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Evaluation of potential herb-drug interactions between oseltamivir and commonly used anti-influenza Chinese medicinal herbs

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Keywords: herb-drug interactions, oseltamivir, Radix Scutellariae, Traditional Chinese medicine, population pharmacokinetics modeling

Chemical compounds studied in this article:
- Oseltamivir (PubChem CID: 78000)
- Oseltamivir acid (PubChem CID: 449381)
- Epoigotin (PubChem CID: 303213)
- Glycyrrhizic acid (PubChem CID: 14982)
- Liquiritin (PubChem CID: 503737)
- Baicalein (PubChem CID: 5281605)
- Baicalin (PubChem CID: 5281703)
- Liquiritin (PubChem CID: 503377)
- Glycyrrhizic acid (PubChem CID: 14982)
- Epigoitrin (PubChem CID: 3032313)
- Oseltamivir acid (PubChem CID: 449381)

ABSTRACT

Ethnopharmacological relevance: According to Traditional Chinese Medicine theory, influenza is categorized as a warm disease or Wen Bing. The Wen Bing formulas, such as Yin-Qiao-San and Sang-Ju-Yin, are still first-line herbal therapies in combating variant influenza virus. To continue our study on the pharmacokinetic and pharmacodynamic interactions between Wen Bing formulas and oseltamivir (OS), the first-line western drug for the treatment of influenza, further interactions between OS and the eight single herbs and their relevant marker components from Wen Bing formulas were investigated in the current study.

Aim of study: To establish an in-vitro screening platform for investigation of the potential anti-influenza herbs/herbal components that may have pharmacokinetic and pharmacodynamic interactions with OS.

Materials and methods: To screen potential inhibition on OS hydrolysis, 1 μg/mL of OS is incubated with herbs/herbal components in diluted rat plasma, microsomes and human recombinant carboxylesterase 1(hCE1) under optimized conditions. MDCK-WT and MDCK-MDR1 cell lines are utilized to identify potential modification on P-gp mediated transport of OS by herbs/herbal components. Caco-2 cells with and without Gly-Sar inhibition are performed to study the uptake of OS via PEPT1 transporters. Modification on OAT3 mediated transport is verified by the uptake of OS on HEK293-MOCK/HEK293-OAT3 cells. Anti-virus effects were evaluated using plaque reduction assay on H1N1 and H3N2 viruses. Potential pharmacokinetic and pharmacodynamic interaction between OS (30 mg/kg) and the selected herb, Radix Scutellariae (RS), at 300–600 mg/kg were carried out on rats. All samples are analyzed by an LC/MS/MS method for the contents of OS and OSA. A mechanistic PK model was developed to interpret the HDI between OS and RS in rats.

Results: Our developed platform was successfully applied to screen the eight herbal extracts and their ten marker components on metabolic inhibition of OS and modification of OS transport mediated by P-gp, OAT3 and PEPT1. Results from six in-vitro experiments were analyzed after converting raw data from each experiment to corresponding fold-change (FC) values, based on which Radix Scutellariae (RS) were selected to have the most HDI potential with OS. By analyzing the plasma and urine pharmacokinetic data after co-administration of OS with a standardized RS extract in rats using an integrated population pharmacokinetics model, it is suggested that RS could inhibit OS hydrolysis during absorption and increase the absorbed fraction of OS, which leads to the increased ratio of OS concentration versus that of OSA in both rat plasma and urine. Never the less, the anti-virus effects of 2.5 h post-dose rat plasma were not influenced by co-administration of OS with RS.

Conclusion: A six-dimension in-vitro screening platform has been developed and successfully applied to find RS as a potential herb that would influence the co-administered OS in rats. Although co-administered RS could inhibit OS hydrolysis during absorption and increase the absorbed fraction of OS, which lead to the increased ratio of OS concentration versus that of OSA in both rat plasma and urine, the anti-virus effect of OS was not influenced by co-administered RS.
1. Introduction

According to the theory of Traditional Chinese Medicine (TCM), influenza is categorized as an exogenous “warm disease” or “Wen Bing” (Koh, 2010). Wen Bing formulas have been used for more than 200 years since their publication in Wen Bing Tiao Bian by Wu Jutong in 1798. These formulas include Yin-Qiao-San (a formula made up by Fructus Forsythiae, Flos Lonicerae Japonicae, Platycodon Radix, Menthae Haplocalycis Herba, Radix et Rhizoma Glycyrrhizae, Herba Schizonepetae, Sojae Semen Praeparatum, Fructus Arctii and Reed Rhizome) and Sang-Ju-Yin (a formula made up by Armeniacae Semen Marrhenae Rhizoma, Fritillariae Thunbergii Bulbus, Platycodonis Radix, Radix et Rhizoma Glycyrrhizae and Reed Rhizome). Up to now, these two formulas are still the first line herb therapies for treatment and prevention of early-stage influenza in China as recommended by National Health Commission, PRC (National Health Commission, 2018). As for middle-stage influenza, the recommended formula is Ma-Xing-Shi-Gan-Tang, which is adapted from Shanghan Lun compiled by Zhang Zhongjing before year 220, at the end of the Han dynasty. This formula is made up by Ephedrae Herba, Armeniaca Semen Amarum, Gypsum Fibrosum, Anemarrhenae Rhizoma, Fritillariae Thunbergii Bulbus, Platycodonis Radix, Radix Scutellariae, Bupleuri Radix and Radix et Rhizoma Glycyrrhizae. Per the pattern differentiation of TCM, more herbs could be added to the original formulas for specific disease presentations. The TCM formula commonly used during the Severe Acute Respiratory Syndrome (SARS) epidemic of 2003 in China is the Sang-Ju-Yin formula adjusted by adding Radix Isatidis, Radix Scutellariae and Radix Astragali (Fraktin, 2010).

