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Baicalin, a prodrug able to reach the CNS, is a prolyl oligopeptidase inhibitor☆

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Abstract—Prolyl oligopeptidase is a cytosolic serine peptidase that hydrolyzes proline-containing peptides at the carboxy terminus of proline residues. It has been associated with schizophrenia, bipolar affective disorder, and related neuropsychiatric disorders and therefore may have important clinical implications. In a previous work, we used ^{19}F NMR to search for new prolyl oligopeptidase inhibitors from a library of traditional Chinese medicine plant extracts, and identified several extracts as powerful inhibitors of this peptidase. Here, the flavonoid baicalin was isolated as the active component of an extract of *Scutellaria baicalensis* roots having prolyl oligopeptidase inhibitory activity. Baicalin inhibited prolyl oligopeptidase in a dose-dependent manner. Inhibition experiments using baicalin analogs showed that the sugar moiety was not necessary for activity. The IC_{50}s of baicalin and its aglycone derivative baicalein were rather similar, showing that the sugar moiety was not involved in the interaction of baicalin with POP. These results were confirmed by saturation transfer difference NMR experiments. To further understand the absorption and transport mechanisms of baicalin and baicalein, we evaluated their transport in vitro through the gastrointestinal tract and the blood–brain barrier using a Parallel Artificial Membrane Permeability Assay. The molecule which potentially crosses both barriers was identified as baicalein, the aglycone moiety of baicalin. Our results show that baicalin is a new prodrug able to inhibit prolyl oligopeptidase. As baicalin is a natural compound with a long history of safe administration to humans, it is a highly attractive base from which to develop new treatments for schizophrenia, bipolar affective disorder, and related neuropsychiatric diseases.

Abbreviations: POP, prolyl oligopeptidase; SZ, schizophrenia; BD, bipolar affective disorder; IP_3, inositol-1,4,5-P_3; TCM, traditional Chinese medicine; STD, saturation transfer difference; DPPIV, dipeptidyl peptidase IV; CNS, central nervous system; FP-Rh, Fluorophosponate-rhodamine; GIT, gastrointestinal tract; BBB, blood–brain barrier; PAMPA, Parallel Artificial Membrane Permeability Assay; Z, benzyloxycarbonyl; AMC, 7-amino-4-methylcoumarin; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; DMSO, dimethylsulfoxide; PVDF, polyvinylidenefluoride; BLM, biomimetic lipid membrane; PBLEP, polar brain lipid extract porcine.

Keywords: Bipolar affective disorder; Prolyl oligopeptidase; Schizophrenia; Traditional Chinese medicine; POP inhibitors, Fluorine NMR; STD NMR; Baicalin; Baicalein; Cognitive disorders.

☆ *Chinese medicinal plants, a new source of prolyl oligopeptidase inhibitors:* The natural flavonoid baicalin was isolated as the active component of an extract of *Scutellaria baicalensis* roots having prolyl oligopeptidase inhibitory activity. Baicalin inhibited prolyl oligopeptidase in a dose-dependent manner, and its aglycone derivative, baicalein, was able to reach the central nervous system. These findings demonstrate the potential of baicalin to be a new prodrug able to inhibit prolyl oligopeptidase.

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1. Introduction

The protease prolyl oligopeptidase (POP; EC 3.4.21.26) is a cytosolic serine protease that hydrolyzes small proline-containing peptides at the carboxy terminus of proline-residues.[1,2] Many bioactive peptides such as substance P, thyroliberin, β-endorphin, and arginine–vasopressin are POP substrates.[3] In our laboratory, POP was recently cloned from human brain RNA, expressed in *Escherichia coli*, and an homology model based on the X-ray structure of porcine POP was obtained.[4]

In recent years, POP has gained importance as a target for the treatment of schizophrenia (SZ), bipolar affective disorder (BD) and cognitive disturbances, such as those present in Alzheimer's disease, mainly due to its involvement in the metabolism of inositol-1,4,5-P_3 (IP_3). IP_3 is a key molecule in the transduction cascade of neuropeptide signaling. Neuropeptides modulate levels of IP_3, which binds to its receptor in the membrane of the

endoplasmic reticulum to induce the release of Ca^{2+}, which is believed to play a crucial role in learning and memory.[5] Recent findings have demonstrated that POP inhibition increases the concentration of IP_3.[6,7] The IP_3 signaling pathway participates in the therapeutic action of several mood-stabilizing drugs (lithium, carbamazepine and valproic acid).[8] Moreover, defects in the mechanisms that regulate IP_3 signaling may underlie BD, suggesting that other small-molecule inhibitors of POP may be useful in the treatment of the disease.[9]

In clinical studies, patients with BD or SZ exhibit abnormally high levels of serum POP activity.[10] SZ affects 1% of the world's population and has an enormous economic impact, as reflected by the fact that up to 90% of patients are unemployed. This impact is largely attributable to the lack of adequate therapeutic agents to treat the key cognitive symptoms; hence, a new class of drugs is required that addresses the cognitive deficits brought on by this disease.[11]

POP inhibitors may prove valuable to treat various clinical conditions of the brain, as indicated by the neuroprotective and cognition-enhancing effects of POP inhibitors in experimental animals.[12–14] The POP inhibitor S-17092-1 has been tested in Phase I trials for its capacity to enhance cognition.[15] However, in spite of promising results, a drug based on POP inhibition has yet to reach the market.

