

Identification and Characterization of Baicalin as a Phosphodiesterase 4 Inhibitor

Kyuhee Park,¹ Jong Suk Lee,¹ Jung Suk Choi,¹ Yeon-Ju Nam,¹ Jong-Heon Han,² Hoo-Dhon Byun,² Myung-Jin Song,¹ Joa-Sup Oh,¹ Sung Gyu Kim^{2,*} and Yongmun Choi^{1,*}

¹Bio-Center, Gyeonggi Institute of Science and Technology Promotion, Suwon, Gyeonggi-do, 443-270, Korea

²Bio Convergence Center, Jeju Technopark, Jeju city, Jeju-do, 699-121, Korea

Asthma is a chronic inflammatory disease of lung airways, and pharmacological inhibitors of cyclic adenosine monophosphate-specific phosphodiesterase 4 (PDE4) have been considered as therapeutics for the treatment of asthma. However, development of PDE4 inhibitors in clinical trials has been hampered because of the severe side effects of non-selective PDE4 inhibitors. Here, screening of a plant extract library in conjunction with dereplication technology led to identification of baicalin as a new type of PDE4-selective inhibitor. We demonstrated that while rolipram inhibited the enzyme activity of a range of PDE4 subtypes in *in vitro* enzyme assays, baicalin selectively inhibited the enzyme activity of PDE4A and 4B. In addition, baicalin suppressed lipopolysaccharide-induced TNF- α expression in macrophage where PDE4B plays a key role in lipopolysaccharide-induced signaling. Furthermore, baicalin treatment in an animal model of allergic asthma reduced inflammatory cell infiltration and TNF- α levels in bronchoalveolar lavage fluids, indicating that the antiinflammatory effects of baicalin *in vivo* are attributable, in part, to its ability to inhibit PDE4. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: phosphodiesterase; asthma; baicalin; TNF- α .

INTRODUCTION

Asthma is a chronic disease of lung airways characterized by allergic inflammation and bronchial hyper-responsiveness that ultimately lead to airway obstruction (Vijverberg *et al.*, 2013; Balhara and Gounni, 2012). The underlying pathophysiological features of asthma involve the interconnecting interaction between numerous types of cells in immune system and inflammatory mediators including histamine, leukotriene, and a range of pro-inflammatory cytokines and chemokines (Cohn *et al.*, 2004). In the acute phase of asthma, histamine and leukotriene, which are released from mast cells upon stimulation by antigens, trigger constriction of the bronchial airways resulting in symptom of breath shortness (Li *et al.*, 2014). Persistent exposure to allergens can lead to chronic inflammation, which is associated with the influx of such inflammatory cells into airway tissue as eosinophil, neutrophil, macrophage, and CD4⁺ lymphocytes (Fireman, 2003).

As inflammation plays a critical role in asthma pathogenesis, strategies have been developed to inhibit the release of inflammatory mediators and to suppress inflammatory reactions. Isoforms of phosphodiesterases¹ (PDEs) that are specific to cyclic adenosine monophosphate (cAMP) have attracted a great deal of attention because inhibition of cAMP-specific PDEs

demonstrated antiinflammatory effects in various cellular contexts (Banner and Press, 2009). PDE4 is among the well-characterized isoforms, and its inhibitors have been considered as novel therapeutics for the treatment of inflammatory diseases including asthma (Kumar *et al.*, 2013). This is attributed to the findings that PDE4 inhibition in inflammatory cells resulted in a reduction of the release of a range of pro-inflammatory mediators including TNF- α (Page and Spina, 2012).

Phosphodiesterase 4 family is comprised of four genes (PDE4A, B, C, and D), and each member has multiple subtypes resulted from the alternative splicing of RNA transcripts and use of distinct promoters. It is not clear why so many PDE4 isoforms exist; however, tissue-specific expression patterns and non-redundant functional roles for PDE4 family members have been reported (Houslay, 2010). This is in line with the observation that most of the non-selective PDE4 inhibitors that are under clinical test have side effects such as nausea and other gastrointestinal disturbances, suggesting that isoform-specific PDE4 inhibitors should be developed to avoid unwanted adverse effects of PDE4 inhibition.

Natural products have been considered a rich source of new drugs for decades (Cragg and Newman, 2013). The efforts at discovering new drugs from natural products have been aided greatly by the availability of natural product-derived libraries (collection of crude mixtures, fractions, and purified compounds) and spectral databases for single purified natural products. In addition, with the advance of technologies involving HPLC–mass spectrometry (MS) systems, hits derived from high-throughput screening of natural product libraries can be easily profiled by dereplication processes (Koehn and Carter, 2005; Nielsen *et al.*, 2011). Here, we report identification of a new type of PDE4 inhibitor using the methods described earlier.

* Correspondence to: Yongmun Choi, Bio-Center, Gyeonggi Institute of Science and Technology Promotion, Suwon, Gyeonggi-do, 443–270, Korea. E-mail: ychoi@gstep.re.kr

¹PDE, phosphodiesterase; LPS, lipopolysaccharide; cAMP, cyclic adenosine monophosphate; TNF, tumor necrosis factor; CRE, cAMP response element; OVA, ovalbumin.

