

Research Article

Baicalin and Baicalein Inhibit Src Tyrosine Kinase and Production of IL-6

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Flavonoids play an important role in the treatment of various diseases, as they are able to inhibit reactive oxygen species, which cause damage to cells and tissues which may lead to increased risk of inflammatory diseases. Baicalin and baicalein, two flavonoids found in the roots of *Scutellaria baicalensis*, in the leaves of *Thymus vulgaris* and *Oroxylum indicum*, were tested for their anti-inflammatory activity as well as for their cytotoxicity. Thereby the two compounds were investigated on Src tyrosine kinase inhibition and inhibition of production of interleukin (IL-6) in lipopolysaccharide- (LPS-) stimulated THP-1 cells. Additionally, the THP-1 cell line was used for the determination of the cytotoxicity. Both baicalin and baicalein showed some anti-inflammatory properties, while baicalein turned out to be the more active compound with higher inhibitory activities on both Src tyrosine kinase and production of cytokine IL-6. Baicalin and baicalein showed no signs of cytotoxicity in the MTS cytotoxicity assay in THP-1 cells.

1. Introduction

Inflammatory mediators (IM) are produced as a response for macrophages exposed to pathogens or other harmful stimuli or trauma. This exposure leads to activation of nitric oxide (NO), reactive oxygen species (ROS), or cytokines including various interleukins [1]. The activation results in inflammation processes in cells or tissues. Consequences are tissue damage, leading to diseases like atherosclerosis and pulmonary fibrosis [2], or autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [3]. IL-6 is a proinflammatory agent produced by various cells, for example, macrophages or neutrophils, and has been considered as a byproduct of ongoing inflammatory processes [4–6]. Together with TNF α and IL-1, IL-6 is also considered a major proinflammatory cytokine important in the protection from pathogens during

an infection. IL-6 is an important modulator of CD4 T cell effector functions thereby shaping the immune response and contributing to inflammation [3]. Recent studies report the inhibitory activity of natural compounds on IL-6 production *in vitro* [7, 8].

Src tyrosine kinase (Src) is a ubiquitously expressed nonreceptor tyrosine kinase belonging to the Src family of kinases [9]. Src family kinases are known for their roles in the progression of cancer; however they are also involved in inflammation-related signaling pathways, proliferation, and chemotaxis [2, 10]. After stimulation of different types of the receptors, Src plays a critical role in recruiting a number of cell signaling molecules, which results in the induction of various cytokines, including IL-6 [11].

Flavonoids as natural polyphenols are a class of secondary plant metabolites with a wide distribution in fruits and

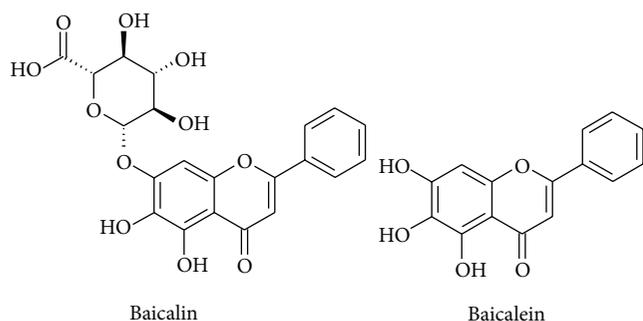


FIGURE 1: Chemical structures of baicalin and baicalein, natural flavonoid compounds, both occur naturally in the roots of *Scutellaria baicalensis* Georgi, as well as in the leaves of *Thymus vulgaris* L. and *Oroxylum indicum* (L.) Benth. ex Kurz.

vegetables [12]. Their antioxidant potential is related to their ability to neutralize free radicals in cells [13]. Additionally, flavonoids inhibit NF κ B and VEGF expression and cause cell toxicity in cancer cells [14, 15]. Baicalein and baicalin are both natural flavonoid compounds. Baicalein belongs to flavones, a subclass of flavonoids, and baicalin, also known as baicalein-7-glucuronide, is its conjugate (Figure 1). Both occur naturally in the roots of *Scutellaria baicalensis* Georgi, as well as in the leaves of *Thymus vulgaris* L. and *Oroxylum indicum* (L.) Benth. ex Kurz [16]. Previous investigations showed antioxidant and anti-inflammatory effects of baicalein and baicalin applying the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, the lipid peroxidation, and tyrosinase inhibition assays [17]. In the present study both substances were tested for their inhibitory activity on Src tyrosine kinase and IL-6 production, as well as for their cytotoxicity in THP-1 cells.