An array of strategies is currently being used to identify POP inhibitors, including the exploration of natural products as a primary source of new inhibitory agents.[16–18] Traditional Chinese medicine (TCM) dates back several thousands of years. China has 12,806 medicinal sources registered, including 11,145 plants.[19] Moreover, 2375 compounds are compiled in the Pharmacopoeia of the People's Republic of China (2000 edition).[20] Compounds with anti-cancer, anti-bacterial, anti-fungal and anti-viral activities have been identified from these medicinal products.[21]

We recently reported a new POP enzymatic assay based on the combined use of ^{19}F NMR and the fluorinated substrate ZGPF-4-CF_3. The assay is fast, reproducible and circumvents the false positives and false negatives inherent to previously reported fluorimetric and colorimetric assays. It may therefore be useful for screening complex natural compound mixtures for new POP inhibitors.[22] We later identified several extracts with powerful POP inhibitory activity, and isolated berberine as the POP inhibitory molecule present in *Rhizoma coptidis* extract.[23] In the present work, we fractionated the extract of *Scutellaria baicalensis* roots and identified baicalin as the POP inhibitory molecule. Inhibition studies using baicalin analogs and saturation transfer difference NMR experiments (STD) showed that the sugar moiety of baicalin was not necessary for POP inhibition. An in vitro study of the absorption pathways through the gastrointestinal tract and the blood–brain barrier (BBB) showed that the molecule which actually reaches the CNS is baicalein. Our results demonstrate that baicalin is a new prodrug able to inhibit POP.

2. Results

We prepared a library of aqueous extracts from 29 plants used in TCM and screened them for their capacity to inhibit POP. Specifically, we focused on plants used to treat neuropsychiatric disorders.[24] To find new POP inhibitors, we concentrated our efforts on one of the extracts with the greatest POP inhibitory capacity, TCM 19, from *S. baicalensis* roots. The extract was fractionated by semi-preparative HPLC, and then all the fractions were collected. After screening each fraction, we selected one active fraction with a purity higher than 99%, as calculated from its HPLC profile (Fig. 1). The retention time of the pure compound was 8.11 min in a gradient of 0–100% MeCN over 15 min. The exact mass of the purified molecule was 447.0922 Da $[M+H]^+$, which corresponded to the formula $C_{21}H_{18}O_{11}$. Further characterization of the purified compound by ^1H and ^{13}C NMR allowed us to identify it as the flavonoid baicalin (Scheme 1).

We constructed a calibration curve with known amounts of baicalin, and determined that the extract of *S. baicalensis* roots contained 28.3% of baicalin. On the basis of this calculation, we estimated that the amount of baicalin in the inhibition assay performed with the plant extract was 63.4 μM. The standard daily dose of *S. baicalensis* roots in TCM is 9 g.[24] From this amount, and on the basis of our yields, 2.8 g of dry weight can be extracted when performing aqueous extracts. Given that the amount of baicalin in the *S. baicalensis* roots extract is 28.3%, we concluded that the dose of baicalin taken in 1 day is 78.4 mg.

Baicalin inhibited POP in a dose-dependent manner (Fig. 2A). The concentration required for half-maximal inhibition (IC_{50}) was 12 ± 3 μM. To study the type of inhibition exerted by baicalin, kinetic experiments were performed. After representing the data following Lineweaver–Burk plot (Fig. 2B), the slope and intercept of the lines showed a non-competitive type of inhibition. These results were confirmed by a different technique where a well-known covalent probe, Fluorophosponate-rhodamine (FP-Rh), which binds to the active site of serine hydrolases,[25] was used to perform a competition experiment with baicalin. This methodology was previously followed to evaluate competition of FP-Rh with several unknown putative inhibitors a brain α/β-hydrolase-6.[26] Thus, POP was incubated for 15 min with increasing amounts of baicalin and afterward FP-Rh was added and the incubation was maintained for an additional 15 min. After resolving the reaction on an SDS–PAGE, the FP-Rh-labeled POP was visualized using a fluorescence scanner. Baicalin did not compete for FP-Rh binding in spite of using an excess of baicalin of 125 times over the probe (Fig. 2C). A control performed with a reference covalent POP inhibitor, *Z*-prolyl-prolinal,[27] clearly showed competition with FP-Rh. On the basis of the kinetic and the competition results, the mechanism of baicalin inhibition can be considered non-competitive. Further experiments are currently underway to elucidate the baicalin-binding site.

