The capability of minor quaternary benzophenanthridine alkaloids to inhibit TNF-α secretion and cyclooxygenase activity

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Abstract

Quaternary benzophenanthridine alkaloids are known to have a wide range of biological effects, including antimicrobial, antifungal, anti-inflammatory, and antitumour activities. However, only sanguinarine and chelerythrine have been studied intensively. The aim of this study was to evaluate the anti-inflammatory potential of the five minor quaternary benzophenanthridine alkaloids sanguilutine, sanguirubine, chelirubine, chelilutine, and macarpine *in vitro* and to compare them with more thoroughly studied sanguinarine and chelerythrine. Before making cell-based assays, the cytotoxicity of the alkaloids was evaluated. The anti-inflammatory potential of the chosen alkaloids was evaluated as for their ability to modulate the lipopolysaccharide-induced secretion of tumour necrosis factor α (TNF-α) in the macrophage-like cell line THP-1. The cyclooxygenase (COX)-1 and COX-2 inhibitory activities were also measured. The results indicate that the presence of a methylenedioxy ring attached at carbon (C)7-C8 is important for reducing the secretion of TNF-α. Interestingly, this effect did not show a simple dependence on concentration. The selected alkaloids showed little or no anti-COX activity. The results obtained from the present experiments may provide additional information useful in understanding the structure-to-activity relationship of the quaternary benzophenanthridine alkaloids. The anti-inflammatory potential and the cytotoxic effect are driven by the presence of a methylenedioxy ring attached at C7-C8 and C2-C3, respectively.

Cyclooxygenase, cytotoxicity, inflammation, TNF-α

Plants and their products have been used since the beginning of human history as remedies for various diseases and disorders. During the 20th century, the natural origin was in most cases replaced by synthetic drugs. Nowadays, we can see a reversing trend; natural medicine reappears in many areas of medicine (Perczel et al. 2016) and some of the used nature-originated drugs belong to alkaloids (e.g., galanthamine, paclitaxel). However, the usage of pure alkaloids has a longer history, e.g. in the case of morphine, codeine, etc. (Atanasov et al. 2015).

Quaternary benzophenanthridine alkaloids (QBAs) are known to have a wide range of biological activities, including antimicrobial, antifungal, anti-inflammatory, and antitumour activities (Walterova et al. 1995). The richest sources of these alkaloids are considered to be *Sanguinaria canadensis* L., *Dicranostigma lactucoides* Hook F. et Thomas, *Chelidonium majus* L., *Macleaya cordata* (Willd.) R.Br, *Macleaya microcarpa* (Maxim.) Fedde, *Stylophorum lasiocarpum* (Oliv.) Fedde, and a few species of the genus *Bocconia.* All of these species belong to the family *Papaveraceae* (Dostal and Potacek 1990). Extracts obtained from these plants, especially *M. cordata* and *S. canadensis*, have been used in traditional medicine for their anti-inflammatory effects, particularly against plaque build-up and gingivitis (Šimánek et al. 2003). Varying amounts of the major alkaloids

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sanguinarine and chelerythrine and the minor ones chelirubine, sanguirubine, chelilutine, sanguilutine, and macarpine have been found in these plants (Suchomelova et al. 2007; Šimánek et al. 2003; Slaninova et al. 2014; Sebrlova et al. 2015).

The biological effects of sanguinarine and chelerythrine have been studied extensively as they are commercially available. The potential of the minor alkaloids has not been fully revealed because their limited availability is a major challenge still to be overcome. However, a few uncommon properties and effects have been found. For instance, whereas sanguilutine and chelilutine exhibit antiproliferative and anti-microtubular activities (Slaninova et al. 2001), sanguirubine, chelirubine, and macarpine are antiproliferative and pro-apoptotic, as has been well documented (Slaninova et al. 2007). Macarpine and sanguirubine show a considerable fluorescence shift when bound to deoxyribonucleic acid (DNA), a property which makes them useful as fluorescent probes (Urbanova et al. 2009). Macarpine seems to be a promising novel cell-permeant DNA dye for live-cell imaging and flow cytometry sorting (Slaninova et al. 2016). Sanguilutine has been found to preferentially induce necroptosis over apoptosis in melanoma cells (Hammerova et al. 2012).

