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Synergistic activity of baicalein with ribavirin against influenza A (H1N1) virus infections in cell culture and in mice

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ABSTRACT

Baicalein is a flavonoid derived from the root of *Scutellaria baicalensis*, a traditional Chinese medicine that has been used for hundreds of years; baicalein has also been demonstrated to have antiviral activity with low toxicity. The synergistic activity of baicalein with ribavirin against influenza virus infections in cell culture and in mice was investigated for the first time in our research. In vitro, maximal synergy at lower concentrations of baicalein (0.125 μg/ml) and ribavirin (12.5 μg/ml) was observed, and the reduced expression of the viral matrix protein (M) gene suggested that drug combinations caused greater inhibition than ribavirin alone, especially the combination of 0.5 μg/ml baicalein and 5 μg/ml ribavirin. In vivo, combinations of baicalein and ribavirin provided a higher survival rate and lower body weight loss. Moreover, fewer inflammatory responses in the lungs of mice infected with virus and treated with baicalein and ribavirin were observed; the mean scores were 1.0, 0.8, and 1.2 with the doses of ribavirin at 50 mg/kg/d combined with baicalein at 100 mg/kg/d, 200 mg/kg/d, and 400 mg/kg/d respectively, while the placebo group had a mean pathology score of 3.2. Thus, the data demonstrates that combinations of baicalein and ribavirin provide better protection against influenza infection than each compound used alone and could potentially be clinically useful.

1. Introduction

Globally, influenza A virus infection is responsible for significant morbidity and mortality, usually causing severe epidemics of respiratory illness; symptoms are numerous and include sore throat, sneezing, fever, headache, muscle fatigue, and inactivity (García-García and Ramos, 2006; van der Sluijs et al., 2004). Influenza A virus infection has the potential to become a much more dangerous disease than it is at present because of easy transmission, rapid emergence of resistance, and the limited efficacy of currently available therapies. Influenza A virus can cause between 3–5 million cases of severe illness in a normal season and up to 500,000 deaths worldwide.

Currently, there are three classes of anti-influenza virus drugs with different modes of action: the M2 channel blockers (e.g., amantadine and rimantadine), neuraminidase (NA) inhibitors (e.g., oseltamivir) and RNA polymerase inhibitors (e.g., ribavirin). However, amantadine and rimantadine have limited usefulness, because of the emergence of drug-resistant variants (Choi et al., 2009; Higgins et al., 2009; Saito et al., 2007). It was reported that 22% of the H1N1 viruses isolated in 2006 in Thailand carried the amino acid substitution S31N of the M2 protein, which confers amantadine-resistance (Bai et al., 2009); furthermore, the frequency of amantadine-resistant H3N2 isolates was much higher than that of amantadine-resistant H1N1 isolates in the 2008–2009 season in South Korea (Choi et al., 2009). Recent oseltamivir-resistant viruses have a histidine (H) to tyrosine (Y) substitution at position 274 (H274Y) of the NA gene, which has also been linked to oseltamivir resistance (Baranovich et al., 2010; Eshaghi et al., 2009; Meijer et al., 2009; Reece, 2007; Triana-Baltzer et al., 2009). Ribavirin has been reported to act through multiple mechanisms affecting both virus replication and host immunoresponse. It is an RNA virus mutagen against HIV, herpes virus etc. to block viral replication as a chain terminator but not of poliovirus polymerase (Crotty et al., 2000). Moreover, ribavirin is a nucleoside analog and its monophosphate inhibits inosine monophosphate dehydrogenase (IMPDH), which is interesting because it potentially explained the broad-spectrum antiviral activity of ribavirin (Streeter et al., 1973). Besides, ribavirin may have immunomodulatory activity via affecting the expression of interleukin 10 in vivo (Tam et al., 2000). According to recent reports regarding ribavirin's mechanism of action, it's proposed that the action against RNA

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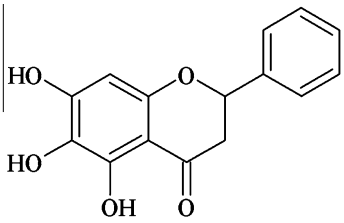

Fig. 1. Molecular structure of baicalein.

virus is mainly via lethal mutagenesis of viral RNA genomes (Conner, 1984; Eggleston, 1987). However, its undesirable toxic side effects have limited its use clinically.