These Wen Bing formulas were also recommended by TCM experts from Healthy Authority (HA) of Hong Kong for the prophylaxis and treatment of influenza with Oseltamivir (OS). In collaboration with the HA, investigations of the pharmacokinetic and pharmacodynamic interactions between OS and HA recommended Chinese formula, namely CMF1 (a combined formula of Yin-Qiao-San and Sang-Ju-Yin) in both animal and human subjects were performed by our group since 2008 (Chang et al., 2014). To explore more, we would like to investigate the interactions between OS and selected single herbs from these Wen Bing formulas. Thus, eight herbs, namely Radix Isatidis (Chen et al., 2017), Radix et Rhizoma Glycyrrhizae (Michaelis et al., 2011; Sekine-Osajima et al., 2009; Wolkersreiter et al., 2009), Radix Scutellariae (Xu et al., 2010), Fructus Forsythiae (Li et al., 2011), Flos Lonicerae Japonicae (Lu et al., 2018), Folium Mori (Savov et al., 2006), Herba Schizonepetae (He et al., 2013) and Fructus Arctii (Hayashi et al., 2010), were selected for further studies based on the flowing cariteria: (i) commonly used in traditional Wen Bing formulas and (ii) the efficacy of the selected herbs in China Pharmacopeia clearly related to anti-influenza effects based on TCM and (iii) the selected herbs/their herbal components having antiviral effects on influenza viruses in modern pharmacological studies. In addition, ten relevant marker components in these eight herbs were also selected to help understand the interaction between OS and herbs: epigallocatechin gallate from Radix Isatidis; glycyrrhizic acid, liquiritin from Radix et Rhizoma Glycyrrhizae; baicalin, baicalein, wogonin from Radix Scutellariae; phillyrin from Fructus Forsythiae; chlorogenic acid from Flos Lonicerae Japonicae; rutin from Folium Mori and arctinin from Fructus Arctii.

OS is the ethyl ester prodrug of oseltamivir acid (OSA, Ro 64-802) a potent and selective inhibitor of the neuraminidases glycoprotein essential for replication of influenza A and B viruses (He et al., 1999). It is currently marketed under the trade name Tamiflu® (Roche), with approval for treatment of influenza in 1999, and for the prophylaxis of influenza in 2000 by U.S. FDA. In 2009, the outbreak of Influenza A virus subtype H1N1, commonly referred to as “swine flu”, which caused fatal infection and over 17,700 deaths in over 213 countries. The World Health Organization (WHO) recommended Tamiflu for early treatment of swine flu. OS is orally well absorbed and metabolism to OSA by cytochrome P450 1B1 in vivo (He et al., 1999; Shi et al., 2006) without interaction with human cytochrome P450 or glucuronosyl-transferase (He et al., 1999). OSA is an anion and has been proved to be the substrate of organic anion tublar secretion transporter (OAT) during its renal secretion (Deguchi et al., 2004). OS, not OSA, is a substrate of P glycoprotein (P-gp). Accordingly, low levels of P-gp activity or drug-drug interactions at P-gp may lead to enhanced brain accumulation of oseltamivir, and this may in turn account for the central nervous system effects of oseltamivir observed in some patients (Morimoto et al., 2008). A recent study suggested that oseltamivir is also a substrate of Peptide transporter 1 (PEPT1) during its intestinal absorption (Ogihara et al., 2009).

OS has limited potential for clinically relevant pharmacokinetics interactions with commonly co-administered drugs, such as paracetamol, cimetidine, amoxicillin, aspirin or antacids (Dutkowski et al., 2003). Comparing with drug-drug interactions of OS, reports on its herb-drug interactions are even fewer. Effects of herbal supplement and traditional medicines (including extracts from six traditional Cree botanicals, a commercially available Echinacea product, Goldenseal and a traditional medicines (including extracts from six traditional Cree botanicals) on the microsomal-mediated metabolism of OS have been evaluated by Liu et al. (2010). To explore more, we would like to investigate the interactions between OS and selected single herbs from these Wen Bing formulas. Thus, eight herbs, namely Radix Isatidis (Chen et al., 2017), Radix et Rhizoma Glycyrrhizae (Michaelis et al., 2011; Sekine-Osajima et al., 2009; Wolkersreiter et al., 2009), Radix Scutellariae (Xu et al., 2010), Fructus Forsythiae (Li et al., 2011), Flos Lonicerae Japonicae (Lu et al., 2018), Folium Mori (Savov et al., 2006), Herba Schizonepetae (He et al., 2013) and Fructus Arctii (Hayashi et al., 2010), were selected for further studies based on the flowing cariteria: (i) commonly used in traditional Wen Bing formulas and (ii) the efficacy of the selected herbs in China Pharmacopeia clearly related to anti-influenza effects based on TCM and (iii) the selected herbs/their herbal components having antiviral effects on influenza viruses in modern pharmacological studies. In addition, ten relevant marker components in these eight herbs were also selected to help understand the interaction between OS and herbs: epigallocatechin gallate from Radix Isatidis; glycyrrhizic acid, liquiritin from Radix et Rhizoma Glycyrrhizae; baicalin, baicalein, wogonin from Radix Scutellariae; phillyrin from Fructus Forsythiae; chlorogenic acid from Flos Lonicerae Japonicae; rutin from Folium Mori and arctinin from Fructus Arctii.

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Our previous findings (Chang et al., 2014) on pharmacokinetic and pharmacodynamic interactions between OS and CMF1 in both animal and human subjects suggested that: (1) Co-administration of OS (30 mg/kg, b.i.d, 5 days) and CMF1 (1.95 g/kg, b.i.d., 5 days) at their human equivalent dose in rats would lead to significantly enhanced
antiviral activities although such combination could significantly reduce the systemic exposure of OS, indicating potential contribution of antiviral activities from CMF1; (2) co-administration of OS (75 mg, b.i.d., 5 days) and CMF1 (10 g, b.i.d., 5 days) in 14 healthy male subjects (Zuo et al., 2011) demonstrated similar trend of reduction in plasma concentration of OS and enhanced antiviral activity against H3N2; (3) metabolism and transport of OS could be significantly inhibited by CMF1/CMF1 components in a dose-dependent manner, among which arctigenin demonstrated to be the strongest inhibitor as well as a strong antiviral activity with IC50 value of 74 μM identified by the in-vitro plaque reduction assay.

Although the absorption and disposition of OS are well identified, the potential effects of herbs/herbal components on these characteristics remain to be investigated. Therefore, we propose to establish an in-vitro screening platform for investigation of the potential herbs/herbal components that may have pharmacokinetic and pharmacodynamic interactions with OS. Such screening platform will be based on available knowledge of absorption, disposition and antiviral activities of OS, and built with our existing models of in-vitro enzymatic reactions of liver microsomes, in-vitro transport using cell lines that over-expressed with P-gp, PEPT1 or OAT, and in-vitro plaque-reduction assay.