2. Materials and Methods

2.1. Reagents. Adenosine 5'-triphosphate disodium salt dehydrate (ATP; A-2383), ethylenediaminetetraacetic acid (EDTA; E-4884), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes; H-3375), magnesium chloride (MgCl₂) (M-8266), ethylene glycol tetraacetic acid (EGTA; E-4884), dimethyl sulfoxide (DMSO) (Hybri-Max™, sterile-filtered, $\geq 99.7\%$; D-2650), Tween 20 (27,434-8), staurosporine (S-4400), LPS from *Escherichia coli* serotype 0111:B4, and bovine serum albumin (BSA; A2153) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), fetal bovine serum (FBS; S04382S1810) was obtained from BioWest, and Immulon 2HB 96-well plates were obtained from Dynex (UK, 3455). LANCE Ultra ULight-TK peptide (TRF0127-M), LANCE® Eu-W1024 anti-phosphotyrosine (PT66; AD0068), LANCE detection buffer 10x (CR97-100), and 384-deep-well Storplate-V (6008590) were purchased from Perkin Elmer. Mouse IL-6 Biotinylated Affinity Purified PAb Goat IgG (antibody with biotin; BAF406) and IL-6 monoclonal antibody (FAB227C) were obtained from R&D (USA), streptavidin from horse radish peroxidase (OR 03L) was obtained from Calbiochem, USA, and Roswell Park Memorial Institute 1640 medium (RPMI; A10491) was

obtained from Gibco. Dithiothreitol (DTT; 161-0611) was purchased from Bio-Rad, and 384-well plates (flat bottom, small volume, white; 784075) were purchased from Greiner BioOne. THP-1 tumor cell lines were obtained from ECACC (ECACC-88081201), Src (08-173) was obtained from Carna Biosciences, and MTS (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Part#G358B, Lot#18824201) was obtained from Promega. The pure compounds baicalin and baicalein were purchased from Sigma-Aldrich.

2.2. THP-1 Cell Cultivation. THP-1 tumor cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere.

2.3. LPS-Induced IL-6 Production in THP-1 Cells. Modulation of IL-6 production in LPS-stimulated THP-1 cells by tested compounds (in a final concentration range of 1000–12 μ g/mL) was exhibited using an enzyme-linked immunosorbent assay (ELISA). An inflammatory response was previously induced by using mitogen LPS (in final conc. of 1 μ g/mL). THP-1 cells (3×10^5 cells/well) were preincubated with the samples for two hours at 37°C, in an atmosphere of 5% CO₂ and 90% humidity, and then treated with LPS overnight at 37°C in 5% CO₂. Compounds are prepared from 10 mM DMSO stock solutions; they are diluted 1:2 in cellular media with 1 mg/mL starting final concentrations. The next day, the supernatant was removed and transferred to the Immulon 2HB 96-well plates. The IL-6 concentration was measured by using sandwich ELISA. IL-6 monoclonal antibody was bound on the well bottom (hIL-6, 2 μ g/mL); samples and standards were transferred and bound to the antibody in the presence of the cytokine. After washing and blocking with 1% BSA, the antibody with biotin, bound to the previously bounded cytokine, was added. After incubation and washing, streptavidin was added. The plates were read using an EnVision 2104 plate reader (Perkin Elmer) at 450 nm. The developed color was proportional to the concentration of cytokines and was quantified using standards. Inhibition (as percentage) was calculated using the following formula: % inhibition = $[1 - (\text{concentration of IL-6 in sample} - \text{concentration of IL-6 in negative control}) / (\text{concentration of IL-6 in positive control} - \text{concentration of IL-6 in negative control})] \times 100$. The positive control refers to LPS-stimulated samples that were not preincubated with the compounds. The negative control refers to unstimulated and untreated samples. Reference control compound for inhibition of IL-6 production was Azithromycin, which showed acceptable standard values for this assay.