Although all of the chosen alkaloids have been shown to possess anti-inflammatory properties and can be used to treat various inflammatory diseases, the mechanism of the anti-inflammatory activity of the minor alkaloids has yet to be described, and only a few studies have described the effects of sanguinarine and chelerythrine. Sanguinarine was found to block tumour necrosis factor-alpha (TNF-α)-induced phosphorylation and the degradation of IκBα, an inhibitory protein of nuclear factor (NF)-κB, as well as inhibiting the translocation of the p65 subunit to a nucleus (Chaturvedi et al. 1997). Other experiments have shown that sanguinarine and chelerythrine are capable of binding to a glucocorticoid receptor and inducing its nuclear translocation, but they lack the capacity to turn on the transcriptional activity of this receptor (Dvorak et al. 2006). The inhibition of two important enzymes, 5- and 12-lipoxygenase, has also been described (Vavreckova et al. 1996). Niu et al. (2012) described significant anti-inflammatory effects of sanguinarine both *in vitro*, in lipopolysaccharide (LPS)-stimulated peritoneal macrophages, and *in vivo*, in acute and chronic inflammatory models. Sanguinarine also attenuated pulmonary histological changes and lung oedema in mice, and reduced the myeloperoxidase activity after LPS stimulation and the neutrophil infiltration in the lung. An immunohistochemical analysis showed that the expression of cyclooxygenase (COX)-2 was significantly suppressed *in vivo* in mice without affecting the inhibition of COX-1 (Li et al. 2014b). The protective effect of chelerythrine on an ethanol-induced gastric ulcer in mice has been described recently (Li et al. 2014a). It significantly reduced the gastric ulcer index, myeloperoxidase activities, and macroscopic and histological score in a dose-dependent manner.

As has been shown, sanguinarine and chelerythrine have interesting anti-inflammatory properties, but nothing is known about the anti-inflammatory activity of other, less common, QBAs. The aim of this paper is to evaluate the anti-inflammatory potential of five minor QBAs sanguilutine (3), sanguirubine (4), chelirubine (5), chelilutine (6), and macarpine (7) *in vitro* and to compare them with the extensively studied sanguinarine (1) and chelerythrine (2) (Fig. 1).

Materials and Methods

Isolation and identification of the selected alkaloids

The benzophenanthridine alkaloids were isolated in the laboratories of the Department of Biochemistry (Faculty of Medicine, Masaryk University) from the plant material of *Macleaya microcarpa* (Maxim.) Fedde, *Dicranostigma lactucoides* Hook. F. et Thomson, *Sanguinaria canadensis* L., and *Stylophorum lasiocarpum* (Oliv.) Fedde as was described previously (Táborská et al. 1978; Dostal et al. 1992). The alkaloids were at least 98% pure according to high-performance liquid chromatography (HPLC) analysis (Suchomelova et al. 2007).

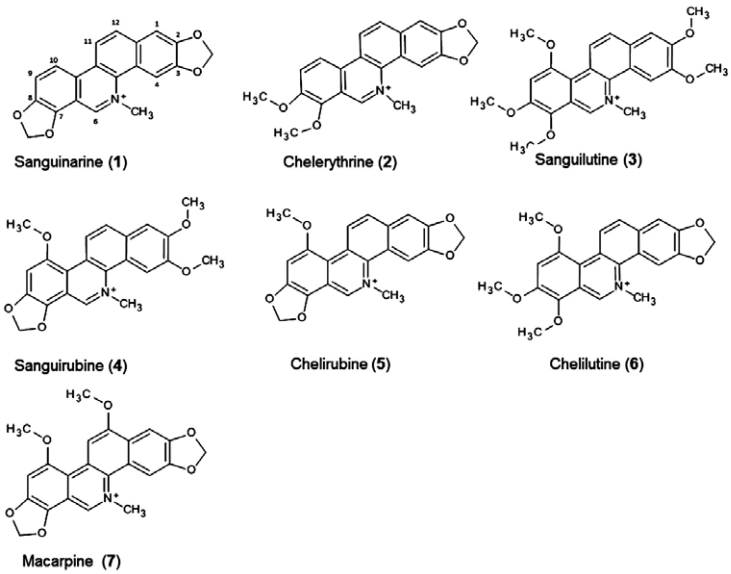

Fig.1. Structures of tested QBAs **1–7**.

Maintenance and preparation of macrophages

The THP-1 human monocytic leukaemia cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). The cells were cultivated at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine (Biosera, Boussens, France), 10% foetal bovine serum (FBS) (HyClone, GE Healthcare, Logan, UT, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Biosera, Boussens, France) in a humidified atmosphere containing 5% CO_2. The culture was split twice a week when cells had reached a concentration of 5–7 × 10^5 cells/ml. The cell number and viability for routine passaging were determined following staining with erythrosin B (Sigma-Aldrich, Steinheim, Germany). Cells were counted manually using a haemocytometer and a light microscope. Stabilized cells (5^{th}–20^{th} passage) were split into multiwell plates to achieve a concentration of 5×10^5 cells/ml, and differentiation into macrophages was induced by phorbol myristate acetate (PMA) (Sigma-Aldrich), as described previously by Pencikova et al. (2012).