2. Materials and methods
2.1. Herbs and herbal components and quality control

Raw herbs, namely Radix Isatidis (H1), Radix et Rhizoma Glycyrrhizae (H2), Radix Scutellariae (H3), Fructus Forsythiae (H4), Flos Lonicerae Japonicae (H5), Folium Mori (H6), Herba Schizonepetae (H7) and Fructus Arctii (H8), were purchased from Hong Wang Herbal Drug Store in Hong Kong. Authentic compounds, epigallocatein (C1), glycyrrhetic acid (C2), liquiritin (C3), baicalin (C4), baicalin (C5), wogonin (C6), phyllityrin (C7), chlorogenic acid (C8), rutin (C9) and arctiin (C10), were purchased from Chengdu Must Bio-technology Co. LTD (Chengdu, China). Content of authentic compounds in the studied herbs were determined using a LC/MS/MS method which employed an Agilent 6430 triple quadruple mass spectrometer (Agilent Technologies) coupled with electrospray ionization (ESI) source. MS parameters and separation conditions were optimized for each herb as shown in Table S1. Contents of C1–C10 in herbs were determined using an external calibration method. Triplicate samples were prepared for each herb and analyzed. Finally, the concentrations of C1–C10 in each gram of herbal extracts were summarized in Table S2. A commercially available, standardized and purified Radix Scutellariae extract (RS) was obtained from Shanghai U-sea Biotech Co., Ltd. (Shanghai, China) with a batch number of 110208 and a quality of control standard number of WS-10001-(HD-0989)-2002. The content (w/w) of C4, C5 and C6 in RS were 2.0%, 48.0% and 0.4%, respectively, as quantified by our LC–MS/MS method.

2.2. Chemicals and reagents

Oselamivir (OS) and oselamivir acid (OSA) (both purity ≥99%) were kindly provided by Roche Company (St. Louis, MO, USA). Albuteorol (as internal standard) was purchased from Sigma-Aldrich Chem. Hygromycin B, glycyrrhetic acid (Gly-Sar) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chem. Co. (Milwaukee, WI, USA). For cell culture, Dulbecco’s modified Eagle’s medium, 0.25% trypsine-EDTA, fetal bovine serum, penicillin-streptomycin, hank’s balanced salt solution (HBSS), sodium butyrate and non-essential amino acids were purchased from Gibco BRL Co. Ltd (Carlsbad, CA, USA) and Life Technologies (Grand Island, NY, USA). Six-well plates with Transwell® inserts (24 mm I.D., 4.71 cm², 0.4μm pore size, polycarbonate filter) were purchase from Corning Costar Corporation (Corning, NY, USA). Biocat poly-D-lysine 24-well plates were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Phosphate buffered saline (PBS), NADP⁺, rat liver microsomes (RLM), recombinant carboxylesterase 1 (hCE1), glucose-6-phosphate dehydrogenase, glucose-6-phosphate and MgCl₂ were purchased from Sigma (St Louis, MO, USA). Rat blank plasma was freshly prepared by sacrificing Sprague-Dawley (SD) rats. Acetonitrile and methanol (Labscan Asia, Bangkok, Thailand) were HPLC grade. All other reagents from BDH Laboratory (Dorset, England) were of analytical grade. Distilled and deionized water was prepared from Millipore water purification system (Millipore, Milford, MA, USA).

2.3. Cell cultures

MDCK-WT and MDCK-MDR1 cells were generously provided by Prof. P. Borst (The Netherlands Cancer Institute). For cell culture, MDCK-WT and MDCK-MDR1 cells were cultured in Dulbecco’s modified Eagle’s medium at 37 °C, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, in an atmosphere of 5% CO₂ and 90% relative humidity. MDCK cells were sub-cultured by 0.25% trypsin-EDTA for 4 min followed by plating onto six-well Transwell® inserts coated with collagen at a density of 2 × 10⁵ cells/well. Trans-epithelial electrical resistance (TER) values were measured to monitor the integrity of the MDCK cell monolayer. MDCK monolayer with TEER above 150 Ωcm² could be employed to the transport study.

Caco-2 cells were purchased from American Type Culture Collection. For cell culture, Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium at 37 °C, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/mL of penicillin, and 100 U/mL of streptomycin in an atmosphere of 5% CO₂ and 90% relative humidity. Caco-2 cells were sub-cultured by trypsinization with 0.25% trypsin-EDTA for 5 min and seeded onto six-well plates at a density of 2 × 10⁵ cells/cm² for around 7 days, followed by uptake study.

Empty vector (mock) transfected and human organic anion transporter 3 (OAT3) transfected HEK293 cells were generously provided by Prof. Dafang Zhong (Shanghai Institute of Materia Medica). For cell culture, HEK293-MOCK and HEK293-OAT3 were cultured in flashes with Dulbecco’s modified Eagle’s medium at 37 °C, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/mL of penicillin, 100 U/mL of streptomycin, and 100 μg/mL of hygromycin B in an atmosphere of 5% CO₂ and 90% relative humidity. The cells were seeded onto 24-well biocat poly-D-lysine-coated plates at a density of 2.0 × 10⁵ cells/well with 0.5 mL per well. To induce the expression of transporters, medium in plates was replaced with fresh culture medium containing 5 mM sodium butyrate 24 h before uptake study.

2.4. Screening of inhibition on OS hydrolysis in plasma and liver by selected herbs/herbal components

2.4.1. Metabolic inhibition on OS hydrolysis in rat plasma by herb/herbal components

Diluted rat plasma (10%) of 400 μL was spiked with 4 μL herb/ herbal components (H1–H8: 50 mg/mL; C1–C10: 10 mg/mL), which were dissolved in DMSO (control group was spiked with only DMSO). These reaction mixtures were pre-incubated for 5 min in a 37 °C water bath. Then, 40 μL of 10 μg/mL OS was added to initialize the reaction. The final mixture was incubated for 30 min and terminated by adding 400 μL ice-cold acetonitrile and 200 μL internal standard (100 ng/mL of albuterol in water). After vertex and centrifugation at 13,000 rpm for 10 min, 2 μL of the supernatant was injected for LC/MS/MS analysis.