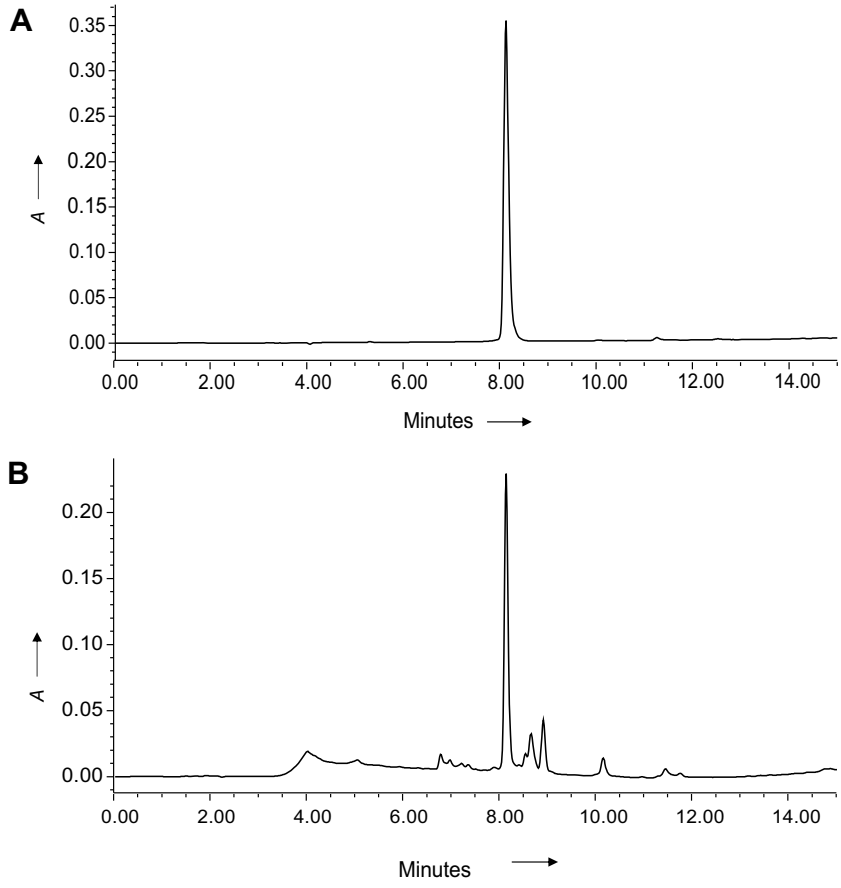
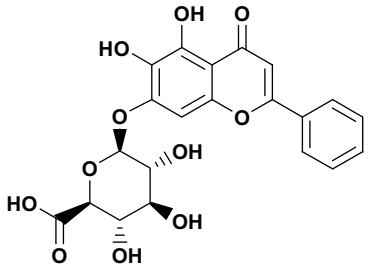


Figure 1. (A) HPLC of purified baicalin obtained from an aqueous extract of *Scutellaria baicalensis* roots. (B) HPLC of the aqueous extract of *S. baicalensis* roots before purification.



Scheme 1. Structure of baicalin.

The specificity of baicalin against POP was studied using the protease dipeptidyl peptidase IV (DPPIV). The IC_{50} of baicalin for DPPIV inhibition was 225 ± 14 μM. To study the binding characteristics of baicalin, a small set of flavonoids was tested to determine their specific inhibition of POP and of DPPIV (Table 1). Quercetin (**5**) and baicalein (**2**) were found to be the best inhibitors. The IC_{50}s of baicalein and its glucoronic derivative baicalin were rather similar, suggesting that the sugar moiety is not involved in the interaction of baicalin with POP. Since the active compounds, especially when losing the sugar moiety, could have low solubility, the possibility of promiscuous inhibition was checked following the protocols described by Fenf and Shoichet.[28] Here an inhibition test of baicalin and baicalein in the presence of the non-ionic detergent Triton X-100 (0.01% final concentration) was performed. Taking into account that based on the behavior of several known aggregators and non-aggregators at 30 μM statistically significant inhibition was previously defined as greater than 23.8%,[28] no significant differences on the inhibition were found in the presence of Triton (Table 2A). Moreover, both baicalin and baicalein retained their inhibitory activities after spinning in a microcentrifuge for 20 min at 14,000*g* (Table 2B). Thus, large particle formation was unlikely. Furthermore, a pre-incubation of 5 min was formerly described to decrease (improve) the IC_{50} of aggregating compounds from 2-fold to more than 50-fold.[29] Nevertheless, inhibition of baicalin and baicalein slightly increased after a 5-min incubation, although a time dependence with longer pre-incubation times was observed (Table 2C). The inhibitory activities of baicalin and baicalein increased with greater pre-incubation time, reaching a maximum at 15 min. However, this time-dependent inhibition does not necessarily imply an aggregation mechanism since these phenomena can be related to the mechanism of inhibition, as described for slow binding inhibitors.[30] In fact, several POP inhibitors are slow binders and it is common to perform a pre-incubation step of 15 min before evaluating their activities.[31,32] Furthermore, the Saturation Transfer Difference (STD) spectrum of baicalin in the absence of POP did not show any effect (see next paragraph), there-