MATERIALS AND METHODS

Chemicals. All of the standard compounds used in this study including rolipram and baicalin were purchased from Sigma-Aldrich Corporation LLC (Korea).

Plant materials and extraction. All plant materials used in this study including *Pourthiaea villosa* (Rosaceae) were collected in Jeju Island (Korea) in May and were identified by curators of Jeju Technopark in Jeju Island. The dried leaf and branch of *P. villosa* were powdered and extracted with 70% ethanol at room temperature for 24 h, and the extracts were filtered and evaporated under reduced pressure. The extraction was followed by successive solvent fractionation using *n*-hexane, ethyl acetate, and *n*-butanol. The resulting samples were evaporated under reduced pressure and freeze-dried to obtain powder.

Phosphodiesterase enzyme assay. All recombinant PDEs were purchased from BPS Bioscience (San Diego, CA, USA), and PDE enzyme activity was measured by using LANCE Ultra cAMP kit (Perkin Elmer, Waltham, MA, USA). In brief, PDE was diluted in reaction buffer [Hank's balanced salt solution (HBSS) 1×, 5 mM HEPES, pH 7.4, 3 mM MgCl₂, 0.1% bovine serum albumin] and incubated with either dimethyl sulfoxide (DMSO) or compounds for 10 min prior to adding 12 nM of cAMP. After a 30-min incubation, the Eu-cAMP tracer and ULight-anti-cAMP were added to the reactions for additional 1 h, and then TR-FRET signal was detected with the EnVision Multilabel reader (Perkin Elmer).

Reporter gene assay. The NIH3T3 cell line stably expressing luciferase reporter genes was a kind gift from Dr. Kyung-Sook Chung at Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The cells were plated out on a 384-well plate at a density of 5000 cells/well in DMEM (Dulbecco's Modified Eagle Medium) containing 5% fetal bovine serum. After a 24-h incubation, the cells were treated with either DMSO or compounds for 6 h, and then luciferase activity was measured by using Dual-Glo® Luciferase assay system (Promega, Madison, WI, USA). For the reporter gene assay in RAW 264.7 cells, the cells (5×10^6 /6-cm dish) were transiently transfected with a 3 µg of DNA construct encoding a firefly luciferase gene under the control of cAMP response elements (CREs). Sixteen hours after transfection, the cells were trypsinized and replated at a density of 20,000 cells/well to a 384-well plate in DMEM supplemented with 10% fetal bovine serum. After a 6-h incubation, the cells were treated with compounds for another 6 h, and then luciferase activity was measured.

UHPLC-HR MS/MS analysis of BuOH fraction. *Pourthiaea villosa* BuOH fraction was analyzed by using a high-resolution LTQ-Orbitrap XL mass spectrometer connected to an Accela UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The column ACQUITY BEH C₁₈ (150×2.1 mm, 1.7 µm; Waters Corporation, Milford, MA, USA) was used for the separation of

various components based on polarity. The flow rate and injection volume were at 0.4 mL/min and 2 µL, respectively. The solvent gradient conditions of binary mobile phases consisted of solvent A (dH₂O with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) were as follows: 5–30% B at 0–20 min (linear gradient), 30–100% B at 20–24 min (linear gradient), and 100% B at 24–27 min (isocratic gradient). The samples and standard compounds were detected with photodiode array at 200–500 nm. The MS conditions in negative electrospray ionization mode were optimized as follows: the spray voltage at 4.0 kV, the capillary voltage at 35 V and the temperature at 300 °C. The Orbitrap analyzer was used for full-scan mass spectra data acquisition with a mass resolving power of 30,000 full width at half maximum at *m/z* 400. Full-scan mass spectra were acquired in an *m/z* range of 100–1000 at negative-ion mode. The MS/MS spectra were acquired in data-dependent mode by collision-induced dissociation. The XCALIBUR software (Thermo Scientific, Waltham, MA, USA) was used for data acquisition and data analysis.

Ovalbumin-induced asthma model and chemical treatment. Five-week-old male BALB/c mice were purchased from Daehan Biolink Co. (Chungbuk, Korea) and maintained in specific pathogen-free conditions. All animal experiments were performed in accordance with the guidelines from Korea Food and Drug Administration and NIH guide for the care and use of laboratory animals. Following 1 week of adaptation period, animals were randomly divided into three groups (*n* = 8 in each group): sham-operated; sensitized and challenged with ovalbumin (OVA-induced asthma model); and asthma model treated with baicalin (40 mg/kg). Mice were sensitized on days 0 and 14 by intraperitoneal injection of 200 µL of phosphate buffered saline (pH 7.4) containing 20 µg of OVA and 1 mg of aluminum hydroxide. On days 28, 29, 30, and 31, mice were exposed to an aerosol of 3% (w/v) OVA in phosphate buffered saline for 30 min using an Ultrasonic Nebulizer (NE-U12, Omron, Japan). Baicalin was administered to mice orally on the same day as they were exposed to an aerosol of OVA. Twenty-four hours after the last challenge, all mice were sacrificed by cervical dislocation following collection of blood from retro-orbital plexus.