2.4.2. Further metabolic inhibition on OS hydrolysis in rat liver microsome by herbal components

Thaw rat liver microsome (RLM) slowly on ice and adjust concentration to 10 mg/mL. The incubation system was composed of: (1) 176 μL of 50 mM PBS; (2) 2 μL of 100 μg/mL OS and 2 μL of 100 μg/mL herb components (C1 to C10); (3) 10 μL of 10 mg/mL RLM. Pre-incubate the above mixture in water bath for 5 min. Initiate the reactions.
with the addition of 10 μL of 20 mM NADP+ regenerating system (NADP+, Glucose-6-phosphate dehydrogenase and Glucose-6-phosphate) and incubate up to 60 min at 37 °C with gentle agitation. Terminate the reactions by the addition of 600 μL of ice-cold acetonitrile and 200 μL H2O. Vortex the samples, and centrifuge at 13,000 rpm for 10 min. Pipette 100 μL supernatant into 50 μL internal standard (100 ng/mL albuterol in water) and 100 μL water. After vortex and centrifugation, 10 μL of supernatant were injected for LC/MS/MS analysis.

2.4.3. Further metabolic inhibition on OS hydrolysis in hCE1 by herbal components

Thaw hCE1 slowly on ice and diluted to 40 μg/mL with 50 mM PBS (pH 7.4). Pre-incubate hCE1 in 37 °C water bath for 5 min. The hydrolysis reaction was initiated by the addition of OS and herbal components (200 μL, final concentration 10 μM) for 60 min. The reaction was terminated by adding 5 μL of 8 mg/mL dichloroethane and then mixed with 600 μL PBS. Vortex the samples, and centrifuge at 13,000 rpm for 10 min. Pipette 100 μL supernatant into 50 μL internal standard (100 ng/mL albuterol in water) and 100 μL water. After vortex and centrifugation, 10 μL of supernatant were injected for LC/MS/MS analysis.

2.5. Screening for inhibition on transport of OS by herb/herbal components

2.5.1. Cytotoxicity assay

The cytotoxicity of OS combined with selective herbs/herbal components to interfere with the growth of MDCK-WT, MDCK-MDR1, Caco-2, HEK293-MOCK and HEK293-OAT3 cells were determined by MTT assay. The cells were seeded onto a 96-well plate at a density of 5 × 10^4 cells/well in DMEM culture medium and cultured at 37 °C in an atmosphere of 5% CO2 and 90% relative humidity for 24 h. Subsequently, the culture medium was replaced with 150 μL of OS with or without herbs/herbal components in HBSS (pH7.4) at different studied concentrations. Concentrations was shown as following: OS was 30 μg/mL, herbal extracts (H1 to H8) were ranged from 0.25 mg/mL to 1 mg/mL and herbal components (C1 to C10) was ranged from 25 μg/mL to 100 μg/mL. Blank HBSS (pH 7.4) was used as negative control. After incubating the 96-well plate at 37 °C for 4 h, 20 μL of 5 mg/mL MTT solution in HBSS was added to each well followed by continuous incubation for another 4 h. The solutions in each well were then removed carefully followed by dissolving the remaining formazan crystals with 200 μL of DMSO. The absorbance was subsequently measured with a microplate reader (Molecular Devices, Downingtown, PA, USA) at 590 nm. The cytotoxicity of OS with or without herbs/herbal components was calculated as the percentage of the absorbance relative to that of the negative control.

2.5.2. Modification on P-gp mediated transport of OS by herbs/herbal components

To identify potential modification on P-gp mediated transport of OS by herbs/herbal components, the A to B transport study of OS with or without herbs/herbal components was carried out in MDCK-WT and MDCK-MDR1 cell lines. Cells were rinsed twice and equilibrated with HBSS at 37 °C before experiment. Test solutions (30 μg/mL OS dissolved in HBSS with or without herbs/herbal components) were loaded into the apical side of chamber (AP, 1.5 mL volume). 400 μL of solution was collected from basolateral side (BL, 2.6 mL in volume) at 30 min, 60 min, 90 min, and 120 min, followed by replacing with equal volume of blank HBSS to the receiver chamber, and finally subjected to analysis for OS by LC/MS/MS.

2.5.3. Modification on PEPT1 mediated transport of OS by herbs/herbal components

Caco-2 cells were used for the screening of herbs/herbal components to determine their effect on the uptake of OS via PEPT1. Caco-2 cells grown on the 6-well plates for 7 days were rinsed with HBSS containing 10 mM Gly-Sar twice and pre-incubated for 20 min before uptake study. After pre-incubation, test solutions (30 μg/mL OS dissolved in HBSS with or without herbs/herbal components) were added to initiate uptake. The uptake of OS increased linearly up to 1 min. Thus, the Caco-2 cells were incubated at 37 °C for 1 min and then washed 5 times with 2 mL of ice-cold blank HBSS to terminate the uptake of OS. The cells were lysed by the multigelation method and the content of OS in cells was then analyzed by LC/MS/MS.

2.5.4. Modification on OAT mediated transport of OS by herbs/herbal components

HEK293-MOCK and HEK293-OAT3 cells were used to illustrate the effect of the herbs/herbal components on the modification of OS via OAT3. Cells were washed twice with HBSS on day 3 or 4 and pre-incubated at 37 °C for 20 min. Experiments were initiated by the addition of HBSS containing OS with or without herbs/herbal components and terminated by removing the solutions and washed 3 times carefully with ice-cold HBSS. Subsequently, the cells were lysed by the multigelation method and the supernatant after processing was injected to LC/MS/MS for analysis of OS.

2.6. Pharmacokinetics and pharmacodynamics interactions between OS and Radix Scutellariae (RS) selected from the in-vitro screening tests