Many studies have been reported using combination therapy. Combination treatments with two or more drugs that target different virus proteins might offer several advantages, such as greater potency and cost-effectiveness, reduction of dosages, and reduction of the risk of respiratory complications. The use of RNA polymerase inhibitors with amantadine or oseltamivir was associated with enhanced survival and was significantly more effective than either drug used alone in vitro (Nguyen et al., 2009, 2010; Smee et al., 2002, 2009) and in vivo (Ilyushina et al., 2008; Smee et al., 2002, 2009, 2010a,b). A combination of an NA inhibitor and an M2 channel blocker provided a survival advantage over single-agent treatment (Galabov et al., 2006; Ilyushina et al., 2007; Nguyen et al., 2009, 2010; Smee et al., 2009). Using combinations of two NA inhibitors, oseltamivir and peramivir, synergistic effects were also reported (Smee et al., 2010a,b).

Baicalein (5,6,7-trihydroxyflavone) (Fig. 1) is a flavonoid derived from the root of *Scutellaria baicalensis*, a traditional Chinese medicine which has been used for hundreds of years. Baicalein has been found to have various beneficial properties, such as antiviral (Ahn et al., 2001; Xu et al., 2010), antibacterial (Chang et al., 2007; Yang et al., 2000), anti-inflammatory (Yang et al., 2008), and antitumor (Ma et al., 2005; Wang et al., 2010) effects. Its ability to inhibit influenza virus in vivo was evaluated in our previous study, and the results suggested that baicalein in BALB/c mice infected with the influenza A/FM1/1/47 (H1N1) virus showed significant effects in preventing death, prolonging survival time, inhibiting lung consolidation, and reducing the viral titers in lungs in a dose-dependent manner (Xu et al., 2010). We further extended our work to describe the efficacy of baicalein's activity against lethal parainfluenza virus (Dou et al., 2011) and found that oral administration of baicalein resulted in significant inhibition of virus in vivo, linked to baicalin in the serum. Baicalin is the other major bioactive compound derived from the same herb. In the present study, we demonstrate for the first time the synergistic activity of baicalein with ribavirin against influenza virus infections in cell culture and in mice.

2. Materials and methods

2.1. Compounds

Baicalein was kindly provided by Prof. Qinglong Guo of the Department of Physiology, China Pharmaceutical University. It was dissolved in dimethyl sulfoxide (DMSO) for in vitro studies or in 0.5% sodium carboxymethyl cellulose (CMC) for oral gavage delivery in mice. Ribavirin was obtained from Sichuan Baili Pharmaceutical Co., Ltd, Sichuan, China.

2.2. Virus and cells

The influenza A/FM1/1/47 (H1N1) virus was maintained at the Department of Microbiology, School of Life Science and Technology, China Pharmaceutical University. Stock virus was grown in the allantoic cavity of 10-day-old embryonated chicken eggs for 2 d at 35 °C. The allantoic fluid was harvested, filtered with a 0.22-μm cellulose acetate membrane, and stored at −70 °C until use.

Dulbecco's modified Eagle's medium containing 10% (v/v) newborn calf serum (NBCS) was used for culturing Madin-Darby canine kidney (MDCK) cells at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. In vitro antiviral studies

Antiviral activities of the combinations of baicalein and ribavirin were determined in confluent cultures of MDCK cells. The assays were performed in 96-well microplates infected with approximately 100 $CCID_{50}$ (50% cell culture infectious doses) of virus, by quantifying virus-induced cytopathic effect (CPE) with crystal violet staining as described previously (Hung et al., 2009; Xu et al., 2010). Briefly, the MDCK cells were seeded onto 96-well microplates and cultured at 37 °C for 24 h. After 1 h for virus absorption, the viral solution was removed and replaced with growth medium (100 μl) containing 0.5% NBCS and baicalein (0.125 μg/ml or 0.25 μg/ml) combined with ribavirin (6.25 μg/ml, 12.5 μg/ml, or 25 μg/ml). After 3 days of incubation at 37 °C, the cells were fixed with 100 μl of 10% formaldehyde solution for 1 h. After removal of the solution, the cells were stained with 0.1% (w/v) crystal violet solution for 15 min at room temperature. Plates were washed, dried, and read for optical density determination at 570 nm.

Drug-drug interactions for three replicate assays were analyzed by the three-dimensional model of Prichard and Shipman (Prichard and Shipman, 1990), using the MacSynergy II software program at 95% confidence limits. The resulting surface diagram appears as horizontal plane at 0% of synergy (antagonism) if the interactions of the two compounds are additive. Any peak above or below this horizontal plane indicates synergy or antagonism, respectively (Kim et al., 2010).