BALF analysis. The main bronchus was ligated, and trachea was cannulated with a 24-gauge needle and lavaged by instilling and aspirating 1 mL of HBSS. The collected bronchoalveolar (BAL) fluid samples were centrifuged at 400 *g* for 10 min at 4 °C, and the supernatants were frozen at –70 °C until use for cytokine analysis. The pellets were suspended in 0.4 mL of HBSS, and the cells were stained with Diff-Quik® reagents (Sysmex Corporation, Japan). The differential counts of eosinophils, lymphocytes, monocytes, and neutrophils were analyzed at 400× magnification.

Statistical analysis. Data were statistically analyzed with analysis of variance techniques by using the software STATVIEW 4.51 (Abacus, CA) and were expressed as either mean ± standard deviation or mean ± standard

error. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Screening of a plant extract library for PDE4 inhibitors

In an effort to discover a new source of lead molecules for PDE4 inhibitors, we first screened a collection of 100 ethanol (EtOH) extracts derived from terrestrial plants in Jeju Island (Korea) for the ability to inhibit enzyme activity of PDE4A1A. The crude extracts were tested at a concentration of 100 µg/mL, and those extracts that inhibited PDE4A1A enzyme activity greater than 80% as compared with DMSO control were subjected to dose–response analysis to confirm the desired activity profile. We have identified six extracts with IC₅₀s (half maximal inhibitory concentration) in the 4- to 30-µg/mL range. Then, we directed our initial efforts toward an extract derived from *P. villosa* to identify major active components because the plant extract has not been previously reported to regulate PDE4 enzyme activity *in vitro*. In addition, the crude extract has been reported to show antiinflammatory and anti-oxidant activities in *in vitro* assays (Ryu *et al.*, 2003; Kim *et al.*, 2007). These observations may suggest that *P. villosa* is a good source for drug discovery endeavors.

As the extract was derived from both leaves and branches of *P. villosa*, we separated two parts, prepared EtOH extracts, and then tested the ability of each extract to inhibit enzyme activity of PDE4A1A. The results showed that the extracts of leaves and branches in isolation retained inhibitory activity against PDE4A1A although each extract displayed slightly reduced activity as compared with the one derived from both leaves and branches (Table 1). Subsequently, the EtOH extracts of leaves or branches were subject to successive solvent fractionation by using *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol (BuOH), and then each fraction was tested for the ability to inhibit PDE4A1A enzyme activity. As shown in Table 1, IC₅₀s of the EtOAc and BuOH fractions derived from EtOH extract of leaves were found to be similar to that of whole extract (leaf + branch), while *n*-hexane and H₂O fractions showed much less inhibitory activity against PDE4A1A with IC₅₀s above 100 µg/mL.

Table 1. Inhibitory activity of samples against phosphodiesterase 4A1A

Sample	IC ₅₀ (µg/mL)
Whole extract (leaf + branch)	28.1
Leaf extract	43.2
Branch extract	21
Leaf_Hexane	>200
Leaf_Ethyl acetate	29.5
Leaf_Butanol	24.9
Leaf_H2O	114.9
Branch_Hexane	>200
Branch_Ethyl acetate	57.7
Branch_Butanol	56.3
Branch_H2O	>200

To test whether the fractions that inhibited PDE4A1A in an *in vitro* enzyme assay are capable of increasing the level of cAMP in tissue culture cells, we used a cell line that expresses both firefly luciferase reporter gene under the control of CREs and renilla luciferase gene whose expression is controlled by cytomegalovirus promoter. Under the system described earlier, increased level of cAMP, which is caused by inhibition of PDE, will lead to stimulation of CRE and transcription of firefly luciferase gene. The cells were treated with 10 µg/mL of either whole extract or each fraction derived from EtOH extract of leaves or branches, and then firefly luciferase activity was measured. The results showed that only BuOH fractions retained the ability to stimulate transcription of reporter gene (Fig. 1), indicating that BuOH fractions inhibited enzyme activity of PDE in tissue culture cells. It is not clear why EtOAc fractions, although they inhibited PDE4A1A in an *in vitro* enzyme assay to the similar extents as BuOH fractions, were found to be inactive in the reporter gene assay. Further analysis with various concentrations of BuOH fractions showed that the BuOH fractions at concentrations above 20 µg/mL acted to decrease the level of firefly and renilla reporter gene expression (data now shown), possibility because of cytotoxicity of the BuOH fractions as determined by WST assay (data not shown).

Identification of PDE4 inhibitors in BuOH fraction derived from *Pourthiaea villosa* extract

In order to identify the active compound(s) in BuOH fractions that inhibits PDE4A1A activity, we first pursued to identify major components in BuOH fractions by metabolite profiling that involves UHPLC-MS/MS analysis (Fig. 2A). The analysis was followed by spectral database search to correlate both high-resolution mass spectrum and MS/MS fragment ions of each component in BuOH fractions with those of known compounds in natural-product databases available online. The database

