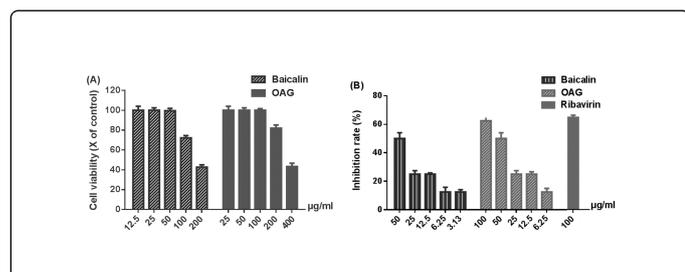


Figure 4: The effect of baicalin and OAG on H3N2-induced cytotoxicity (A) The cytotoxicity of baicalin and OAG in MDCK cells. Cell viability was measured by MTT method. The concentration of baicalin was increased from 12.5 µg/ml to 200 µg/ml. The concentration of OAG was increased from 25 µg/ml to 400 µg/ml. The data represent the mean ± S.D. (B) Inhibition of H3N2 in Vero cells by baicalin and OAG. MDCK cells were cultured in the presence of 100, 0, 25, 12.5, 6.25, 3.13 µg/ml baicalin (50, 25, 12.5, 6.25, 3.13 µg/ml), OAG (100, 50, 25, 12.5, 6.25 µg/ml) or ribavirin (100 µg/ml). Data are presented as mean ± S.D. of three experiments.

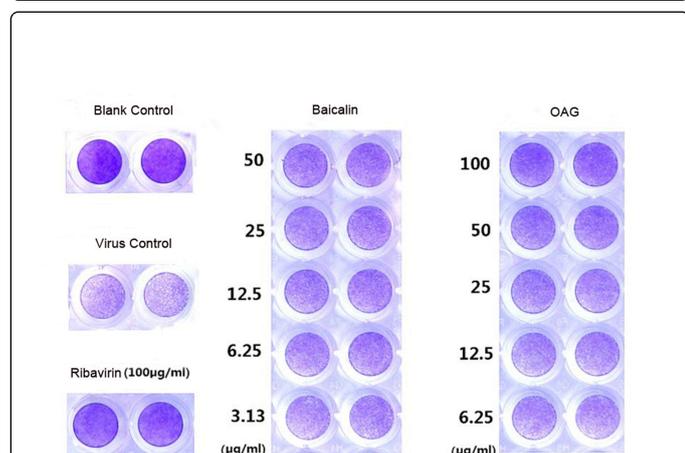


Figure 5: The effect of baicalin and OAG on H3N2 plaque assay. MDCK cells were inoculated with influenza virus H3N2 for 2 h at 37°C. After viral adsorption, the cell monolayer was covered with overlay medium containing baicalin and OAG and further cultured at 37°C for 48 h. Then, the overlay medium was removed, and the cell monolayer was fixed with 10% formalin, stained with 1% crystal violet, and plaques were counted.

OAG and baicalin inhibition of H3N2 infection was further examined by plaque reduction assay (PRA). As shown in Figure 5, Both OAG and baicalin inhibited IAV H3N2-induced plaque formation in a dose-dependent manner. Consistent with the CPE assay results, OAG had a similar inhibitory effect to ribavirin at 100 µg/ml.

Discussion

In this study, we evaluated the antiviral activity of OAG and baicalin against HBV, HSV and IAV, and the by which these compounds inhibit the HBV life cycle were preliminarily explored.

The anti-HBV activity of baicalin was previously reported [25-27]. Here, both OAG and baicalin exhibited anti-HBV activity, inhibiting secretion of HBsAg and HBeAg from HBV-infected HepG2 2.2.15 cells by 74.50% and 22.43% respectively, for OAG and c47.175% and 25.56% for baicalin, respectively. OAG and baicalin also affected the release of viral DNA. OAG may have inhibited HBsAg release more potently as the glucose receptor is known to be present on HepG2 2.2.15 cells.

Little or no anti-HSV activity was noted in a previous study of baicalin [16,28]. However, in this study, we observed baicalin's anti-HSV activity, and found that it can inhibit HSV-induced cytotoxicity by 25% at 12.5 µg/ml. Furthermore, 12.5 µg/ml OAG inhibited HSV-induced cytotoxicity by 50.0%. These results suggested that OAG was more effective than the parental baicalin at inhibiting HSV-induced cytotoxicity. We hypothesize that a methylation group of C-6 of flavonoid nucleus or hydroxyl group of C-6' of glycoside may enhance the anti-HSV potency OAG.

Outbreaks of influenza virus infection remain one of most severe threats to public health [28]. Baicalin was previously reported to have anti-viral activity [29,30]. In our study, we evaluated the anti-viral activity of baicalin against IAV H3N2 by measuring the virus CPE. The results suggested that OAG can inhibit IAV H3N2 replication by 62.5% at 100 µg/ml and by 50% at 50 µg/ml. Baicalin also inhibited H3N2 replication by 50% at 50 µg/ml. Thus, OAG can be used at higher concentrations with more potent effects without causing cytotoxicity.

In summary, this is the first report of the broad antiviral function of OAG, which was produced by transformation of baicalin. OAG inhibited HBV and HSV replication more potently than baicalin. OAG appears to be a promising drug candidate with broad antiviral potential. Our findings provide a basis for further work to characterize OAG's antiviral function.

Acknowledgements

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