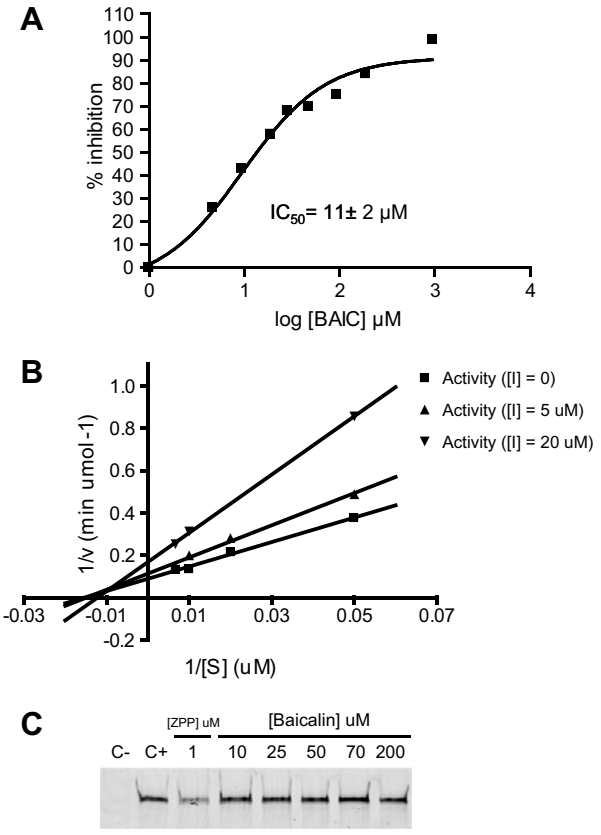


Figure 2. (A) Baicalin IC_{50} calculation curve using ZGP-AMC as substrate; results are the means ± standard deviations of three independent experiments. (B) Lineweaver–Burk plot of the kinetic data. (C) Competition experiment with the probe FP-Rhodamine. The image shows a fluoresce scan of an SDS–PAGE. Increasing amounts of baicalin were pre-incubated with POP before adding the probe. (C−) Negative control of POP without probe. (C+) Positive control of POP incubated with the probe alone. ZPP, *Z*-prolyl-prolinal.

by confirming the absence of large aggregates of baicalin. All these results indicate that the formation of aggregates that would hypothetically capture the enzyme is not likely to be the mechanism of action of either baicalin or baicalein.

To analyze the binding mode of baicalin when interacting with POP STD NMR spectroscopy was used.[33] An STD spectrum of a sample containing baicalin in a 50-fold excess over POP was recorded. Strong STD effects (up to 18%) were observed, clearly indicating a protein–ligand interaction (Fig. 3). The corresponding STD spectrum of the ligand in absence of the protein did not show any effect. Since the T_1-relaxation times of the individual protons are very different, direct analysis of the STD value often leads to incorrect binding epitopes. This problem can be solved by recording saturation build-up curves, in which the initial slopes correspond directly to the proximity of the proton to the protein.[34] Here, baicalin was added in a 9-fold excess over protein and the saturation time was varied from 500 ms to 2 s. The saturation build-up curves of the protons of baicalin showed different initial slopes (supporting information). The analysis of the binding epitope indicated that the protons of the γ-chromenone, and those of the phenyl ring, participated in the binding epitope, whereas the contribution of the saccharide moiety to the binding was smaller (Fig. 4). The results were confirmed by a titration experiment, in which the ligand excess was increased from 5:1 to 24:1. The aromatic protons showed strong chemical shift perturbations, while no significant effects were observed for the saccharide protons (Fig. 5).

To further understand the absorption and transport mechanisms of baicalin and baicalein, we evaluated their transport in vitro through the gastrointestinal tract (GIT) and the blood–brain barrier (BBB) using a Parallel Artificial Membrane Permeability Assay (PAMPA).[35,36] Delivery to the brain is complex as compounds must cross the BBB. This barrier is a natural defence mechanism designed to keep harmful substances out of this organ. The PAMPA assay uses an artificial membrane in the form of filter-supported phospholipid bilayers. In the case of mimicking the BBB, a porcine polar lipid extract is used to coat the filter. The phospholipid membrane mimics the cell membrane but has no means for active or paracellular transport of drug molecules and is therefore a convenient tool to evaluate the transport of compounds by passive diffusion.

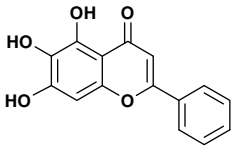
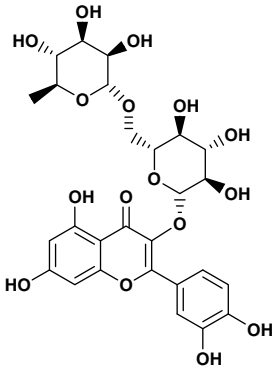
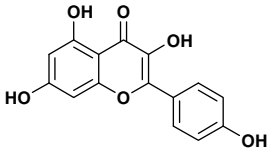
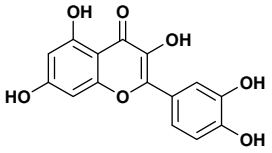
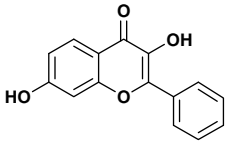
The molecule which potentially crosses both barriers was identified as baicalein, the aglycone moiety of baicalin (Table 3); hence, the sugar does not cause baicalin to undergo passive diffusion through the BBB.