2.4. Analysis of M gene amplification by RT-PCR

The M gene (1027 bp) encodes two proteins, M1 (nucleotide positions 26 to 784) and M2 (positions 26 to 51 and 740 to 1007) (Lamb et al., 1981). The M2 protein comprises 97 amino acids and has ion channel activity. The functions of M1 proteins are usually multifaceted, and not completely understood. They are, however, known to be involved in viral assembly and envelope stabilization (Lyles et al., 1992).

MDCK cells were infected with 100 $CCID_{50}$ of the influenza A/FM1/1/47 (H1N1) virus for 1 h in the presence or absence of drug, and total RNA was isolated after 36 h cultivation using TRIzol (Beijing TransGen Biotech Co., Ltd., Beijing, China). For first-strand cDNA synthesis, 5 μl of total RNA was primed with oligo $(dT)_{15}$ (Invitrogen) at a final concentration of 25 ng/μl. Reverse transcription was performed with 10 U of Moloney murine leukemia virus (M-MLV) RT/μl (Beijing TransGen Biotech Co., Ltd., Beijing, China) in the presence of 1.25 U of Rnasin/μl (Beijing TransGen Biotech Co., Ltd., Beijing, China). Reverse transcription was carried out at 42 °C for 15 min, then at 95 °C for 5 min. Thereafter, 4/20 volume of cDNA was used as a template for PCR (30 cycles). A thermal cycler was programmed as follows: 95 °C for 5 min; cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 20 s; and 72 °C for 10 min. An aliquot of 6 μl from each PCR product was loaded onto an agarose gel (2%) containing ethidium bromide (0.5 μg/ml) and separated by electrophoresis. A partial segment of the M1 gene from the H1N1 virus was amplified in this reaction using the following primer sets: 5′-TTCTAACCGAGGTCGAAAC-3′ and 5′-AAGCG TCTACGCTGCAGTCC-3′. The internal control GAPDH was amplified

using primers 5′-CACTCACGGCAAATTCAACGGCAC-3′ and 5′-GAC TCCACGACATACTCAGCAC-3′.

2.5. Animal experiment design

Female ICR mice (6 weeks in age) weighing approximately 18 to 20 g were purchased from Laboratory Animal Centre, Nantong University. Animals were treated according to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health; protocols were approved by the Ethics Committee of China Pharmaceutical University, and experiments were carried out in strict accordance. Mice were anesthetized by inhalation of diethyl ether, followed by intranasal infection with a 50 μl suspension of 8 MLD_{50} (50% murine lethal dose) of influenza A/FM1/1/47 (H1N1) in phosphate-buffered saline (PBS). Groups of 10 mice were then given baicalein (400 mg/kg/day) or ribavirin (50 mg/kg/day) by oral gavage twice daily for 5 days starting 24 h before virus challenge. Drug combinations were administered with baicalein (100, 200, or 400 mg/kg/day) and ribavirin (50, 25, 12.5 mg/kg/day) on the same schedule. Virus-infected mice received 0.5% CMC as a placebo. Survival was observed daily for 14 days. Mean day to death values were statistically analyzed and mice were weighed every other day.

Lung index and lung index inhibition were determined in one experiment using six mice per group. Mice were weighed and killed on day 5 after inoculation, and lungs were removed and weighed. The lung index and the lung index inhibition were calculated by the following equation using the obtained values:

$$\text{Lung index} = (A/B) \times 100\%$$

$$\text{Lung index inhibition}(\%) = (C - D)/C \times 100\%$$

where A is lung weight, B is body weight, C is the mean lung index of the placebo control group, and D is the mean lung index of the drug-treated group.

Lung consolidation scores ranging from 0 (a normal lung with no inflammation) to 4 (diffuse bronchointerstitial pneumonia) were assigned. At least three mice were formalin-fixed and processed for histopathology.

2.6. Statistical analysis for animal experiments

Data from the in vivo studies are presented as mean ± SD. Comparisons were made between placebo and treated groups, and between combination treatments versus single drug treatment. Differences in the mean day to death and lung indices were evaluated by the unpaired, two-tailed t-test. The Kaplan–Meier method was used to estimate the probability of the survival of the mice, and survival estimates were analyzed by the log-rank test.

3. Results

3.1. Antiviral activity in cell culture

In vitro cytotoxicities of baicalein, ribavirin, and their combinations were investigated in cell culture for 72 h through MTT assay. The results showed that no cytotoxicities were observed in combinations of 32 μg/ml ribavirin and 0.25 μg/ml baicalein. The inhibition activity against influenza A/FM1/1/47 (H1N1) virus in vitro was determined by measuring the inhibition of virus-induced cytopathic effect in MDCK cells, as determined by crystal violet staining. A three-dimensional MacSynergy plot of the data shows the synergistic (antagonistic) effect of the combination of baicalein and ribavirin on cell survival (Fig. 2).