Cytotoxicity testing

THP-1 cells (floating monocytes, 5×10^5 cells/ml) were incubated with 100 µl of a serum-free RPMI 1640 medium, which does not support the cell proliferation, and seeded into 96-well plates in triplicate at 37 °C. Measurements were taken 24 h after treatment with increasing concentrations (0.04–10 µM) of the test alkaloids 3–7 dissolved in dimethylsulphoxide (DMSO). The viability of cells after compounds application was measured by the Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland), according to the manufacturer's manual. The amount of formazan created (which corresponds to the number of metabolically active cells in the culture) was calculated as a percentage of the control cells, which were treated only with DMSO and were assigned as 100%. The cytotoxicity of sanguinarine (**1**) and chelerythrine (**2**) has been measured previously (Pencikova et al. 2012).

Evaluation of cytokine secretion

Differentiated macrophages (500 000 cells/ml) were pre-treated for 1 h with solutions of the test compounds (20–500 nM) or prednisone (1 µM) dissolved in DMSO or with DMSO alone (the final DMSO concentration was 0.1% in all wells); the concentrations of the test compounds used lack of cytotoxic effects (cell viability >94%). The inflammatory-like response was triggered by adding 1 µg/ml lipopolysaccharide isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich) and dissolved in water to the pre-treated macrophages; the control cells were left without LPS treatment. Macrophages were incubated with LPS for the next 24 h. After this period, the medium was collected and the concentration of TNF-α was measured using an instant enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA), according to the manufacturer's manual. Each experiment was run in triplicate.

Detection of cyclooxygenase inhibition

The inhibitory potential of the seven alkaloids tested was determined by the cell-free model by using a COX colorimetric inhibitor screening assay kit (Cayman Chemical Co.,Ann Arbor, MI, USA), which measures the peroxidase component of COXs. The kit includes both ovine COX-1 and human recombinant COX-2 enzymes in order to screen isoenzyme-specific inhibitors.The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine at 590 nm. The COX-1 and COX-2 tests were performed in two independent experiments with three replicates. At least four concentrations were used to calculate the IC_{50} values of the tested compounds. All of the samples were dissolved in DMSO.

Statistical analysis

All experiments were performed in a triplicate, and results are presented as mean values, with error bars representing the standard error (SE) of the mean. GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA) was used to perform the analysis.

The IC_{50} values were calculated from the curve fitting using four parameters logistic (4PL) regression. A one-way ANOVA test followed by a Tukey's *post hoc* test for multiple comparisons was used for statistical analysis of TNF-α secretion. A value of $P < 0.05$ was considered to be significant.

Results

Before making cell-based assays, the cytotoxicity of the alkaloids **3-7** was evaluated [alkaloids **1** and **2** had been measured previously (Pencikova et al. 2012)] (Table1). All of the selected alkaloids showed IC_{50} (half maximal inhibitory concentration) values in the range of 0.4–5.8 μM. Chelirubine (**5**), sanguinarine (**1**), and macarpine (**7**) were noted as the most cytotoxic compounds (IC_{50} < 1 μM). Less cytotoxic were alkaloids **2** and **6**, with the IC_{50} values of 3.5 μM and 1.7 μM, respectively. The lowest cytotoxicity was observed for alkaloids **3** and **4** (IC_{50} = 5.6 μM and 5.8 μM, respectively) (Fig. 2). Based on the results of the cytotoxicity evaluation, the concentration of 100 nM was selected as non-toxic for further *in vitro* tests.

Table 1. Cell viability expressed as IC_{50} values of compounds **1-7** obtained for the THP-1 cell line.

	IC_{50}	95% CI
Sanguinarine (**1**)	0.8 μM *	n.d.
Chelerythrine (**2**)	3.5 μM *	n.d.
Sanguilutine (**3**)	5.6 ± 1.3 μM	3.5–9.1 μM
Sanguirubine (**4**)	5.8 ± 1.3 μM	3.5–9.9 μM
Chelirubine (**5**)	0.4 ± 1.2 μM	0.3–0.6 μM
Chelilutine (**6**)	1.7 ± 1.5 μM	0.8–4.0 μM
Macarpine (**7**)	0.9 ± 1.1 μM	0.7–1.1 μM

*Data were published previously (Pencikova et al. 2012). IC_{50} – the half maximal inhibitory concentration; 95% CI – 95% confidence interval; n.d. – not determined.