3. Discussion

There is ever increasing interest in the exploitation of natural products for medical applications. However, little is known about the mechanisms of action of their active ingredients.[37] After fractionation of the *S. baicalensis* roots extract, we identified baicalin as one of the constituent POP inhibitors. To our knowledge, this is the first report of POP inhibition by the flavonoid baicalin, which has a diverse pharmacological profile, including inhibition of HIV-1 replication in vitro,[38] inhibition of the formation of α-synuclein fibrils,[39] anxyolitic-like effects,[40] and anti-cancer activity.[41]

Recent studies have demonstrated that baicalin has a protective effect against brain edema and cerebral ischemic damage.[42–44] *S. baicalensis* roots is one of the main components of *Huang Lian Jie Du Tang* (*Oren-gedoku-to*), a traditional herbal prescription that protects against neuronal death induced by cerebral ischemia[45] and impairment of learning and memory induced by cerebral ischemia in mice.[46] In a previous work, we reported berberine as the main POP inhibitory molecule present in an extract of *Rhizoma coptidis*, which is also an ingredient of the *Huang Lian Jie Du Tang* formulation. In this work, we also identified another component with powerful POP inhibitory action, baicalin. We showed that baicalin directly inhibits POP in a dose-dependent manner; thus, the effects observed when baicalin is administered could be the result of POP inhibi-

Table 1. IC_{50} of baicalin analogs against POP and DPPIV

Compound	Compound structure	IC_{50} against POP (μM)	IC_{50} against DPP IV (μM)
2		36 ± 6	>200
3		>200	145 ± 15
4		>200	132 ± 9
5		13 ± 7	130 ± 23
6		>200	>200

tion. A combination of several POP inhibitors in *Huang Lian Jie Du Tang* could explain its therapeutic efficacy, although the presence of other molecules that interact with distinct targets can not be ruled out.

Regarding the potency of baicalin when compared with typical reference compounds such as S-17092-1,[15] it is well known than S-17092-1 is a covalent inhibitor that reacts with the serine in the enzyme active site, whereas baicalin is probably a non-covalent inhibitor. It is logical that the potencies of a covalent and a non-covalent inhibitor differ by several orders of magnitude. However, the POP IC_{50} of berberine (145 ± 19 μM)[23] is much higher than that of baicalin (12 ± 3 μM). Furthermore, the natural product baicalin has other advantages, such as water solubility and the capacity to reach the CNS.

An important issue when developing a CNS drug is its ability to cross the BBB. In the case of many flavonoids it is understood that aglycones are absorbed directly through the gut wall, whereas flavonoid glycosides are usually absorbed only after being hydrolyzed to the corresponding aglycones by enterobacterial enzymes. It has been reported that baicalin is transformed to baicalein by gut β-glucoronidase prior to absorption from the rat gastrointestinal tract.[47] Also, in a pharmacokinetic study in rats, it was shown that baicalin cannot cross the BBB, whereas baicalein was detected inside the brain after baicalin administration.[48] These results are in agreement with the PAMPA assays performed in the present work, which suggest that the molecule which reaches the CNS and which is responsible for the CNS-related effects of baicalin is indeed the aglycone derivative, baicalein. After ingestion, the sugar moiety of baicalin is cleaved by enzymes present in the intestinal tract to yield baicalein. As baicalein is more lipophilic, it can cross the GIT, enter the bloodstream, cross the BBB, and ultimately reach the CNS—the principal location of POP. Moreover, the sugar moiety of baicalin confers solubility to the molecule and allows aqueous extraction during infusion. All these properties indicate that baicalin could be used as a pro-drug and therefore, due to its POP inhibition effect, may be of therapeutic use for SZ, BD and cognitive disorders.

4. Conclusions

We have demonstrated that the alkaloid baicalin and its aglycone, baicalein, inhibit POP activity in a dose-dependent manner. Baicalin can be considered a pro-drug, as its sugar moiety is cleaved in the GIT to yield baicalein, the compound which crosses the GIT and the BBB. Since baicalin is a natural product with a proven record of safe human administration, it is a highly attractive starting point from which to develop treatments for SZ and BD. Indeed, baicalin may be of particular interest for therapies designed to improve the cognitive deficits of patients with these diseases.

5. Experimental

Solvents for RP-HPLC were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid was supplied from KaliChemie (BadWimpfen, Germany). ZGP-AMC and ZG-AMC were obtained from Bachem (Bubendorf, Switzerland). Other chemicals, including porcine DPPIV, baicalin and baicalein analogs, were purchased from Sigma–Aldrich (Deisenhofen, Germany).

HPLC was performed using a Waters Alliance 2695 (Waters, Massachusetts, USA) chromatography system with a PDA 995 detector, a reverse-phase Symmetry C_{18} (4.6 × 150 mm) 5-μm column, and mobile phases

Table 2.

	Baicalin % I (50 μM)	Baicalein % I (50 μM)
(A) Effect of Triton X-100 on baicalin and baicalein inhibition		
Control	63 ± 9	60 ± 6
0.01% Triton X-100	60 ± 6	51 ± 4
(B) Effect of previous centrifugation of the stock inhibitor solutions on POP inhibition		
Control	57 ± 4	63 ± 4
Centrifuged	61 ± 3	64 ± 2
Pre-incubation time (min)		
(C) Effect of pre-incubation time on POP inhibition		
0	52 ± 5	53 ± 7
5	67 ± 7	60 ± 6
10	81 ± 5	81 ± 6
15	83 ± 2	77 ± 15
30	87 ± 3	84 ± 7

Values are the mean of three independent experiments ± standard deviation. %, percentage of inhibition at a given inhibitor concentration.

of H_2O with TFA (0.045%), and MeCN with TFA (0.036%). Semi-preparative HPLC was performed using a Waters Controller 600 chromatography system with a Fraction Collector II, Simple Manager 2700 auto-injector, and a 2478 UV/VIS detector. The column used was a Symmetry C_{18} (30 × 100 mm) at a flow rate of 10 mL min^{-1}. The mobile phases used were H_2O with TFA (0.1%), and MeCN with TFA (0.1%). Exact mass spectra were recorded on an ESI-TOF spectrometer (Bruker Microtof, Bremen, Germany). NMR spectra were recorded at 298 K on a Bruker Avance DRX 600 MHz spectrometer equipped with a 5-mm cryogenic inverse triple-resonance probe head using DMSO-d_6 as solvent. Fluorescence was measured using a Bio-Tek FL600 fluorescence plate reader (Bio-Tek Instruments, Vermont, USA).