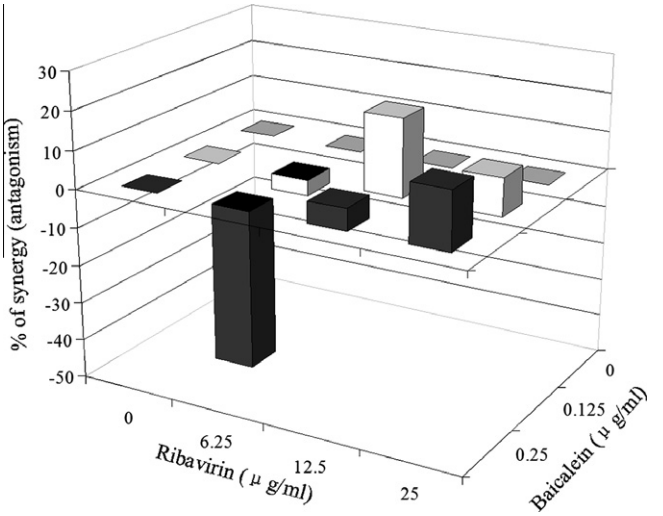

Fig. 2. Three-dimensional plots showing the interaction of baicalein and ribavirin and the effect of treatment on cell survival rate. The synergy or antagonism for the various drug combinations was significant at the 95% confidence level.

A region of synergy was generated with ribavirin (12.5 μg/ml or 25 μg/ml) combined with baicalein (0.125 μg/ml or 0.25 μg/ml), giving a synergy volume of 50.1. However, when ribavirin (6.25 μg/ml) was combined with each concentration of baicalein, the results were indicative of antagonism with a volume of −44.3. Maximal synergy was observed at lower concentrations of baicalein (0.125 μg/ml) and ribavirin (12.5 μg/ml), and the combination of baicalein (0.25 μg/ml) and ribavirin (25 μg/ml) was slightly smaller. The net effect across the entire surface was a volume of synergy of 5.8.

3.2. Suppression of viral RNA synthesis in the cells inoculated with the combination of drugs

After virus infection and treatment with baicalein and ribavirin, the total RNA of cells was extracted in each well using Trizol, as described above. The inhibitory effects of M gene synthesis were confirmed by RT-PCR analysis. Specific amplified products of M1 (229 bp) and GAPDH by the two pairs of primers were identified as described in "Section 2" (Fig. 3A). The combination of ribavirin and baicalein caused greater inhibition than ribavirin used alone,

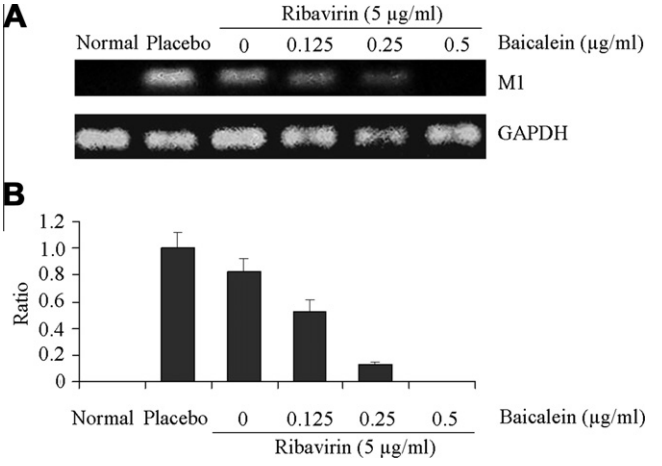

Fig. 3. RT-PCR analysis of the M1 gene. Cells infected with the influenza A/FM1/1/47 (H1N1) virus were subjected to RT-PCR. Housekeeping gene GAPDH was an internal reference. (A) RT-PCR products of M1 and GAPDH; (B) the ratio of M1 expression level in the combination groups to that of the placebo group.

especially the combination of 0.5 μg/ml baicalein and 5 μg/ml ribavirin. No significant difference in the expression of housekeeping gene GAPDH was observed among different groups. At the same time, the result from the placebo group indicated active replication of the virus in the MDCK cells following viral inoculation. Band intensity was analyzed with a Bio-Imaging System and Quality One 1-D analysis software (Bio-Rad, USA). The ratio of the M1 peak value for the combination groups to that of the placebo group was calculated by the software (Fig. 3B). The ratio decreased with an increased dose of baicalein (at 5 μg/ml ribavirin) after virus infection, and the ratios for ribavirin (5 μg/ml) combined with baicalein (0.125, 0.25, and 0.5 μg/ml) were 0.53, 0.13, and 0.00, respectively.