2.6.1. Animal grouping and treatment

The experiments were carried out after approval by the Animal Ethics Committee of The Chinese University of Hong Kong (Ref No. 14-171-MIS). Male Sprague-Dawley (SD) rats (weighting ~250 g) used in the experiment were provided by Laboratory Animal Services Center of The Chinese University of Hong Kong (Hong Kong SAR, People’s Republic of China). Solutions of oseltamivir (OS, 7.5 mg/mL) were freshly prepared before each experiment by dissolving OS in deionized water. RS extract was prepared as a suspension (Low dose: 75 mg/g; High dose: 150 mg/g) in water containing 5% (w/w) PEG400. A total of 60 rats were randomly divided into 6 groups (G1 to G6), with 10 rats in each group, to receive various oral drug treatments for 5 days. On 9AM and 6PM of each day, rats in G1 to G3 received a dose of 30 mg/kg OS and rats in G4 to G6 were given water only at the same time. On 11AM and 8PM of each day, the doses administered to rats were water for G1 and G6, 300 mg/kg RS for G2 and G4, and 600 mg/kg RS for G3 and G5, respectively. On Day 4, 1 h after receiving the 11AM-dose, a jugular vein cannulation surgery was performed on rats under light anesthesia. After surgery, the rats were placed individually into metabolic cages and allowed to recover, with overnight fasting but free access to water. After the 9AM dosing on Day 5, 10 blood samples (0.2 mL each) were obtained via the catheter at 0, 15, 30, 60, 90, 120, 180, 240, 360 and 480 min post dose. After each blood sampling, 0.2 mL of normal saline containing 20 units/mL of heparin were immediately injected into the catheter to flush the catheter. The urine samples were collected over 8 h post-dose and combined with the volume of water used for rinsing the metabolic cage. The collected urine samples were stored at ~80 °C until analysis. At 30 min following the 8PM dose on Day 5, the rats were anesthetized and about 10 mL of blood were collected from rats’ inferior vena for determination of antiviral activity. The blood samples collected in G6 (group with no treatment) served as control.

2.6.2. Sample analyses

Rat plasma or diluted urine samples were purified with a Waters Oasis MCX extraction cartridge (1 cc, 30 mg) per the following procedure: 1) Equilibrate the cartridge with 1 mL methanol and 1 mL H2O containing 2% formic acid (FA) in sequence; 2) Load the sample mixture (containing 100 μL rat plasma/diluted urine, 100 μL standard solution of OS/2%FA-H2O and 50 μL 200 ng/mL of caffeine in H2O as internal standard) onto the cartridge; 3) Wash with 1 mL water; 4) Apply 1% ammonium hydroxide in methanol as elution solvent and let
it flow through by gravity before switching on the vacuum pump; 5) Evaporate the eluent to dryness under reduced pressure (40 °C); 6) Reconstitute with mobile phase (40% methanol and 60% 0.1%FA in H₂O) and analyze with the following LC-MS/MS method.

An Agilent 6430 triple quadruple mass spectrometer (Agilent Technologies) coupled with electrospray ionization (ESI) source was employed for detecting OS and OSA in the above-mentioned bio-samples. MS parameters and separation conditions were optimized for each kind of bio-samples. Generally, nitrogen was used as the de-solvation (10 L/min) and nebulizer gas (50 psi) and the source parameters were: ESI (+), temperature 350 °C, source voltage 4000 V and delta EMV 400 V. Chromatographic separations were achieved by a SunFire C18 column (4.6 × 150 mm i.d., 5 μm) equipped with a guard filter. Mobile phases consisted of eluents A (0.2%formic acid in water) and B (acetonitrile). A 2–10 μL aliquot of the samples was injected and eluted off the column with a flow rate of 0.8 mL/min and with an isocratic elution program (30% eluent B). The temperatures of the auto-sampler and column were set to be 10 °C and ambient, respectively. MRM scans were used for quantification of all the analytes: OS: m/z 313.2 → 166.0, Fragmentor 90 V, Collision energy 15; OSA: m/z 285.2 → 138.0, Fragmentor 80 V, Collision energy 15; Caffeine (internal standard): m/z 195.1 → 138.0, Fragmentor 110 V, Collision energy 16.

2.6.3. Evaluation of antiviral effect via plaque reduction assay

The MDCK SIAT-1 cell line was grown in DMEM, high glucose, pyruvate supplemented with 10% fetal bovine serum, 2 mM L-glutamine (all from Thermo Fisher Scientific, Massachusetts, USA) at 37 °C in 5% CO₂. A batch of clinical isolates (H1N1 and H3N2) collected from the Prince of Wales Hospital was evaluated for their replication and then plaque formation in MDCK SIAT-1 cells. Viruses were propagated in MDCK SIAT-1 cells at 33 °C with maintenance medium (DMEM, high glucose, pyruvate supplemented with 1% fetal bovine serum, 2 mM L-glutamine, 0.75 μg/mL TPCK-treated trypsin). Replication ability was assessed by immune-fluorescent staining of cell culture harvest using influenza A-specific antibodies. Virus isolates showing satisfactory growth ability were selected for further assessment on plaque forming ability by methods as detailed below. Quantified stocks of the most appropriate H1N1 and H3N2 isolates with known number of plaque forming unit (PFU) were kept at −70 °C for further use in plaque reduction assay.

MDCK SIAT-1 cells were seeded at 6.25 × 10⁴ cells per well in a 24-well plate and then incubated at 37 °C overnight to reach 80–90% confluence. Virus stocks at concentration of 200 PFU/mL for H1N1 or 100 PFU/mL for H3N2 were used to prepare a 500-μL mixture containing 250 μL of viruses and 250 μL of rat plasma samples. A different optimal number of PFU was used because the H1N1 and H3N2 isolates generated different sizes of plaque. The mixtures of viruses and test compounds were incubated at 37 °C under 5% CO₂ for 1 h with gentle shaking several times to allow even mixing. Cells were washed with DMEM containing 0.75 μg/mL TPCK-treated trypsin and then incubated with 1 mL medium-agar overlay for 48 h. The agar overlay, containing an equal volume of 0.8% melted agarose and 2X MDCK maintenance medium, was kept at 42 °C until adding to wells. After 48 h of incubation at 37 °C, the cell monolayers were fixed with 1% formaldehyde overnight at room temperature, and stained with 0.4% crystal violet for 30 min at room temperature. The number of plaques formed were examined with naked eyes with assistant of a magnifying glass when necessary.

2.7. Data analyses

2.7.1. Effects of herb/herbal components on hydrolysis, transport and uptake of OS

For inhibition of OS hydrolysis, OSA formation rate were calculated by the molar amount of OSA divided by reaction time and volume of rat plasma (or protein amount of RLM and hCE1). For P-gp transport study, apparent permeability coefficient (Papp) was obtained as we described before (Li et al., 2012) to estimate the potential modification of OS by herbs/herbal components. For uptake study, the cell uptake amount of OS was obtained and compared between the groups in the absence and presence of herbs/herbal components.

Selection of herb for verification of the potential herb-drug interaction (HDI) in rats.