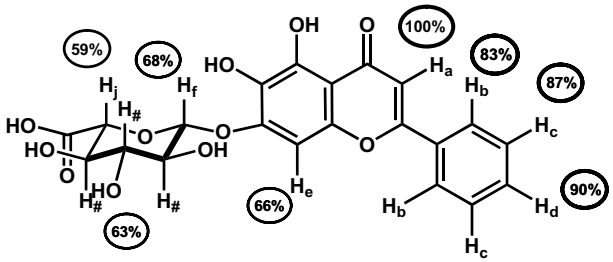


Figure 4. Binding epitope of baicalin. $H_\#$ The average of the STD effects on these protons was determined since the corresponding signals were not sufficiently resolved.

5.1. Methods

5.1.1. TCM extract preparation. Plants used in TCM were obtained from Herbasin (Shenyang, China). Dried plant material (30 g) was extracted with H_2O (400 mL) under reflux for 5 h using a Soxhlet apparatus. The volume of aqueous extracts was reduced in vacuo, and subsequently freeze-dried and stored at −20 °C. Before use, an aqueous stock solution (5 mg mL^{-1}) was prepared.

5.1.2. Purification and quantification of baicalin. Purification of the crude *S. baicalinalensis* roots extract was performed by semi-preparative HPLC. First, the extract was fractionated using a gradient of MeCN (20–100%) for 30 min, and then all the fractions were collected. After identifying the fractions that showed POP inhibitory activity (t_R = 18–19 min), those with a purity above 99% were collected and freeze-dried. Analytical data for baicalin are as follows: ^{1}H NMR (DMSO-d_6, 600.13 MHz): δ *3.37(t, 1H,* J = 9.0 Hz), 3.43 (m, 2H), 4.05 (d, 1H, J = 9.02 Hz), 5.23 (d, 1H, J = 7.89 Hz), 5.26 (s, 1H), 5.48 (s, 1H), 7.00 (s, 1H), 7.05 (s, 1H), 7.61 (m, 3H), 8.08 (d, 2H), 8.65 (s, 1H), 12.6 (s, 1H) ^{13}C NMR (DMSO-d_6 150.90 MHz) δ 71.8, 73.3, 75.7, 75.9, 94.2, 100.5, 105.2, 106.6, 126.8, 129.6, 131.1, 131.3, 132.4, 147.2, 149.6, 151.7, 164.0, 170.5, 182.9.

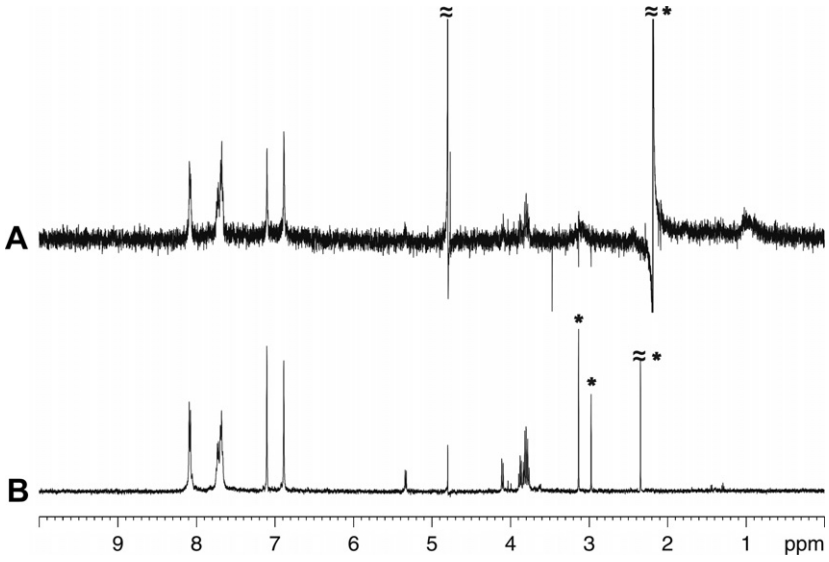


Figure 3. (A) Six hundred mega-Hertz ^{1}H STD spectrum of baicalin (500 μM) in the presence of POP (10 μM) recorded at 308 K. Suppression of the protein signals was achieved by applying a $T_1\rho$-filter. (B) ^{1}H-reference spectrum of baicalin. *Signals arising from sample impurities.