3.3. Effect of baicalein and ribavirin on survival of mice lethally challenged with influenza A (H1N1) virus

The toxicity of baicalein in vivo is very low, and the maximum tolerated dose of baicalein is 15.4 g/kg in mice (Table 3). No adverse effects were observed in terms of body weight loss or appearance of mice when uninfected mice were treated with ribavirin (100 mg/kg/d) or baicalein (800 mg/kg/d) alone, or in combinations using this dose of each inhibitor for 5 days (data not shown). To examine whether baicalein is synergistic with ribavirin against influenza A/FM1/1/47 (H1N1), ICR mice were pretreated i.g. with baicalein and ribavirin 24 h before lethal influenza A/FM1/1/47 (H1N1) infection. Results of combination treatments of a lethal infection in mice with baicalein and ribavirin are reported in Table 1. A dose of Ribavirin (50 mg/kg/d) was used in combination with baicalein to treat infections in mice caused by A/FM1/1/47 (H1N1). Ribavirin alone at 50 mg/kg/d was 50% protective, and baicalein alone at 400 mg/kg/d was 20% protective. The 50 mg/kg/d dose of ribavirin combined with 100 mg/kg/d, 200 mg/kg/d, and 400 mg/kg/d doses of baicalein resulted in 90%, 80%, and 100% protection, respectively; the combined treatment significantly improved the survival rate ($P < 0.001$) compared to the placebo group. To evaluate the efficacy of baicalein synergistic with ribavirin at lower dosages, we administered the 25 and 12.5 mg/kg/d of drug to mice and inoculated them with influenza A (H1N1) virus. Mice that received 25 or 12.5 mg/kg/d of ribavirin combined with baicalein had higher survival rates and mean days to death than those that received placebo. However, compared to the drugs used alone, the synergistic was not obvious.

Table 1
Treatment of influenza A/FM1/1/47 (H1N1) virus infection in mice with ribavirin and baicalein, either alone or in combination ($n = 10$).

Baicalein (mg/kg/day)[a]	Ribavirin (mg/kg/day)	No. survivors/total no.	MDD ± SD[b]
400	0	2/10	8.1 ± 3.1
0	50	5/10	10.8 ± 3.4**
400	50	10/10	14.0 ± 0.0***,ϕ,ψψψ
200	50	8/10	12.5 ± 3.2***,ψψ
100	50	9/10	13.3 ± 2.2***,ψψψ
400	25	4/10	11.2 ± 3.4***
200	25	4/10	10.2 ± 3.0*
100	25	2/10	9.1 ± 2.8
400	12.5	4/10	10.9 ± 2.5***
200	12.5	1/10	9.5 ± 3.7
100	12.5	0/10	8.1 ± 2.0
Placebo		0/10	7.1 ± 1.5

[a] Oral treatments were given twice a day for 5 days starting 24 h prior to virus exposure.
[b] Mean day to death of mice dying prior to day 14.
* $P < 0.05$ compared to placebo controls.
** $P < 0.01$ compared to placebo controls.
*** $P < 0.001$ compared to placebo controls.
ϕ $P < 0.01$ compared to ribavirin used alone.
ψψ $P < 0.01$ compared to baicalein used alone.
ψψψ $P < 0.001$ compared to baicalein used alone.

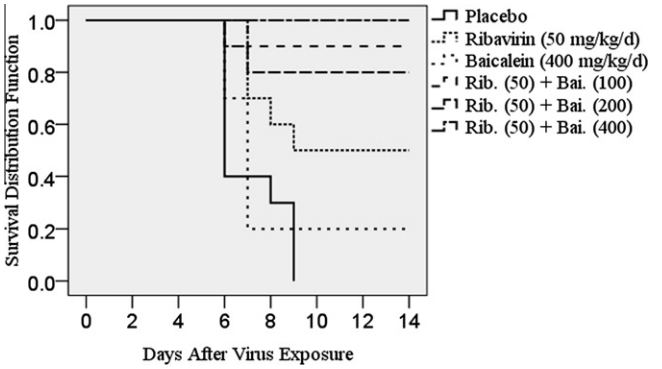

Fig. 4. Mouse Survival: ICR mice were infected with 8 MLD_{50} of influenza virus (H1N1) and orally treated with ribavirin and baicalein, used alone or in combination. Oral treatments were given twice a day for 5 d, starting 24 h before virus exposure. Ten mice per group were observed for 14 d for clinical signs of infection or death ($n = 10$).