2.4. Src Tyrosine Kinase Assay. A time-resolved fluorescence resonance energy transfer (TR-FRET) tyrosine kinase assay was used for testing the inhibitory activity of the samples [18]. The Src kinase assay was performed in low-volume 384-well test plates. A mother plate (Storplate-384-deep-well-V plate, Perkin Elmer) with 1:3 serial dilutions (1 mg/mL as starting concentrations) of the samples in pure DMSO was prepared from 100 mg/mL compound stock solutions on the Janus automatic pipetting workstation (JANUS Integrator

Platform, 8 tips, AJI8001, Perkin Elmer). 100 nL of the samples was transferred from the mother plate to the test plate by using a nanovolume liquid handling instrument Mosquito (3019-0002, TTP Labtech). The first step initiated the kinase reaction by mixing 5 μ L of kinase with 5 μ L of combined peptide substrate (2.5 μ L) and ATP (2.5 μ L) and incubating for 3 hours at 20°C \pm 2°C. Src (0.5 nM), peptide substrate (50 nM), and ATP (200 μ M) were diluted in kinase buffer (50 mM HEPES, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, and 0.01% Tween 20). Final concentrations of compounds in the test plate were in a range from 1 mg/mL to 0.02 μ g/mL. In the second step, 5 μ L of EDTA (10 mM) and 5 μ L of Europium-anti-phospho antibody (1 nM) (both prepared in LANCE detection buffer) were added and incubated for 1 hour at 20°C \pm 2°C and the plates were read at Ex340/Em615-665 with an EnVision (Xcite Multilabel Reader, 2104-0020, Perkin Elmer) plate reader. The concentrations given in brackets refer to final concentrations used in the assay. Total percentage of DMSO in the kinase reaction was 1%. Staurosporine was used as a reference control compound.

2.5. Cytotoxicity Assay. Cytotoxicity of the samples was estimated on human leukemia cells (THP-1) by a modified version of Mosmann [19]. A mother plate (Storplate-384-deep-well-V plate, Perkin Elmer) with 1:3 serial dilutions in triplicate (1 mg/mL as starting concentrations) of the samples in pure DMSO was prepared from compound stock solutions on the Janus automatic pipetting workstation (JANUS Integrator Platform, 8 tips, AJI8001, Perkin Elmer). 500 nL of the samples was transferred from the mother plate to the test plate by using a nanovolume liquid handling instrument Mosquito (3019-0002, TTP Labtech). Cells were distributed in 384-well plates, in concentrations of 2×10^4 cells per well, in 50 μ L of cell culture medium. Cells were incubated in presence of the tested compound for 24 h at 37°C. Cell growth was estimated by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] MTS assay. After adding 5 μ L of MTS, the plates were incubated for 3 hours. Absorbance at 492 nm was measured with a plate reader. The total percentage of DMSO in the cytotoxicity assay was 1%. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH, produced by dehydrogenase enzymes in metabolically active cells. This enzyme turns the MTS into a different color by cutting the tetrazolium ring. Reference control compound for MTS assay was Azithromycin, which showed acceptable standard values for this assay.

2.6. Data Handling. Calculation of IC₅₀ data, curves, and QC analysis was made by using Excel tools and GraphPad Prism software, v. 5.03. Briefly, individual concentration-effect curves were generated by plotting the logarithm of the tested concentration of tested compounds (*X*) versus corresponding percent inhibition values (*Y*) using least squares (ordinary) fit. Best fit IC₅₀ values were calculated using log(inhibitor) versus normalized response-variable

slope equation, where $Y = 100 / (1 + 10^{((\log IC_{50} - X) * HillSlope)})$. QC criteria parameters (Z' , S : B , R^2 , HillSlope) were checked for every IC₅₀ curve.