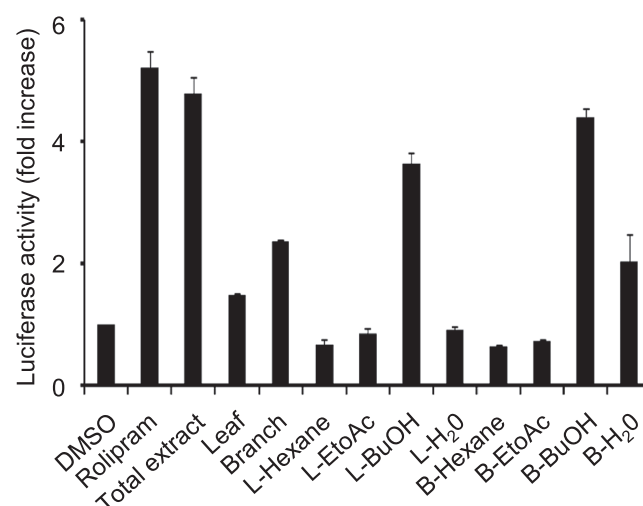


Figure 1. Effects of *Pourthiaea villosa* extracts on cyclic adenosine monophosphate response element-driven transcription of reporter gene. The cells were treated with each extract at a concentration of 10 µg/mL for 6 h, and then luciferase activity was measured. Rolipram (20 µM) was used as a reference compound. The results are shown as a fold increase over the luciferase activity in dimethyl sulfoxide (DMSO)-treated cells. L and B represent leaf and branch, respectively. Each column represents the mean ± SD, *n* = 3.

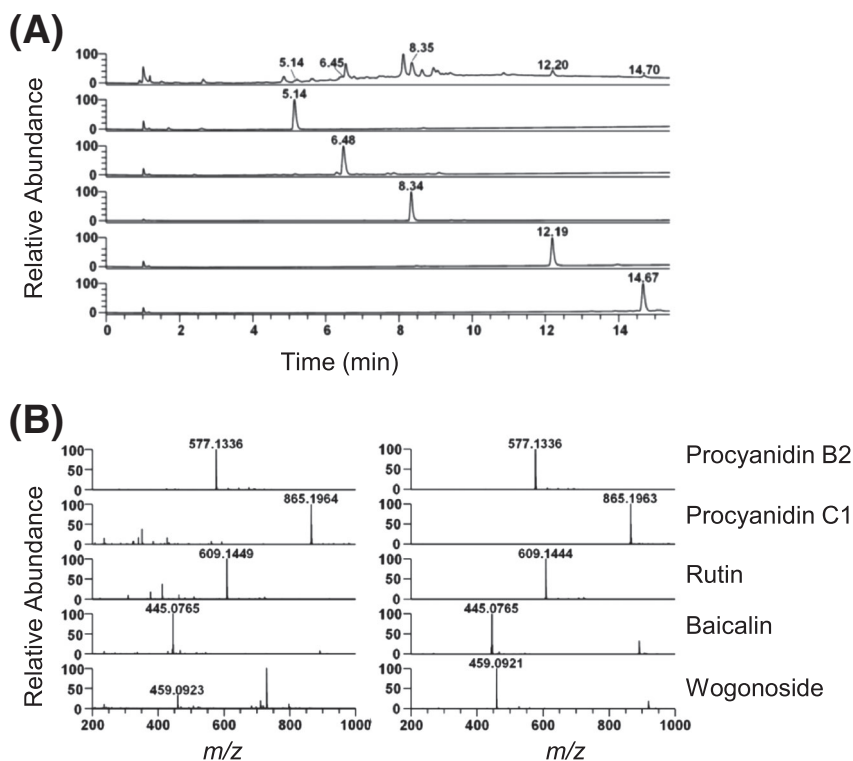


Figure 2. Ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) analyses of BuOH fraction derived from *Pourthiaea villosa* leaf and standard compounds. (A) The photodiode array chromatogram of BuOH fraction is shown. (B) The high-resolution mass spectra of BuOH fraction (left) are compared with those of five standard compounds (right).

search yielded five relevant hits for the corresponding components in the BuOH fraction derived from leaf extracts (Table 2). The profiling results were confirmed by comparing the high-resolution MS and MS/MS spectral characteristics of the phenolic compounds with those of commercial standard compounds (Fig. 2B). Compounds with the retention times 5.16, 6.45, 8.35, 12.20, and 14.70 min were verified as procyanidin B2, procyanidin C1, rutin, baicalin, and wogonoside, respectively (Table 2). Similarly, the BuOH fraction derived from branch extracts was found to contain the same compounds, except for rutin.

The identified compounds were tested for the ability to inhibit enzyme activity of PDE4A1A in an *in vitro* enzyme assay. To our surprise, although they are not as potent as rolipram, most of the flavonoid compounds, except for procyanidin B2 showed inhibitory activity against PDE4A1A to some extents with IC_{50} s in the 5- to 40- μ M range (Table 3). Foremost among the compounds that inhibited PDE4A1A enzyme activity was baicalin, and the compound was reported to have antiinflammatory and anti-allergic activities in a mouse model of allergic asthma (Ma *et al.*, 2014). Interestingly, the chemical structure of wogonoside is highly similar to

that of baicalin, while its inhibitory activity against PDE4 is much less than baicalin, indicating that the position of hydroxyl group and the number of substituents in the flavonoid backbone greatly affects compound's biological function.