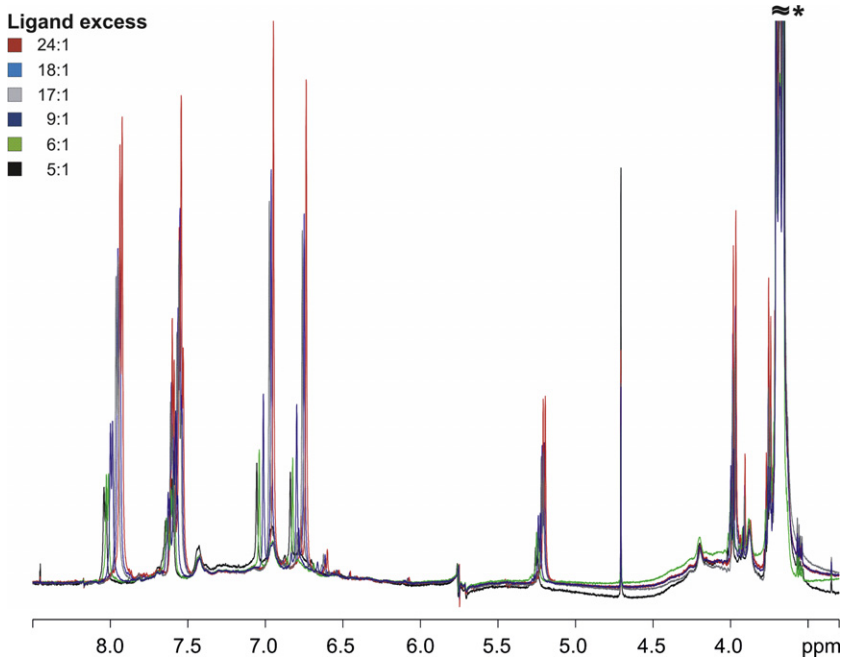


Figure 5. Titration of baicalin in the presence of 20 μM POP. *Signal arising from Tris buffer, that remained after protein purification covers some of the saccharide signals.

Table 3. Baicalin and baicalein permeability through GIT and BBB determined by PAMPA

	P_e (10E−6) (cm/s)	Log P_e
GIT PAMPA		
BAIC	1.639	−5.785
BAIN	0.382	−6.418
BBB PAMPA		
BAIC	0.0455	−7.343
BAIN	5.247	−5.292

The baicalin in the plant extract was quantified by C_{18} RP-HPLC using a calibration curve. Different solutions of baicalin were prepared (1–100 μM). These solutions were analyzed using a gradient of MeCN (0–100%) in H_2O over 15 min at 254 nm (baicalin t_R = 8.11 min).

5.1.3. Expression and purification of POP. POP was obtained by expression in *E. coli* and affinity purification using a His tail fusion according to a literature procedure.[22]

5.1.4. POP inhibition assays. POP activity was determined following the method described by Toide et al.[12] The reactions were performed in 96-well microtiter plates, which allowed simultaneous monitoring of multiple reactions. For each reaction, activity buffer (131 μl, 100 mM Na/K phosphate buffer, pH 8.0) was pre-incubated for 15 min at 37 °C with POP (7 nM) and the corresponding inhibitor solution (3 μl). A stock solution of inhibitor was prepared in DMSO-d_6 (100 mM), and dilutions were prepared from this stock solution with DMSO-d_6. A control with the same concentration was also prepared in DMSO-d_6. After pre-incubation (15 min at 37 °C), ZGP-AMC (10 μl, 3 mM in 40% 1,4-dioxane) was added, and the reaction was incubated for 1 h at 37 °C. The reaction was stopped with sodium acetate (150 μl, 1 M, pH 4) and the formation of AMC was measured fluorimetrically. The excitation and emission wavelengths were 360/40 and 485/20 nm, respectively. The IC_{50} value was defined as the concentration of compound required to inhibit 50% of POP activity.

Kinetic experiments were performed using the POP activity procedure described above with the following conditions. The substrate concentrations used were: 20, 50, 100 and 150 μM. The inhibitor concentrations used were: 5 and 20 μM. After a 5-min pre-incubation of POP with baicalin, the substrate was immediately added and fluorescence measures were performed every 5 min. The reaction was incubated at 37 °C. Data were analyzed using Graph-Pad Prism 4 software. Initial velocity was calculated and fluorescence activity units were converted to μmols of released AMC using an AMC calibration curve.

The effect of Triton X-100 on inhibition was evaluated using the protocol already described.[28] Briefly, Triton X-100 was added to POP activity buffer at a final concentration of 0.01% before introducing the enzyme. The inhibitory compounds were then added and after a 5-min pre-incubation the reaction was started by addition of the substrate. The rate of inhibition was calculated as described above.

The effect of centrifugation on inhibition was assessed by centrifuging the stock inhibitory solution (50 μM in DMSO) for 20 min at 14,000*g*. Afterwards, the inhibition reaction was performed with 5-min pre-incubation as described above.

Time-dependent inhibition was evaluated using the protocol described above but the pre-incubation time of the inhibitory samples as well as the control samples was varied (0, 5, 10, 15, and 30 min).

Competition with the probe FP-Rh was performed as described[26] with the following modifications. Fifty nanograms of POP in 10 μl of Tris–HCl 50 mM, pH 8, buffer was pre-incubated with 2 μl of baicalin dissolved in DMSO during 20 min at RT. Several baicalin stock solutions were prepared to achieve the following final concentrations (10, 25, 50, 70, and 200 μM). Then, 0.4 μl of FP-Rh at 50 μM was added and incubation was performed during 20 min at RT. The control with *Z*-prolyl-prolinal was performed under the same conditions at a final concentration of 1 μM. Afterwards, SDS–PAGE loading buffer was added and the whole samples were analyzed on a 10% SDS–PAGE.