To comprehensively compare the influential of herbs/herbal components on the inhibition of OS hydrolysis and the transport/uptake of OS, the results from each experiment were expressed as a fold change (FC) by the following equation:

\[ FC = \frac{M_i}{M_c} - 1 \]

where M_i is the results from OS plus herb/herbal components experiments and M_c is that from OS only (or OS plus inhibitor in PEPT1 cell study) experiments. Then, FC values in each experiment would be converted into z-scores by the following equation:

\[ z_i = \frac{x_i - \bar{x}}{s} - 1 \]

where z_i is z-transformed sample observations, x_i is FC values, \( \bar{x} \) is mean of FC values and s is standard deviation of FC values in one experiment.

2.7.2. Population pharmacokinetics modeling of OS and OSA in rats

Nonlinear mixed-effects modeling was performed with NONMEM (version 7.4, ICON Development Solutions, Ellicott City, Maryland, USA) and Perl-speaks-NONMEM (version 4.8.1; Uppsala University, Uppsala, Sweden). The structural model building was performed under ADVAN 6 with the first order conditional method with interaction (FOCE + I), and the variance-covariance matrix was calculated using the default method to estimate the relative standard error (RSE) of each parameter. The final model was established in a stepwise manner per the following criteria: (1) minimum of objective function value (OFV); (2) successful both for minimization and covariance calculation; (3) RSE < 50%; (4) only accepting additional parameter when the dropped OFV > 3.84 (p < 0.05); (5) shrinkage < 50% for accepting the addition of an inter-individual variability (IVV) of one parameter. All models were also visually qualified based on goodness-of-fit of plasma concentration–time profiles and accumulative urine amount of OS and OSA on an individual and population level. A visual predictive check (VPC) and bootstrap procedure (n = 1000) were used as internal validation tools to ensure the ruggedness and predictability of the final model. From the bootstrap estimation, the median and 2.5th and 97.5th percentiles were obtained to represent the non-parametric 95% confidence intervals (CI).

3. Results

3.1. In-vitro inhibition of OS hydrolysis by studied herbs/herbal components

Inhibition effects of the eight herb extracts (H1 to H8) and their ten marker components (C1 to C10) on the hydrolysis of OS were evaluated in rat plasma, RLM and hCE1 by incubating OS in presence and absence of studied herb/herbal components. The incubation system for rat plasma contains 1 μg/mL of OS and 10% rat plasma since the super-fast reaction rate of 100% rat plasma resulted in no discrimination for nearly all the studied herb/herbal components. The results were demonstrated in Fig. S1. The formation rate of OSA could be significantly suppressed by all the herb/herbal components. Among all the selected herbs, H4 (Fructus Forsythiae) and H8 (Fructus Arctii) can almost completely inhibit the hydrolysis of OS (> 97% OS remained after 60 min incubation). For the herbal components, C4 and C6, which are the marker compounds from Radix Scutellariae, could inhibit more than 50% of OS hydrolysis. Results from RLM and hCE1 showed a similar trend for the ten marker components (Figs. S2 and S3), in which both
C1 and C2 significantly suppressed the OS hydrolysis. This suggests the hydrolysis of OS in RLM are mainly mediated by an analogue of hCE1.

3.2. Modification on transport of OS by herbs/herbal components

Cytotoxicity test of herbs/herbal components on MDCK cells (MDCK-WT and MDCK-MDR1) and HEK293 cells (HEK293-MOCK and HEK293-OAT3) did not show significant cytotoxicity at concentrations up to 1 mg/mL. In addition, the corresponding ten tested herbal components did not show significant cytotoxicity on MDCK-WT, MDCK-MDR1, HEK29-MOCK and HEK293-OAT3 cells at concentrations up to 100 μg/mL. The viability of all cells was more than 80% in presence of both oseltamivir (30 μg/mL) and herbs/herbal components at the studied highest concentrations. The maximum concentrations of herbs and herbal components for transport and uptake studies were therefore set at 1 mg/mL and 100 μg/mL, respectively, for MDCK-WT, MDCK-MDR1, HEK29-MOCK and HEK293-OAT3 cells.

As shown in Fig. S4, the P_app values of OS were significantly increased when co-incubated with C4 (Baicalin), H2 (Radix et Rhizoma Glycyrrhizae) and C2 (Glycyrrhizic acid). It suggested that they could inhibit the efflux effect of P-gp, and thus promoted the absorption of OS. Since transfected PEPT1 cells are not available, we used a PEPT1-inhibitor, Gly-Sar, to inhibit PEPT1 and to check if co-incubation with herbs/herbal components could decrease the uptake of OS. The inhibition results are shown in Fig. S5. When adding the inhibitor, the uptake amount of OS was significantly lowered. However, when co-incubated with herbs or herbal components, no significant reduction of OS uptake was observed. On the contrary, the herbs (H1 to H7) and herbal components (C1, C3, C4) could increase the uptake of OS in Caco-2 cell. It seems that the uptake through PEPT1 transporter is not the dominant way in the absorption of OS. The increased uptake may be due to the inhibition of P-gp. As shown in Fig. S6, no difference was observed between HEK293-Mock and HEK293-OAT3 cells for the uptake of OS. The inhibition results are shown in Fig. S5. When adding the inhibitor, the uptake amount of OS was significantly lowered. However, when co-incubated with herbs or herbal components, no significant reduction of OS uptake was observed. On the contrary, the herbs (H1 to H7) and herbal components (C1, C3, C4) could increase the uptake of OS in Caco-2 cell. It seems that the uptake through PEPT1 transporter is not the dominant way in the absorption of OS. The increased uptake may be due to the inhibition of P-gp. As shown in Fig. S6, no difference was observed between HEK293-Mock and HEK293-OAT3 cells for the uptake of OS.

3.3. Selection of herb for verification of the potential herb-drug interaction (HDI) in rats

The above in-vitro experiments suggested that there should be some potential HDI between OS and these anti-influenza herbs/herbal components during absorption and metabolism. Since testing HDI in rat models is expensive and time-consuming, we prefer to select one herb, having the most possibility of HDI, to perform in-vivo experiments. To make it comparable for the results from the six in-vitro experiments, original values were converted to fold-change (FC) of the control group (no herb/herbal component interference) and then, the FC values in each experiment were converted into z-scores using z-transform. After z-transform, results from different experiments would be comparable since the mean and standard deviation of z-scores is always zero and one, respectively. The z-scores of each experiment were given in Fig. 1. The sum values of absolute z-scores of each herb/herbal component were given on the top of Fig. 1, right above each sample’s results. Based on the sum values of absolute z-scores, H3 (Radix Scutellariae), having a highest score (14.6) among all the studied herbs, was selected for further in-vivo experiments.