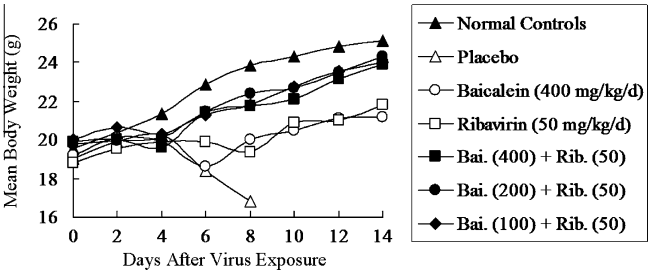

Fig. 5. Effects of murine treatment with ribavirin and baicalein, used alone or in combination, on mean body weight during an influenza A/FM1/1/47 (H1N1) virus infection. Oral treatments were given twice a day for 5 d, starting 24 h before virus exposure ($n = 10$).

Moreover, the 50 mg/kg/d dose of ribavirin combined with 100 mg/kg/d and 400 mg/kg/d doses of baicalein significantly improved the survival rate ($P < 0.001$), compared to the baicalein group used alone. The 50 mg/kg/d dose of ribavirin combined with the 400 mg/kg/d dose of baicalein also statistically improved the survival rate ($P < 0.01$).

Mean days to death for the experiment are also shown in Table 1. The treatments at all doses increased the mean day to death compared to the placebo group, except for baicalein used alone. The combination drug treatments resulted in a longer delay than the drugs used alone in the mean day to death; especially when ribavirin (50 mg/kg/d) was combined with baicalein (400 mg/kg/d), mice were completely protected (the mean day to death was 14 d). Mortality of the placebo group occurred on day 6 after virus exposure and all died on day 9, for which the combination of ribavirin (50 mg/kg/d) and baicalein (200 mg/kg/d) was 1 d later than in the placebo group (Fig. 4).

The combination treatment of 50 mg/kg/d dose of ribavirin combined with 100 mg/kg/d, 200 mg/kg/d, and 400 mg/kg/d doses of baicalein significantly protected mice from lethal challenge with no significant weight loss (Fig. 5), whereas all of the mice that received CMC treatment demonstrated significant weight loss (Placebo). In addition, the mice with combination treatments recovered their weight on day 4, compared with single-drug on day 8.

3.4. Effect of baicalein and ribavirin on influenza A (H1N1) virus replication in the lung

To further examine whether baicalein is synergistic with ribavirin against influenza A/FM1/1/47 (H1N1), we assayed the lung index and lung index inhibition of mice that received the various test

Table 2
Effect of ribavirin and baicalein used alone or in combination on lung parameters of mice infected by influenza A/FM1/1/47 (H1N1) virus ($n = 6$).

Compoud 1 (mg/kg/day)	Compoud 2 (mg/kg/day)	Mean lung parameters		
		Score ± SD	Lung index ± SD (%)	Lung index inhibition (%)
Normal		0.0 ± 0.0	0.67 ± 0.04	–
Placebo		3.2 ± 0.8	2.15 ± 0.04	–
Ribavirin (50)		1.3 ± 0.5*	1.32 ± 0.09*	39
Baicalein (400)		2.8 ± 0.5	1.92 ± 0.19	11
Ribavirin (50)	Baicalein (100)	1.0 ± 0.0$^{***,\psi\psi\psi}$	1.15 ± 0.04$^{*,\varphi,\psi\psi}$	47
Ribavirin (50)	Baicalein (200)	0.8 ± 0.8$^{***,\psi\psi\psi}$	1.03 ± 0.04$^{**,\varphi\varphi,\psi\psi}$	53
Ribavirin (50)	Baicalein (400)	1.2 ± 0.8$^{**,\psi\psi\psi}$	1.00 ± 0.09$^{**,\varphi,\psi\psi}$	54

* $P < 0.05$ compared to placebo.
** $P < 0.01$ compared to placebo.
*** $P < 0.001$ compared to placebo.
$^{\varphi}$ $P < 0.05$ compared to ribavirin used alone.
$^{\varphi\varphi}$ $P < 0.01$ compared to ribavirin used alone.
$^{\psi\psi}$ $P < 0.01$ compared to baicalein used alone.
$^{\psi\psi\psi}$ $P < 0.001$, compared to baicalein used alone.

compounds. Lung parameter data (Table 2) showed that treatment with ribavirin and baicalein in combination decrease the lung index significantly and similarly, compared to baicalein used alone ($P < 0.01$). Importantly, the combination of ribavirin (50 mg/kg/d) and baicalein (200 mg/kg/d) statistically reduced the lung index ($P < 0.01$), compared to the ribavirin alone. The lung index inhibition scores were 47%, 53%, and 54%, with ribavirin at 50 mg/kg/d combined with baicalein at 100 mg/kg/d, 200 mg/kg/d, and 400 mg/kg/d each; groups treated with ribavirin or baicalein alone had inhibition scores of 39% and 11%, respectively.