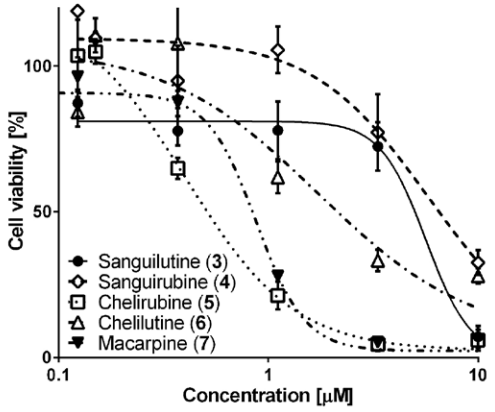

Fig. 2. Dose-dependent cytotoxicity of compounds **3–7**.

To evaluate the anti-inflammatory potential of the minor QBAs, the human macrophage-like cell line THP-1 was selected as a model of the inflammatory response *in vitro*. Cells pre-treated with the selected alkaloids were stimulated by bacterial lipopolysaccharide, and the production of the pro-inflammatory cytokine TNF-α was measured. A noticeable but not significant reduction in the secretion of TNF-α was observed for alkaloids **1**, **4**, and **7**; compounds **2**, **3**, **5**, and **6** had no effect (Fig. 3a). Because compounds **1** and **4** showed the greatest ability to reduce the secretion of TNF-α at a concentration of 100 nM, they were

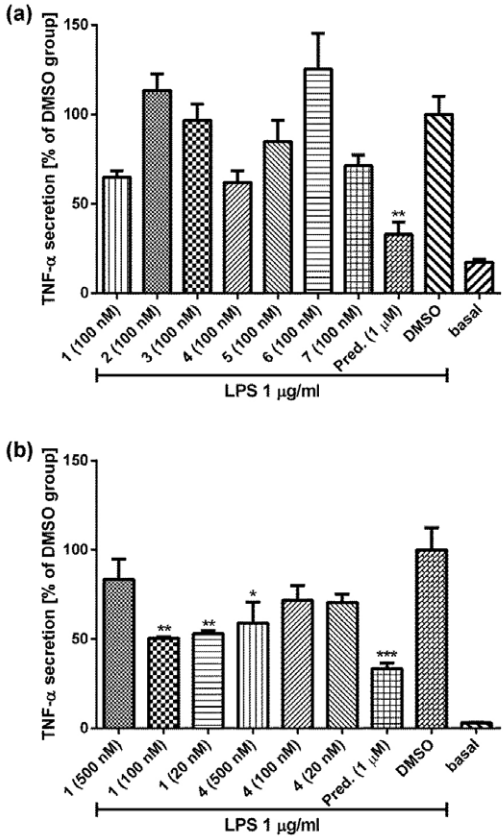

Fig. 3. The effect of QBAs **1–7** (a), and **1** and **4** (b) on the secretion of TNF-α. THP-1 macrophages were pre-treated with compounds **1–7** and prednisone (Pred.) at the indicated concentrations or with DMSO only (DMSO and basal) for 1 h. Subsequently, LPS (1 μg/ml) was added [except for the control cells (basal)] to trigger the secretion of the pro-inflammatory cytokine TNF-α. After 24 h, the amount of TNF-α was evaluated by ELISA. The results are expressed as the mean ± S.E. for three independent experiments.
*Indicates a significant difference in comparison with the DMSO-treated cells $P < 0.05$, ** indicates a significant difference in comparison with the DMSO -treated cells $P < 0.01$, and *** indicates a significant difference in comparison with the DMSO-treated cells $P < 0.001$. DMSO – dimethylsulphoxide; ELISA - enzyme-linked immunosorbent assay; LPS – lipopolysaccharide; TNF-α – tumour necrosis factor α.

selected to evaluate the dependence of this effect on concentration (500, 100, and 20 nM) (Fig. 3b]. The greatest inhibition effect was observed at the concentration of 500 nM, lower for concentrations of 100 and 20 nM of **4**, where the effect was comparable. The same profile for the expression was observed for compound **1** at concentrations of 100 and 20 nM, but at the highest concentration (500 nM), sanguinarine (**1**) displayed a lower activity than for 100 and 20 nM.

The potential for directly modulating the activities of COX-1 and COX-2 was also examined in this paper. Macarpine (**7**) showed a considerable ability to inhibit COX-2 with an IC_{50} value of 148 μM. The results demonstrated that macarpine (**7**) preferentially inhibited COX-2 rather than COX-1. Other compounds were inactive or showed a non-significant inhibitory potential at a concentration of 150 μM, with the efficiencies presented in Table 2.

Table 2. IC_{50} values of compounds **1-7** obtained for COX-1 and COX-2 inhibition.