3.4. Pharmacokinetic interaction between OS and RS in rats

Per the above in-vitro screening in cells and enzymes, we found that H3 (Radix Scutellariae) and its marker components (C4-C6) have a clear hint to interact with OS during the absorption, transportation and metabolism processes. Thus, a standardized herbal extract of Radix Scutellariae (RS) was utilized to perform an herb-drug interaction study with OS in rats. After 5 days’ administration of the proposed dosages as described in Section 2.6, the plasma-concentration profiles of OS and OSA in groups OS + W (G1), OS+1H (G2) and OS+2H (G3) (Fig. 2, right) and their corresponding 8-h accumulated amount in urine were obtained. These data were analyzed using non-compartmental analysis (Table S3). The results suggested that co-administration with RS could significantly increase the systemic exposure of OS and decrease that of OSA (Fig. S7). This can further be verified by the lowered OSA/OS ratio in rat urine samples after co-administration with RS (Fig. S8).

To better understand the interaction mechanism between OS and RS during the absorption and metabolism processes, a population pharmacokinetics (pop-PK) model was developed using both the plasma and urine PK data from groups OS + W, OS+1H and OS+2H. As shown in Fig. 2 (left), the final model was a four compartmental PK model (two compartments for OS and OSA distribution, and another two to represent the reservoirs of OS and OSA in urine) with two absorption compartments (one for directly absorbed amount of OS and one for first-pass eliminated amount of OS). OS was absorbed from deposit compartment into the central compartment via a first-order process (k_a) and the disposition of OS and OSA could be described by a one-compartment model. The reported volume of distribution for OS and OSA in rats were 3.1 and 1.3 L/kg, respectively, with a ratio of 2.42 (equal to 3.1 divided by 1.3). Thus, in the final model the distribution volume of OS is V_d and that of OSA is assumed to be V_d/2.42. The formation of OSA from OS was either from first-pass metabolism or from the OS in the central compartment via CL_mec. To determine if co-administration with RS could lead to different structural parameters (k_a, CL_mec, V_d, FP, etc.)
BIO, $k_{e,\text{OS}}$, $k_{e,\text{OSA}}$), in the modeling development process, models with identical or different structural parameters among the three groups were tried and compared. Only when the dropped OFV is bigger than 3.84 ($p < 0.05$), these structural parameters were thought to be significantly different after co-administration with RS (Table 1). It was found that co-administration with RS had no effect on the absorption rate constant ($k_a$) and metabolic clearance ($CL_{\text{met}}$) of OS, but would decrease $V_d$ and increase the remaining structural parameters (FP, BIO, $k_{e,\text{OS}}$, $k_{e,\text{OSA}}$). Inter-individual variability (IV) were successfully estimated for parameters $CL_{\text{met}}$, $V_d$ and $k_a$. However, for other structural parameters, IV tend to be zero with a shrinkage close to 100%. In the error model, both additive and proportional errors were added to the observed values from plasma and urine, respectively. Due to the RSE of additive errors were high than 50%, the additive errors were fixed to be zero and only proportional errors were included in the final model. We also tried to use only one proportional error parameter for both plasma and urine data, but this led to an increased OFV of 8.2.

Goodness-of-fit plots showed good agreement between predicted and observed concentrations/amounts with no apparent bias in residual (Figs. S9 and S10). The VPC result for the covariate analysis revealed that the observed and predicted concentrations/amounts with no apparent bias in residual and urine data, but this led to an increased OFV of 8.2.

By observing the microscopic morphological changes of MDCK SAIT-1 cells after incubating with serial dilutions of diluted rat plasma for 48 h, it showed that the maximum concentration of plasma preparation could be used for plaque reduction assay was 1:80. This plasma dilution was used for plaque reduction assay. The virus plaque reduction results of groups 1–6 (G1 to G6) are shown in Table 2. Results were mean values from 3 rats with each plasma sample tested in triplicate. The no. of plaque obtained from the G6 (no herb and no oseltamivir administration) was used as reference. The herb only groups (G4 and G5) did not show significant reduction in virus plaque of either H1N1 or H3N2. A significant reduction in the number of virus plaque (both H1N1 and H3N2) was observed for oseltamivir treated groups (G1, G2 and G3) and co-administration of one/two times HED of RS extracts with OS did not increase the total antivirus effect.

### Table 1

| Parameters | Estimation (RSE%) [shr.:%] | Bootstrap median [95% CI] (bias%) |
|------------|---------------------------|----------------------------------|
| $k_a$ (1/h) | 0.211 (6%) | 0.211 [0.179, 0.231] (0%) |
| $CL_{\text{met}}$ (mL/h) | 99.8 (37%) | 99.5 [31.8, 163] (0.7%) |
| $V_d$ (mL) | 104 (27%) | 104 [59.0, 168] (0%) |
| FP | 0.343 (10%) | 0.342 [0.281, 0.400] (0.3%) |
| BIO | 0.236 (8%) | 0.236 [0.197, 0.275] (0.7%) |
| $k_{e,\text{OS}}$ | 1.49 (22%) | 1.46 [1.03, 2.36] (0.5%) |
| $k_{e,\text{OSA}}$ | 0.851 (21%) | 0.857 [0.615, 1.331] (0.7%) |
| IV $CL_{\text{met}}$ (%) | 23.4% (34%) | 23.1% [17.5, 46.8] (0.5%) |
| IV $V_d$ (%) | 16.8% (22%) | 16.5% [7.5, 24.8] (0.8%) |
| IV $k_a$ (%) | 21.8% (16%) | 21.2% [14.0, 37.3] (0.8%) |
| Prop. error (plasma) | 0.358 (6%) | 0.355 [0.321, 0.398] (0.8%) |
| Prop. error (urine) | 0.271 (14%) | 0.259 [0.011, 0.327] (1.4%) |

$k_a$: absorption rate constant of Oseltamivir (OS); $k_{e,\text{OS}}$: elimination rate constant of OS; $k_{e,\text{OSA}}$: elimination rate constant of oseltamivir acid (OSA); $CL_{\text{met}}$: metabolic clearance of OS; $V_d$: volume distribution of OS; BIO: fraction of OS absorbed into the body; FP: remaining fraction after first-pass elimination; IV: inter-individual variability; Add.: additive; Prop.: proportional; RSE: relative standard errors; shr.: shrinkage; CI: confidence interval; bias% = (bootstrap median – estimation) *100/estimation.
and the dotted lines are the 5th and 95th percentiles of the observed data. The shaded areas are the 95% confidence intervals for the median, 5th percentile and 95th percentiles of the simulated data. Observed concentrations are displayed as open circles. OS: oseltamivir; OSA: oseltamivir acid.