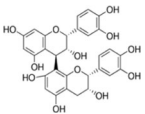
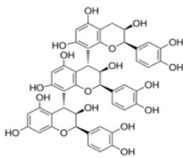
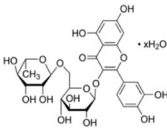
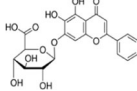
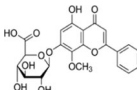
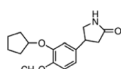
Next, we asked a question whether baicalin was able to inhibit PDE activity in tissue culture cells, especially in cells implicated in inflammatory reactions. To address this issue, RAW 264.7 macrophage cells were transfected with a construct encoding firefly luciferase gene under the control of CRE, treated with compounds, and then luciferase activity was measured. As shown in Fig. 3A, baicalin was capable of increasing the luciferase activity in a dose-dependent manner, suggesting that it raised intracellular cAMP level through inhibition of PDE activity in inflammatory cells. The increased luciferase activity was not just as a result of non-specific transcriptional activation of luciferase gene without regarding for CRE, because the luciferase transcription under the control of basal promoter (TATA box) was not affected by baicalin (Fig. 3B). The other identified compounds, procyanidin C1, rutin, and wogonoside, at concentrations up to 40 μ M had little, if any, effects on CRE activation (data not shown).

Table 2. Identification of five flavonoid compounds from *Pourthiaea villosa* leaf

RT (min)	m/z ($[M - H]^-$)	Calculated formula	Δ ppm	MS/MS (m/z)	Identification
5.14	577.1339	C ₃₀ H ₂₅ O ₁₂	-0.183	289, 407, 425, 451	Procyanidin B2
6.45	865.1964	C ₄₅ H ₃₇ O ₁₈	-1.249	287, 407, 577, 695, 739	Procyanidin C1
8.35	609.1449	C ₂₇ H ₂₉ O ₁₆	-0.182	301	Rutin
12.20	445.0765	C ₂₁ H ₁₇ O ₁₁	0.005	269, 175	Baicalin
14.70	459.0924	C ₂₂ H ₁₉ O ₁₁	0.440	283, 175	Wogonoside

RT, retention time; MS, mass spectrometry.

Table 3. Inhibitory activity of compounds against phosphodiesterase 4A1A

Compound	Structure	IC ₅₀ (μM)
Procyanidin B2		>100
Procyanidin C1		24.3
Rutin		21.2
Baicalin		5.0
Wogonoside		36.8
Rolipram		0.4

PDE selectivity of baicalin

Comprehensive analysis of inhibitor selectivity against PDE isoforms appears to be necessary, because unwanted side effects of PDE4 inhibitors correlate with non-selective inhibition of PDE isoforms. We profiled the activity of baicalin against PDE isoforms using an *in vitro* enzyme assay and used rolipram as a reference compound in order to take selectivity into account. The results showed that while rolipram inhibited the enzyme activity of a range of PDE4 subtypes, baicalin at a concentration of 20 μM only inhibited the enzyme activity of PDE4B in addition to PDE4A1A and had little effects on the other PDE isoforms (Fig. 4). The IC₅₀s of baicalin and rolipram against PDE4B was determined to be 4.8 and 0.16 μM, respectively. The enzyme activities of PDE4C and PDE4D were not inhibited by baicalin at concentrations up to 100 μM (data not shown).

Antiinflammatory effects of baicalin

It has been reported that PDE4 is the major cAMP-specific PDE isoforms in monocyte/macrophage and a targeted disruption of PDE4B gene but not the paralogs PDE4A or PDE4D resulted in an attenuation of lipopolysaccharide (LPS)-induced TNF-α expression (Jin *et al.*, 2005; Jin and Conti, 2002). To test whether baicalin affect TNF-α expression in macrophage, RAW 264.7 cells were

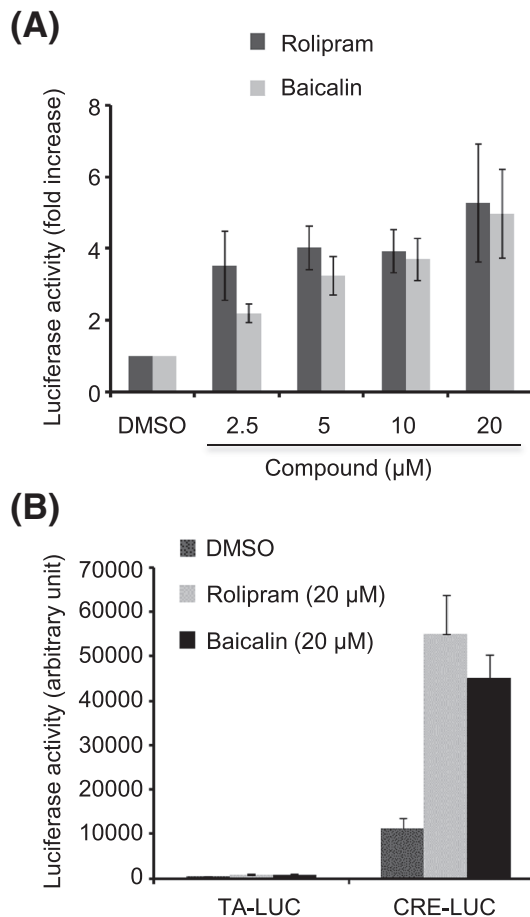


Figure 3. Inhibition of phosphodiesterase activity by baicalin in RAW 264.7 cells. (A) RAW 264.7 cells were transiently transfected with a DNA construct encoding firefly luciferase gene under the control of cyclic adenosine monophosphate response elements and treated with either dimethyl sulfoxide (DMSO) or compounds for 6 h. The results are shown as a fold increase over the luciferase activity in DMSO-treated cells. Each column represents the mean ± SD, *n* = 3. (B) RAW 264.7 cells were transiently transfected with a DNA construct encoding firefly luciferase gene under the control of either basal promoter (TA-LUC) or cyclic adenosine monophosphate response elements (CRE-LUC). The luciferase activity was measured after treating the cells with either DMSO or compounds for 6 h. Each column represents the mean ± SD, *n* = 3.