Table 3
Selectivity indexes (SI) of baicalein, baicalin and ribavirin on influenza A/FM1/1/47 (H1N1) virus in vitro and in vivo.

Drugs	In vitro (MDCK cells)			In vivo (ICR mice)		
	TC_{50} (μg/ml)	IC_{50} (μg/ml)	SI	Tolerated dose (mg/kg)	ED_{50} (mg/kg)	SI
Baicalein	0.5	>0.5	<1	15400	<100[a]	>154
Baicalin	16	0.70 ± 0.09	22.9	–	–	–
Ribavirin	64	4.10 ± 0.13	15.6	–	–	–

[a] Stands for baicalein only, which is one of the compounds of baicalein (100 mg/kg/d) and ribavirin (50 mg/kg/d).

The lungs of mice were examined for histopathologic changes caused by influenza A (H1N1) virus at 6 d.p.i. by hematoxylin–eosin staining (Fig. 6). The bronchial epithelial cells were necrotic in mice infected with virus (Placebo drug), with thickened alveolar walls and alveolar spaces filled with moderate inflammatory infiltrates of neutrophils, macrophages, and lymphocytes. The placebo group had a mean pathology score of 3.2. No signs of lung inflammation or pathological changes were seen in the control group (Normal), which had a mean pathology score of 0.0. However, the lungs of mice treated with the combinations of baicalein and ribavirin had less inflammatory responses, and the mean scores were 1.0, 0.8, and 1.2 with 50 mg/kg/d ribavirin combined with baicalein at 100 mg/kg/d, 200 mg/kg/d, or 400 mg/kg/d (Table 2). The combination treatment groups had a statistically lower mean pathology score ($P < 0.001$) compared to baicalein use alone.

4. Discussion

This study is the first to assess the effectiveness of baicalein–ribavirin combination chemotherapy against the influenza A/FM1/

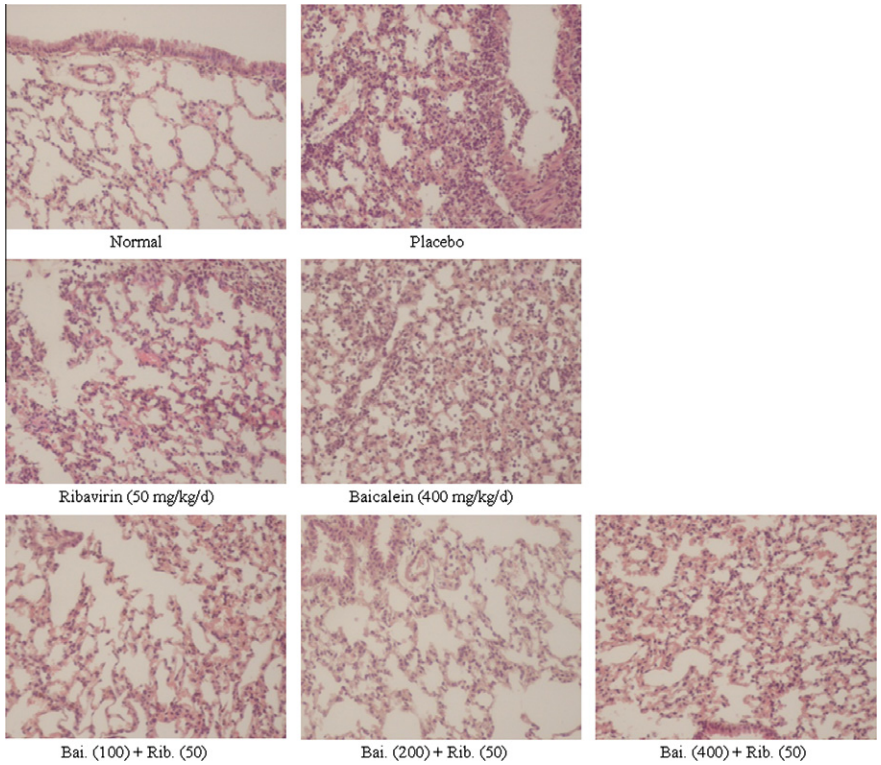

Fig. 6. Pathologic changes in lungs of mice infected with A/FM1/1/47 (H1N1). The mice were killed 6 d after infection. The lungs were removed and rinsed with sterile PBS. After fixing in 10% formalin, the lungs were sectioned for histologic examinations ($n = 3$).