	Concentration [μM]	% of inhibition		IC_{50} [μM]	
		COX-1	COX-2	COX-1	COX-2
Sanguinarine (**1**)	450	47.4	84.9	> 450 307.4	
	150	8.0	11.5		
Chelerythrine (**2**)	450	58.6	16.6	362.4 ± 2.4	> 450
	150	43.7	0		
	45	18.9	0		
Sanguilutine (**3**)	450	49.3	33.2	> 450	> 450
	150	3.4	46.0		
Sanguirubine (**4**)	450	14.8	0	> 450	> 450
	150	15.9	1.0		
Chelirubine (**5**)	450	29.2	3.9	> 450	> 450
	150	1.8	0		
Chelilutine (**6**)	450	56.2	49.8	399.7 ± 2.5	> 450
	150	45.5	0		
	45	6.3	0		
Macarpine (**7**)	450	40.0	100	> 450	148.1 ± 9.3
	150	33.8	50.8		
	45	0	6.5		
Indomethacin				1.9 ± 0.6 *	2.5 ± 0.9 *

*Data were published previously (Hošek et al. 2011). COX – cyclooxygenase; IC_{50} – the half maximal inhibitory concentration.

Discussion

QBAs possess significant cytotoxic activity. The type of substituent affects the structure and influences their cytotoxicity. Slaninova et al. (2001) observed reduced cytotoxicity in HeLa cells for QBAs with a lower number of methylenedioxy rings (Slaninova et al. 2001). Other studies in various cell lines led to an assumption that methylenedioxy groups attached at carbon (C)2-C3 and C7-C8 probably guarantee greater alkaloid toxicity (Slunska et al. 2010; Slaninova et al. 2007). These findings are in agreement with our results. The most cytotoxic compounds chelirubine (**5**), sanguinarine (**1**), and macarpine (**7**) contain methylenedioxy rings attached at C2-C3 and C7-C8. Less cytotoxic were compounds **2** and **6**, with the IC_{50}> 2 μM. These alkaloids each have a single methylenedioxy ring attached at C2-C3, with methoxy groups at C7 and C8. The lowest cytotoxicity was observed for compounds **3** and **4** (IC_{50}>5.5 μM). These molecules include either a single methylenedioxy ring attached at C7-C8 (**4**) or none at all (**3**). These findings support the assumption that a methylenedioxy ring attached at C2-C3 may be crucial for the cytotoxic effect of QBAs.

Previous studies have indicated a promising anti-inflammatory effect for sanguinarine (**1**) and chelerythrine (**2**) both *in vitro* and *in vivo* (Niu et al. 2011; Niu et al. 2012; Pencikova et al. 2012). The results obtained in this study indicate that the presence of a methylenedioxy ring attached at C7-C8 is important for reducing the secretion of TNF-α. The only exception is compound **5**, which possesses a methylenedioxy ring attached at C7-C8 but only minutely diminished the expression of this cytokine. Furthermore, the presence of a single methylenedioxy ring attached at C2-C3, but not at C7-C8, rather increased the production of TNF-α (compounds **2** and **6**). Compounds **1** and **4** showed

the greatest ability to reduce the secretion of TNF-α and they were selected to evaluate the dependence of this effect on concentration. Interestingly, at the highest concentration (500 nM), sanguinarine (**1**) displayed a lower activity than for 100 and 20 nM. This could be ascribed to the greater cytotoxic effect of compound **1**, which could have led to the loss of the antiphlogistic potential in the sub-toxic concentration. However, this effect was not observed in murine primary macrophages (Niu et al. 2012). It could be explained by the varying sensitivity of different cell lines to the alkaloids, as described previously (Malikova et al. 2006).

Previous papers have shown the ability of some benzophenanthridine alkaloids to inhibit the production of the prostaglandin PGE_2 by inhibiting the expression of COX-2 (Niu et al. 2011; Li et al. 2014b). However, except for macarpine (**7**), other test compounds were inactive or showed an insignificant inhibitory potential on COXs. These findings together with previously published results indicate that these alkaloids act by inhibiting the expression of COXs rather by their directly inactivating them.

The results obtained from the presented experiments contribute to our understanding of the structure-to-activity relationship of QBAs. The anti-inflammatory potential and the cytotoxic effect are driven by the presence of methylenedioxy rings attached at C7-C8 and C2-C3, respectively. Combining the presence or absence of such rings may enable a search for compounds with cytotoxic activity (potential anti-cancer drugs), with antiphlogistic effects (potential anti-inflammatory drugs), or with both of these features.

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Declaration of Interest

The authors declare no conflict of interest.

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