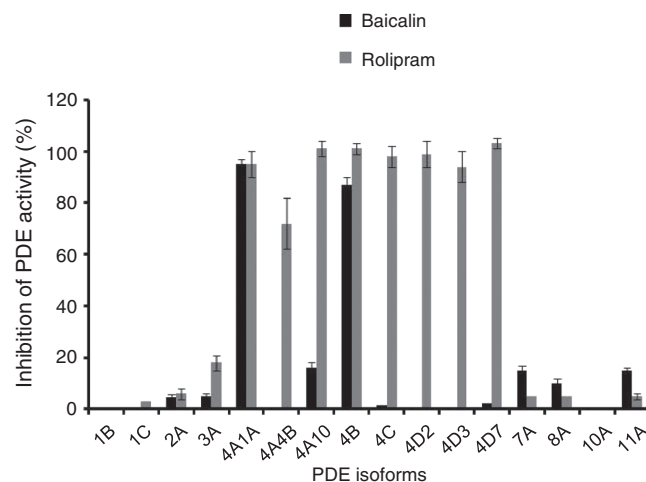


Figure 4. Evaluation of baicalin for phosphodiesterase (PDE) selectivity. Baicalin (20 μM) and rolipram (5 μM) were tested in duplicate for the ability to inhibit the enzyme activity of PDE isoforms, and the results are shown as percent inhibition of enzyme activity compared with dimethyl sulfoxide control (0% inhibition).

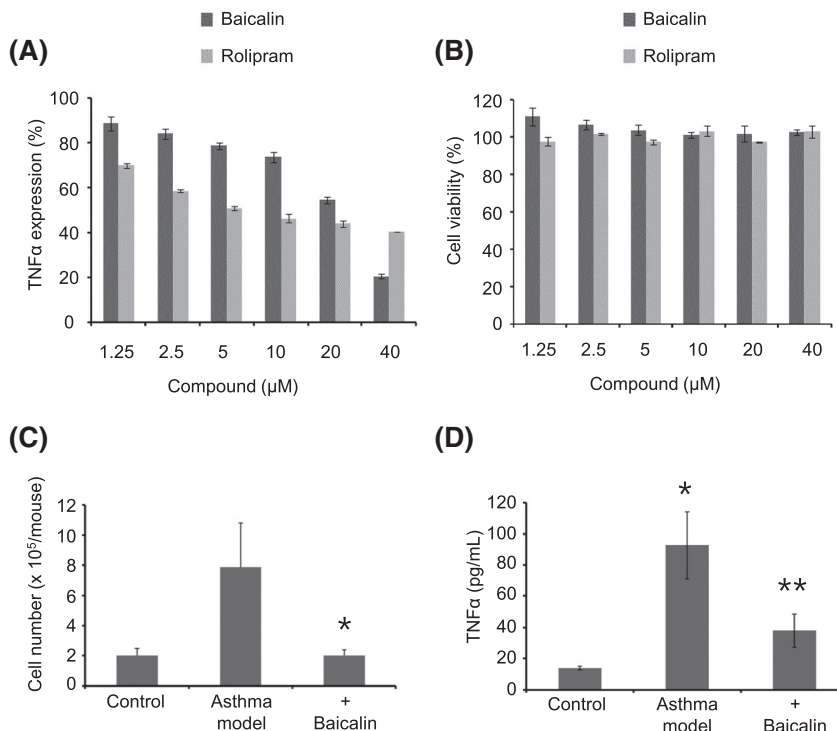


Figure 5. Evaluation of antiinflammatory effects of baicalin. (A) RAW 264.7 cells were pretreated with compounds for 1 h and then incubated with LPS for another 6 h. The conditioned media were analyzed for TNF- α by ELISA. Data are expressed as percentage of dimethyl sulfoxide controls (mean $\pm$ SD; $n = 3$). (B) The cell viability was evaluated by WST assay. Data are expressed as percentage of dimethyl sulfoxide controls (mean $\pm$ SD; $n = 3$). (C) Evaluation of baicalin in an ovalbumin-induced asthma model. Bronchoalveolar fluids were analyzed for monocyte count. Data are expressed as a mean $\pm$ SE ($n = 8$); * $p < 0.05$ vs asthma model. (D) Bronchoalveolar fluids were analyzed for TNF- α by ELISA. Data are expressed as a mean $\pm$ SE ($n = 8$); *, $p < 0.01$ vs control; **, $p < 0.01$ vs asthma model.

exposed to LPS for 6 h following pretreatment with baicalin for 1 h, and then the level of secreted TNF- α was measured by ELISA. As shown in Fig. 5A, baicalin in a dose-dependent manner inhibited LPS-induced expression of TNF- α , while it had little effects on the viability of RAW 264.7 cells (Fig. 5B). These results strongly support our finding that baicalin is a new type of PDE4 inhibitor, and may extend the previous observation that selective inhibition of PDE4 activity in macrophages leads to a decrease in the expression level of inflammatory cytokine TNF- α .