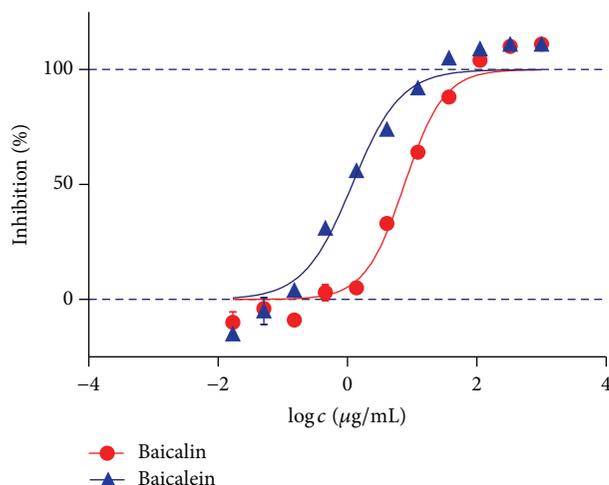
3. Results and Discussion

The process of inflammation is involved in an increasing number of diseases. For this reason it is necessary to develop effective and safe treatments. Steroids are widely prescribed “gold standard” drugs which reduce inflammation by binding to the glucocorticoid receptor. However, due to their known side effects they are often avoided, if possible. Nonsteroidal anti-inflammatory drugs (NSAIDs), which act mainly through the cyclooxygenase (COX) enzyme inhibition, are the most common “nonsteroidal” way to treat various inflammatory diseases. However, their mode of action does not include direct inhibition of cytokine production, and besides their therapeutic effects, they also cause various side effects, also due to inhibition of COX enzymes [20]. Facing these problems, it is necessary to find substances with maximum inhibitory activity of various inflammatory mediators and minimum toxicity and side effects for the patients.

Flavonoids are potent antioxidant compounds with the ability of opposing free radicals damage to DNA, lipids, proteins, and other biomolecules. It is believed that these properties are attributed to the anticarcinogenic, antiproliferative, and antiangiogenic activities of flavonoids [21–23]. Summarizing the activities of flavonoids, they may be considered as an alternative and natural approach for the treatment of inflammatory diseases. In the present study, baicalin and baicalein were tested for their anti-inflammatory activity in the IL-6 cytokine and Src tyrosine kinase inhibition assays, as Src is involved in the inflammation process as well as in cancer development [2, 11]. Both baicalin and baicalein showed significant Src inhibitory activities. Aglycone baicalein inhibited Src tyrosine kinase with IC₅₀ values of 1.2 μ g/mL (or 4 μ M, if results are presented in molar concentrations), while the glycoside baicalin inhibited Src with a slightly higher IC₅₀ value of 7.6 μ g/mL (or 17 μ M) (Figure 2). The obtained results should be taken into consideration for the possible evaluation of baicalin and baicalein for anticancer treatment as well.

Concerning the inhibition of cytokine production, especially the inflammation marker IL-6, baicalin and baicalein were investigated by measuring the IL-6 production in LPS-stimulated THP-1 cells. The results are displayed in Figure 3. Full inhibitory activity of the level of IL-6 could be observed for baicalein for the three highest tested concentrations (1000, 333, and 111 μ g/mL), which would give IC₅₀ value of 88 μ g/mL (or 326 μ M), whereas baicalin inhibited IL-6 production more significantly only at a highest tested concentration (1000 μ g/mL), which would give IC₅₀ value of 578 μ g/mL, or 1295 μ M. Therefore, this means that baicalin is about 4 times less potent than baicalein also in this assay.

Additionally, cytotoxicity of the two flavonoids was evaluated by using THP-1 cells. The results showed that both baicalin and baicalein had no cytotoxic effect on the THP-1 cells after 24 h at concentrations up to 1000 μ g/mL, in



	Baicalin	Baicalein
log(inhibitor) versus normalized response-variable slope		
HillSlope	1.42	1.16
IC ₅₀ /µg/mL	7.6	1.2
R ²	0.977	0.963

FIGURE 2: Dose-response curves and IC₅₀ values of Src kinase inhibition for baicalin and baicalein. Error bars are representing standard deviations of triplicate average values. If results are presented in molar concentrations (for better comparison of compounds with different MWs), IC₅₀ values would be 17 µM for baicalin and 4 µM for baicalein.