Table 2
Effect of plasma samples obtained from different treatment groups on influenza A H1N1 and H3N2 virus plaque formation (n = 3).

| Groups     | Average no. of plaque | Plaque reduction (%) |
|------------|------------------------|----------------------|
|            | H1N1                  | H3N2                 |
| OS + W     | 12.5 ± 1.1**          | 6.8 ± 1.8**          | 61.6  | 65.7 |
| OS + 1H    | 12.6 ± 1.9**          | 6.7 ± 2.3**          | 61.3  | 66.5 |
| OS + 2H    | 13.5 ± 2.4**          | 7.8 ± 0.8**          | 58.6  | 60.7 |
| W + 1H     | 22.9 ± 5.2            | 16.3 ± 2.5           | 29.7  | 18.0 |
| W + 2H     | 24.2 ± 3.7            | 17.4 ± 1.5           | 25.8  | 12.6 |
| W + W      | 32.6 ± 3.8            | 19.9 ± 3.8           | –     | –    |

Note: OS: oseltamivir, W: water, 1H: 300 mg/kg of Radix Scutellariae, 2H: 600 mg/kg of Radix Scutellariae. Student t-test were calculated between W + W (control group) and other groups (**p < 0.01). — not applicable.

inhibition of OS hydrolisis in rat plasma, but no inhibition in microsomes and hCE1 systems. Among the herbal components, herbal component C1 was the strongest inhibitor for OS hydrolisis in microsomes and hCE1 while in rat plasma it ranked behind C4, C6 and C10. The composition and enzymes in rat plasma are much more complex than those in microsomes and hCE1. Major esterases in plasma are butyr- ylcholinesterase (BChE), paraoxonase (PON), and carboxylesterase (CES) (Bahar et al., 2012). The most abundant plasma protein is al- ylcholinesterase (BChE), paraoxonase (PON), and carboxylesterase those in microsomes and hCE1. Major esterases in plasma are butyr-

Based on the in-vitro screening results, RS extract was co-admini-

score of an herb/herbal component effect on OS would be proportional to its level of HDI relevancy with OS. Thus, the sum of all the absolute z-scores for each herb/herbal component in the six in-vitro experiments would be an indicator for their HDI potential.

For PK studies, plasma concentration data from 10 time-points were used, while only one time-point at 2.5 h after oseltamivir administra-

z-transformation to make the results with different nature comparable between experiments. Fold change is a measure describing how much a quantity changes going from an initial (control) to a final value and is often used in analysis of gene expression data in microarray and RNA-Seq experiments, for measuring change in the expression level of a gene (Tusher et al., 2001).

In our experiments, FC could be considered as the contribution of herbs/herbal components in each experiment. The more FC is far from one (bigger or less than 1), the more likely the studied herbs/herbal components have an HDI with OS. However, FC still could not be compared between experiments due to varied FC ranges in each experiment. In- spiried by multivariate statistical methods (Varmuza and Filzmoser, 2009), we chose z-transformation, a common normalization method, to normalize the FC values in each experiment to make them having the same distribution of N(0,1). After such a normalization, the absolute z-score of an herb/herbal component effect on OS would be proportional to its level of HDI relevancy with OS. Thus, the sum of all the absolute z-scores for each herb/herbal component in the six in-vitro experiments would be an indicator for their HDI potential.

Since the data from the six in-vitro experiments have different units and intensity ranges, they cannot be compared directly. Our current proposed method is to first convert the raw values of each experiment to fold change (FC) of their control groups and then normalize the FC values of each experiment with z-transformation to make the results with different nature comparable between experiments. Fold change is
A platform including a six-dimension in-vitro screening, an in-vivo pharmacokinetics in rats and an ex-vivo pharmacodynamic evaluation, has been developed and successfully applied to find RS as a potential herb that would influence the co-administered OS in rats. Our work sets a good example to demonstrate the feasibility and advantage of using multidisciplinary techniques including experimental science, informatics and pharmacometrics on the investigation of herb-drug interactions. Potential herb-drug interaction (HDI) between OS and herb/herbal components observed in the current project serve as a good platform to identify the potential interacted herbs and related mechanism for future clinical monitoring of OS during its integrative medicine practice in human subjects.

5. Conclusions

Our six-dimension in-vitro screening identified RS as the herb to have the most HDI potential with OS. Co-administered RS could inhibit OS hydrolysis during absorption and increase the absorbed fraction of OS, which lead to the increased ratio of OS concentration versus that of OSA in both rat plasma and urine. Never the less, the anti-virus effects of OS were not influenced by co-administered RS.

Herbs studied in this article

Radix Scutellariae (Root of Scutellaria baicalensis Georgi, Huangqin).
Radix Isatisidis (Root of Isatis indigotica Fort., Banlangen).
Radix et Rhizoma Glycyrrhizae (Rhizome of Glycyrrhiza uralensis Fisch., Gancao).
Fructus Forsythiae (Fruit of Forsythia suspensa (Thunb.) Vahl, Lianqiao).
Flos Lonicerae Japonicae (Flower of Lonicera japonica Thunb., Jinyinhuai).
Folium Mori (Leaf of Morus alba L., Sangye).
Herba Schizonepetae (Aerial part of Schizonepeta tenuifolia (Benth.) Briq., Jingjie).
Fructus Arctii (Fruit of Arctium lappa L., Niubangzi).

Conflicts of interest

There are no conflicts of interest among the authors.

Author contribution

Yufeng Zhang, Rat PK, modeling and Manuscript drafting; Chunming Lyu, In-vitro metabolic inhibition and transporter study; Sophia Fong, Transporter study; Qian Wang, Rat PK; Chenrui Li, Rat PK; Nicolas James Ho, Manuscript proof reading; Kay Sheung Chan, Experimental design and plaque reduction assays; Xiaoyu Yan, PK modeling; Zhong Zuo, Experimental design, data analyses and manuscript drafting

Acknowledgments

This work is supported by Health and Medical Research Fund (Reference number: 11120451) from the Food and Health Bureau of the Government of the Hong Kong Special Administrative Region.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2019.112097.

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