Although the effects of baicalin on asthmatic parameters in a mouse model of allergic asthma have already been reported by other group (Ma *et al.*, 2014), we tested baicalin in an OVA-induced asthma model in an effort to observe any additional effects of baicalin. Our independent investigation led to confirmation of most of the published results; baicalin suppressed OVA-induced increases in inflammatory cell count in BAL fluid (data not shown). However, while the previous works by Ma *et al.* failed to observe OVA-induced increase in monocyte count in BAL fluids, our results showed that the number of monocytes in BAL fluid was significantly increased upon OVA challenge as compared with that derived from control animals and that baicalin treatment resulted in a suppression of monocyte influx into BAL fluid (Fig. 5C). In addition, the OVA-induced increase in TNF- α level in BAL fluid was attenuated by baicalin (Fig. 5D).

DISCUSSION

Upon screening of a plant extract library for PDE4 inhibitors, we identified *P. villosa* as a source of lead

compounds that can be exploited for drug discovery. Baicalin turned out to be an active component in the extracts of *P. villosa* and showed inhibitory activity against PDE4A1A and PDE4B isoforms in *in vitro* enzyme assays. In support of these findings, we demonstrated that baicalin treatment resulted in an inhibition of TNF- α expression by LPS in macrophage (RAW 264.7) where PDE4B plays a key role in LPS-induced TNF- α expression. Furthermore, the increase in the influx of inflammatory cells into the airways in a mouse model of allergic asthma was significantly attenuated by baicalin treatment, suggesting that the antiinflammatory effects of baicalin *in vivo* are attributable, in part, to its ability to inhibit PDE4B enzyme activity. This speculation is also supported by the previous reports that targeted disruption of PDE4B gene but not that of PDE4A and PDE4D resulted in a significant decrease in inflammatory cell recruitment to the lung tissue in OVA-challenged animals (Jin *et al.*, 2010; Hansen *et al.*, 2000).

Ma *et al.* previously reported the effects of baicalin on asthmatic parameters in an OVA-induced asthma model. Our data from the independent investigation of baicalin in a mouse model of allergic asthma are in good concert with the published results except for the change in monocyte cell number in BAL fluids. While in the previous work by Ma *et al.*, OVA challenge in animals had little effect on the change in monocyte cell number in BAL fluids, our investigation observed an increase in monocyte cell number in BAL fluids collected from OVA-challenged animals as compared with that derived from control animals. In addition, baicalin treatment resulted in a suppression of monocyte influx into lung airways. A key difference between the two investigations

lies in the amount of OVA that was used to challenge the sensitized animals: We exposed the animals with an aerosol of 3% (w/v) of OVA for 4 days, while Ma *et al.* used 1% (w/v) of OVA for 3 days. Considering that monocyte count tends to increase transiently in lung airways at early stage of OVA challenge (Gonzalo *et al.*, 1998), longer exposure of animals with higher concentration of OVA could end up with monocytes being present in lung airways for an extended period of time. This speculation might explain why we were able to observe an increase in monocyte cell number in BAL fluids obtained from challenged animals.

Baicalin has been reported to have various biological effects including antiinflammatory properties (Chen *et al.*, 2014; Zhu *et al.*, 2015; Sun *et al.*, 2013). However, its molecular targets and mechanism of action have remained to be elusive. In this study, we demonstrate that one of the molecular targets of baicalin is PDE4, and our data from the reporter gene assay involving a luciferase gene under the control of CREs support that antiinflammatory effects of baicalin are mediated through inhibition of PDE activity and thereby raising intracellular cAMP level in cells. It would be of interest to establish whether the ability of baicalin to inhibit PDE4 enzyme activity is linked to its biological effects other than antiinflammatory properties.

The biological effects of baicalin have been investigated in comparison with structurally those of similar polyphenol compounds such as wogonoside, rutin, and their aglycones (baicalein, wogonin, and quercetin) (Chen *et al.*, 2014; Lee *et al.*, 2015). Those compounds differ by the position of hydroxyl group and other substituents including sugar moieties. In most cases, the sugar moiety does not seem to affect the activity of baicalin *in vitro*. However, biotransformation of baicalin

to its aglycone baicalein appears to be necessary to cross both the gastrointestinal tract and the blood–brain barrier *in vivo* (Tarrago *et al.*, 2008). These observations opened the door for the use of medicinal chemistry to optimize the structure of baicalin by substitution of the sugar at 7-OH position with proper chemical moieties (Luo *et al.*, 2014).