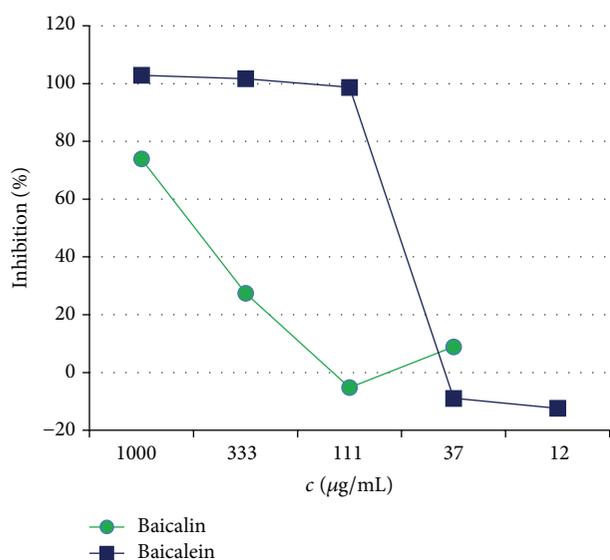


FIGURE 3: Inhibition values of IL-6 production in LPS-stimulated THP-1 cells for the flavonoid compounds baicalin and baicalein. Full inhibitory activity of the level of IL-6 could be observed for baicalein for the three highest tested concentrations (1000, 333, and 111 µg/mL, which would give IC₅₀ value of 88 µg/mL, or 326 µM), whereas baicalin inhibited IL-6 production only at a highest tested concentration (1000 µg/mL, which would give IC₅₀ value of 578 µg/mL, or 1295 µM).

comparison to the reference compound staurosporine, used as a cytotoxic positive control of the assay. IC₅₀ values for inhibition of THP-1 cell lines were >1000 µg/mL for both baicalin (>2.2 mM in molar concentrations) and baicalein (>3.7 mM in molar concentrations). Quite the contrary, a significant level of stimulation of mitochondrial activity of the cells could be observed in this study after 24 h incubation of cells, but only after incubation with baicalein. This effect

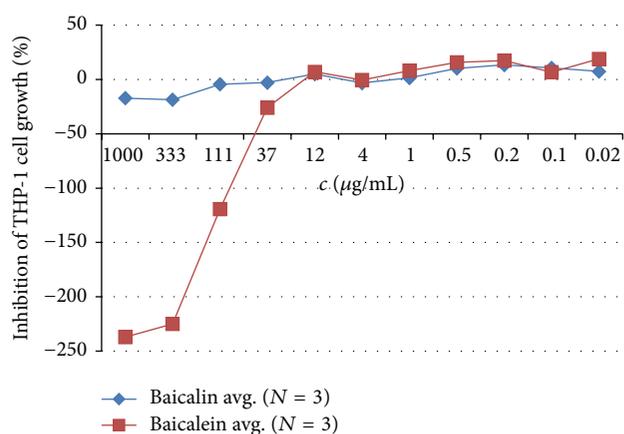


FIGURE 4: Influence of baicalin and baicalein on the viability of THP-1 cell lines. Untreated cells and media only as controls represented 0 or 100% of inhibition. Significant level of stimulation of mitochondrial activity of the cells could be observed in this study after 24 h incubation of cells, but only after incubation with baicalein. EC₅₀ value for stimulation of mitochondrial activity for baicalein can be calculated from the dose-response curve, and result is 111 µg/mL, or 411 µM.

is visible in concentrations within 1000 µg/mL–37 µg/mL (3700 µM–137 µM), in a dose-response manner. Since MTS assay was used to evaluate mitochondrial activity of cells, by measuring succinate hydrogenase activity [19], negative values of the tested inhibition of the cell growth represent, in fact, stimulation of cellular mitochondrial activity (Figure 4). Interestingly, this stimulatory effect is visible only with baicalein, which is a compound with more pronounced anti-inflammatory activity. EC₅₀ value for stimulation of mitochondrial activity for baicalein can be calculated from the dose-response curve, and result is 111 µg/mL, or 411 µM (Figure 4).

Summarizing the results, it can be concluded that both the aglycone baicalein and the glycoside baicalin showed inhibitory activities in the two different anti-inflammatory assays, on inhibition of Src tyrosine kinase and production of IL-6 cytokine in LPS-stimulated THP-1 cells, with baicalein being much more potent in both assays. These differences may be related to the structure of the flavonoids, since baicalin has the sugar moiety attached to the flavonoid ring, which could be of significant steric hindrance in ligand-receptor interactions, in comparison to the baicalein flavonoid ring. These results can be useful for recognizing important orientation of possible docking/binding of the flavonoids to their receptors. The cytotoxicity profile of both compounds indicates no cytotoxic properties. The results obtained from the present study confirm the anti-inflammatory activities of baicalein and baicalin and therefore will be beneficial to further investigation of these flavonoids as an effective new possible treatment option for inflammatory or cancer diseases.

Competing Interests

The authors have declared no conflict of interests.

Acknowledgments

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