5.1.5. DPPIV inhibition assay. DPPIV activity was determined following the method described by Checler et al.[49] The reactions were performed in a 96-well microtiter plate. For each well, the reaction mixture contained activity buffer (131 μl, 100 mM Na/K phosphate buffer, pH 8.0), 0.7 nM DPPIV, and 3 μl of the corresponding inhibitor solution (in DMSO-d_6). After pre-incubation (15 min at 37 °C), GP-AMC (10 μl, 3 mM in 40% 1,4-dioxane) was added, and the reaction was incubated for 1 h at 37 °C. Finally, the reaction was stopped with sodium acetate (150 μl, 1 M, pH 4) and the formation of AMC was measured fluorimetrically. The excitation and emission wavelengths were 360/40 and 485/20 nm, respectively. The IC_{50} value was defined as the concentration of compound required to inhibit 50% of DPPIV activity.

5.1.6. Acquisition of STD spectra. All NMR samples were prepared in phosphate buffer (20 mM, pH 7 in 100% D_2O). STD spectra were recorded at a temperature of 308 K with a spectral width of 10 ppm on a Bruker Avance DRX 600 MHz spectrometer equipped with a 5-mm cryogenic inverse triple-resonance probe head. Selective saturation of the protein was achieved by a train of Gauss-shaped pulses of 50 ms length each, truncated at 1%, and separated by a 1-ms delay. The on-resonance irradiation of the protein was performed at a chemical shift of 0 ppm. Off-resonance irradiation was set at 80 ppm, where no protein signals are present. The spectra were recorded using different memory buffers for on- and off-resonance. Total scan number in the STD experiments was 2k. Water suppression was achieved by excitation sculpting. For the spectra, which were recorded with a T1ρ-filter, spin lock pulses of 30 ms were applied. NMR spectra were multiplied by an exponential line-broadening function of 0.5 Hz prior to Fourier transformation.

The saturation build-up curves were recorded with a 9:1 baicalin-to-POP ratio (20 μM POP). Saturation times of 0.5, 1, 1.5, and 2 s were applied. The data were fitted to the monoexponential equation: $STD = STD_{max}(1 - \exp(-k_{sat}t))$. The initial slope is given by the product of k_{sat} and STD_{max}.[34]

5.1.7. Parallel Artificial Membrane Permeability Assay (PAMPA). PAMPA assays were carried out in a 96-well plate (pION, Inc.). Donor and acceptor wells were separated by a polyvinylidenefluoride (PVDF) membrane coated with different mixtures of phospholipids. For the GIT PAMPA, the PVDF membrane was coated with a mixture known as biomimetic lipid membrane (BLM) (pION, Inc.), which contained phosphatidylcholine in dodecane (20% w/v). For the BBB PAMPA, the membrane was coated with polar brain lipid extract porcine (PBLEP) (Avanti Polar Lipids, Inc.) which contained phosphatidylcholine (12.6%), phosphatidylethanolamine (33.1%), phosphatidylserine (18.5%), phosphatidylinositol (4.1%), phosphatidic acid (0.8%), and cerebrosides and pigments (30.9%). For the GIT and BBB PAMPA assays, the PVDF membrane was coated with either BLM or PBLEP, respectively, in an amount equivalent to 300 bilayers. GIT PAMPA assay was done at three different pHs of the donor well: pH 5.0, 6.2, and 7.4 that are the main pHs along the GIT. In BBB PAMPA assay all the donor wells were at pH 7.4. Donor wells were filled with *S. baicalensis* roots extract (195 μl) at a concentration of 200 μM of the major compound present in the extract (baicalin), and acceptors wells were filled with system solution (pION, Inc.), (195 μl, pH 7.4). All PAMPA measures were done by triplicate. The assays were performed in a Gut-Box device (pION, Inc.). For both assays, the PAMPA plate was incubated for 4 h in a humidity-saturated atmosphere. For the GIT assay, the stirring rate was equivalent to 100 μm thickness of unstirred water layer (UWL), whereas for the BBB assay, the rate was equivalent to 25 μm thickness of UWL (device minimum). After 4 h, the donor and acceptor wells were analyzed by HPLC in a gradient of 0–100% MeCN over 15 min, and the compounds were detected between 210 and 315 nm. Baicalin and baicalein were identified according to their retention times (baicalin $t_R = 8.11$ min, and baicalein $t_R = 10.16$ min) and UV spectra. After HPLC analysis, the effective permeability (P_e) values for baicalin and baicalein were calculated according to the following expression: $P_e = (-218.3/t)\,\mathrm{Log}[1 - (2 \times C_A(t)/C_D(0)] \times 10^{-6}\ \mathrm{cm\ s^{-1}}$. Whereby t is the experiment time (incubation); $C_A(t)$ is the concentration of the compound in the acceptor well at the end of the assay; and $C_D(0)$ is the concentration of the compound in the donor well at the beginning of the assay (time zero).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.04.067.

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