Four PDE4 subfamilies (A, B, C, and D) share a high degree of structural similarity in the active site. Therefore, it has been a challenge to design a PDE4 subtype-selective inhibitor. In this respect, it is of note that baicalin inhibited the enzyme activity of only PDE4A1A and PDE4B and displayed substantially reduced activity against PDE4C and PDE4D. These observations suggest that baicalin may exploit either regions immediately adjacent to the highly conserved active site or a site distant from the active site, also referred to as the allosteric site (Goodey and Benkovic, 2008). A comprehensive analysis of protein structure of PDE4B in complex with baicalin is likely to provide valuable information for structure-based design of PDE4 subtype-selective inhibitors.

Acknowledgements

This work was supported by the Ministry of Trade, Industry and Energy (MOTIE), Korea Institute for Advancement of Technology (KIA), through the inter-ER Cooperation Projects (R0002016).

Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

- Balhara J, Gounni AS. 2012. The alveolar macrophages in asthma: a double-edged sword. *Mucosal Immunol* **5**: 605–609.
- Banner KH, Press NJ. 2009. Dual PDE3/4 inhibitors as therapeutic agents for chronic obstructive pulmonary disease. *Br J Pharmacol* **157**: 892–906.
- Chen H, Gao Y, Wu J, *et al.* 2014. Exploring therapeutic potentials of baicalin and its aglycone baicalein for hematological malignancies. *Cancer Lett* **354**: 5–11.
- Cohn L, Elias JA, Chupp GL. 2004. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* **22**: 789–815.
- Cragg GM, Newman DJ. 2013. Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta* **1830**: 3670–3695.
- Fireman P. 2003. Understanding asthma pathophysiology. *Allergy Asthma Proc* **24**: 79–83.
- Gonzalo JA, Lloyd CM, Wen D, *et al.* 1998. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* **188**: 157–167.
- Goodey NM, Benkovic SJ. 2008. Allosteric regulation and catalysis emerge via a common route. *Nat Chem Biol* **4**: 474–482.
- Hansen G, Jin S, Umetsu DT, Conti M. 2000. Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci U S A* **97**: 6751–6756.
- Houslay MD. 2010. Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* **35**: 91–100.
- Jin SL, Conti M. 2002. Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF- α responses. *Proc Natl Acad Sci U S A* **99**: 7628–7633.
- Jin SL, Lan L, Zoudilova M, Conti M. 2005. Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J Immunol* **175**: 1523–1531.
- Jin SL, Goya S, Nakae S, *et al.* 2010. Phosphodiesterase 4B is essential for T(H)2-cell function and development of airway hyperresponsiveness in allergic asthma. *J Allergy Clin Immunol* **126**: 1252–1259, e1212.
- Kim SS, Hyun CG, Lee J, Lim J, Kim JY, Park DH. 2007. In vitro screening of Jeju medicinal plants for cosmeceutical materials. *J Appl Biol Chem* **50**: 215–220.
- Koehn FE, Carter GT. 2005. The evolving role of natural products in drug discovery. *Nat Rev Drug Discov* **4**: 206–220.
- Kumar N, Goldminz AM, Kim N, Gottlieb AB. 2013. Phosphodiesterase 4-targeted treatments for autoimmune diseases. *BMC Med* **11**: 96.
- Lee W, Ku SK, Bae JS. 2015. Anti-inflammatory effects of Baicalin, Baicalein, and Wogonin in vitro and in vivo. *Inflammation* **38**: 110–125.
- Li S, Aliyeva M, Daphtary N, Martin RA, Poynter ME, *et al.* 2014. Antigen-induced mast cell expansion and bronchoconstriction in a mouse model of asthma. *Am J Physiol Lung Cell Mol Physiol* **306**: L196–L206.
- Luo R, Wang J, Zhao L, *et al.* 2014. Synthesis and biological evaluation of baicalein derivatives as potent antitumor agents. *Bioorg Med Chem Lett* **24**: 1334–1338.
- Ma C, Ma Z, Fu Q, Ma S. 2014. Anti-asthmatic effects of baicalin in a mouse model of allergic asthma. *Phytother Res* **28**: 231–237.
- Nielsen KF, Mansson M, Rank C, Frisvad JC, Larsen TO. 2011. Dereplication of microbial natural products by LC-DAD-TOFMS. *J Nat Prod* **74**: 2338–2348.
- Page CP, Spina D. 2012. Selective PDE inhibitors as novel treatments for respiratory diseases. *Curr Opin Pharmacol* **12**: 275–286.
- Ryu JH, Ahn H, Kim JY, Kim YK. 2003. Inhibitory activity of plant extracts on nitric oxide synthesis in LPS-activated macrophages. *Phytother Res* **17**: 485–489.

- Sun J, Li L, Wu J, *et al.* 2013. Effects of baicalin on airway remodeling in asthmatic mice. *Planta Med* **79**: 199–206.
- Tarrago T, Kichik N, Claasen B, *et al.* 2008. Baicalin, a prodrug able to reach the CNS, is a prolyl oligopeptidase inhibitor. *Bioorg Med Chem* **16**: 7516–7524.
- Vijverberg SJ, Hilvering B, Raaijmakers JA, Lammers JW, Maitland-van der Zee AH, Koenderman L. 2013. Clinical utility of asthma biomarkers: from bench to bedside. *Biologics* **7**: 199–210.
- Zhu HY, Han L, Shi XL, *et al.* 2015. Baicalin inhibits autophagy induced by influenza A virus H3N2. *Antiviral Res* **113**: 62–70.