1/47 (H1N1) virus in vitro and in vivo. The data demonstrated that combination treatment can have a greater antiviral effect than monotherapy in vivo.

Baicalein and baicalin, two of the major bioactive compounds found in the Chinese herb *S. baicalensis*, have been shown to be effective against cancer and bacterial diseases, among others (Ahn et al., 2001; Chang et al., 2007; Ma et al., 2005; Wang et al., 2010; Xu et al., 2010; Yang et al., 2008, 2000). In our previous study, we found that oral administration of baicalein resulted in significant inhibition of influenza A/FM1/1/47 (H1N1) virus in vivo, and linked to baicalin's antiviral activity in the serum (Xu et al., 2010), and related to NA inhibition of baicalin (IC_{50} = 3.22 μg/ml) (Dou et al., 2011). However, according the data obtained here, baicalein is less effective against virus in vitro. This is because baicalein has low solubility and marked cytotoxicity for MDCK cells in vitro (TC_{50} = 0.5 μg/ml) (Table 3), and the high dosage of baicalein used in combination with ribavirin was as low as 0.25 μg/ml, which may influence the potenty of antiviral activity and synergistic activity. This may explain why a combination of baicalein with ribavirin was less potent in vitro, with the net effect across the entire surface was a volume of synergy of 5.8. Actually, baicalein shows an effective antiviral activity in vivo with 100% protection at the 50 mg/kg/d dose of ribavirin combined with 400 mg/kg/d of baicalein, which is mainly due to its metabolite baicalin, where baicalein is converted to baicalin in the serum by UDP-glucuronosyltransferases and baicalin has the inhibition of neuraminidase (NA) (Xu et al., 2010; Dou et al., 2011).

Our animal experiments showed that combinations of baicalein and ribavirin could provide a prolonged survival time and rate for infected mice, as well as lung index inhibition. The virus titer was too low to be detected in the lungs of infected mice, suggesting a synergistic activity of baicalein and ribavirin against influenza A (H1N1) virus infection in mice, especially at the combination of baicalein (400 mg/kg/d) and ribavirin (50 mg/kg/d). The combination of drugs with different antiviral mechanisms may have clinical potential in the treatment of drug-resistant virus, which requires further study. As we know, ribavirin requires high drug doses for clinical treatment, and significant side effects of ribavirin have limited its use (Crotty et al., 2002). The toxicity of baicalein is very low in vivo with a maximum tolerated dose of 15.4 g/kg in mice, and selectivity index of baicalein which is one of the compounds of baicalein (100 mg/kg/d) and ribavirin (50 mg/kg/d) is more than 154 (Table 3).

The combination of baicalein and ribavirin could reduce the dosage of ribavirin in clinical treatment, leading to fewer side effects of ribavirin and higher effective antiviral activity.

Some effects of baicalein on viruses and its mechanism have been previously reported. For example, baicalein inhibited the activity of HIV-1 reverse transcriptase (RT) (Kitamura et al., 1998; Ono et al., 1989, 1990). Baicalein binds to the hydrophobic region of the HIV-1 integrase catalytic core domain, inducing a conformational change in the enzyme (Ahn et al., 2001). It was also reported that the primary mechanism of action for baicalein's antiviral activity against human cytomegalovirus (HCMV) was to prevent virus entry by targeting epidermal growth factor receptor (EGFR) (Evers et al., 2005). However, little is known about the mechanism of baicalein against influenza virus. In our previous study, we found that baicalin in the serum to some extent inhibited neuraminidase activity of sendai virus, with a mean IC_{50} of 3.22 μg/ml in a neuraminidase inhibition (NI) assay (Dou et al., 2011).

The exact mechanism by which baicalein and ribavirin synergistically reduce influenza virus infection in mice is interesting. As we know, ribavirin has multiple mechanisms affecting virus replication and immunomodulatory activity (Browne, 1978; Crotty et al., 2002; Parker, 2005; Vignuzzi1 et al., 2005); its primary antiviral mechanism is via lethal mutagenesis of RNA virus genomes. The antiviral activity of baicalein is by NA inhibition, which is different from ribavirin. Thus, it is an interesting challenge to elucidate which of the mechanisms of ribavirin is responsible for the synergy with baicalein. From the data obtained in our study, it is reasonable to speculate that different antiviral mechanisms may be important in the synergistic activity of baicalein with ribavirin against influenza A (H1N1) virus infections in mice.

In summary, these experiments demonstrated the synergistic activity of baicalein with ribavirin against influenza A (H1N1) virus infections in both cell culture and mice, which could be of clinical use.

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