PHYTOTHERAPY RESEARCH
Phytother. Res. **16**, 84–87 (2002)
DOI: 10.1002/ptr.969

SHORT COMMUNICATION

Quaternary Benzo[c]phenanthridine Alkaloids as Inhibitors of Aminopeptidase N and Dipeptidyl Peptidase IV

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Chelerythrine, sanguinarine and an alkaloid extract from *Macleaya cordata*—sanguiritrin—were found to be inhibitors of aminopeptidase A and dipeptidyl peptidase IV, while fagaronine inhibited dipeptidyl peptidase IV only. At 50 μM, chelerythrine, sanguinarine and sanguiritrin inhibited aminopeptidase N by 82%, 82%, 88%, DPP IV by 38%, 62%, 57%, and fagaronine by 34%, respectively. When bovine serum albumin (500 μg/mL) was added, the inhibition of both proteases by quaternary benzo[c]phenanthridine alkaloids (QBA) (50 μM) was significantly diminished. Strong interaction of chelerythrine and sanguinarine with bovine and human serum albumin was proved by electrophoretic determination of their respective conditional binding constants. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: benzo[c]phenanthridines; *Macleaya cordata*; C6 rat glioma cells; aminopeptidase N; dipeptidyl peptidase IV; albumin; binding constants.

INTRODUCTION

Quaternary benzo[c]phenanthridine alkaloids (QBA) are widely spread in the Papaveraceae, Fumariaceae and Rutaceae families (Šimánek, 1985) where they act as allelochemicals with a broad activity spectrum (Wink *et al.*, 1998; Verpoorte, 1998). Several plants containing QBA are used in traditional medicine and homeopathy (Dostál and Slavík, 2000). The standardized QBA containing extracts, sanguinaria extract (from *Sanguinaria canadensis* L., Papaveraceae) and sanguiritrin (from *Macleaya cordata* (Wild.) R. Br., Papaveraceae) are used as ingredients in oral hygiene products because of their promising antimicrobial and antiinflammatory effects (Tenenbaum *et al.*, 1999). The physiological effect of QBA is pleiotropic (Walterová *et al.*, 1995). They affect basic molecular targets common to all cells, an effect depending mainly on the reactivity of the iminium bond of the benzo[c]phenanthridine nucleus (Dostál and Potáček, 1990). Many observed biological effects of chelerythrine and sanguinarine involve the formation of a labile covalent bond between the SH groups of a cell component and an electrophilic C6 carbon (Walterová *et al.*, 1981). QBA are strong DNA intercalators (Walterová *et al.*, 1995). The *in vitro* and *in vivo* activities of QBA versus the safety of phytopreparations containing plant extracts with QBA has been an unending history of controversial discussion (Elvin-Lewis and Lewis, 1995).

Aminopeptidase N (APN; EC 3.4.11.2, identical to CD13) and dipeptidyl peptidase IV (DPP- IV; EC 3.4.14.5, identical to CD26) are typical representatives of cell membrane proteases; both of them are known to have soluble counterparts in blood serum. The complex array of cell membrane protease roles makes these molecules attractive as a model for the study of QBA interactions with non-SH proteins. APN and DPP-IV are involved, by proteolytic as well as by nonproteolytic mechanisms, in a number of physiological functions such as limited proteolysis of biologically active peptides, cell–cell or cell–matrix adhesion and signalling (Šedo *et al.*, 1996). APN inhibitors could interfere in some steps of the pathogenetic cascade of inflammation, tumour penetration through the basal membrane and human coronavirus infection (Riemann *et al.*, 1999). DPP-IV plays a crucial role in cell proliferation, DNA synthesis and cytokine production, not only in T cells, but also in other systems, and the enzymatic activity seems to be an important prerequisite for at least some of DPP-IV molecule functions; it has been well demonstrated that DPP-IV inhibitors block the above mentioned processes (DeMester *et al.*, 1999).

Since APN and DPP-IV are important enzymes in the processes of cell activation and differentiation (Šedo *et al.*, 1998; Lendeckel *et al.*, 1998), compounds regulating their activity are of interest as subjects in both enzymological studies and for future practical applica-

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Contract/grant sponsor: Ministry of Education, Youth and Sports; Contract/grant number: J14/98: 151100003; Contract/grant number: J13/98: 444400004.
Contract/grant sponsor: Grant Agency of Charles University; Contract/grant number: 58/1999/C.

Received 15 November 1999
Accepted 15 June 2000

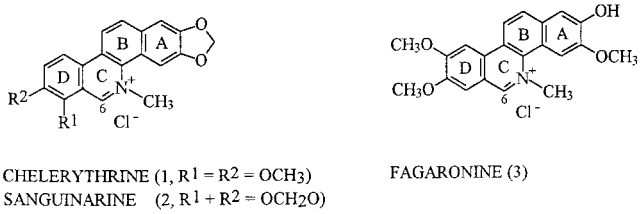

Figure 1. Structures of studied alkaloids.

tion. In this paper, the inhibitory effect of chelerythrine (**1**), sanguinarine (**2**), fagaronine (**3**) (Fig. 1), and sanguiritrin (a natural mixture of **1** and **2**) on APN and DPP IV is described. In serum, the inhibition of these proteases by QBA is suppressed by albumin. To explain these phenomena the mode of QBA binding with albumin is investigated here.

MATERIALS AND METHODS

Alkaloids. Sanguiritrin, QBA extract from *Macleaya cordata* (Wild.) R. Br., Papaveraceae, was purchased from CAMAS Technologies, Inc. (Broomfield, USA), it contained **1** and **2** as chlorides in the ratio 1:3 (Ševčik *et al.*, 2000). Chelerythrine (**1**) and sanguinarine (**2**) chlorides were isolated from sanguiritrin using column chromatography on alumina (Dostál *et al.*, 1992); (**1**) in 95% purity, MP 200 °–204 °C (lit. 202 °–203 °C, Southon and Buckingham, 1989), UV (EtOH) λ_{max} (log ε): 226 (4.39), 230 (4.42), 343 (sh, 4.34), 352 (4.35), 431 (3.70), and (**2**) in 98% purity, MP 279 °–282 °C (lit. 277 °–280 °C, Southon and Buckingham, 1989), UV (EtOH) λ_{max} (log ε): 229 (4.35), 241 (4.35), 266 (4.31), 350 (sh, 4.33), 361 (4.34), 463 (3.67) were obtained. Fagaronine (**3**) chloride was synthesized by Šmidrkal (Šmidrkal, 1988), MP 203 °– 206 °C (lit. 202 °C, Southon and Buckingham, 1989), UV (EtOH) λ_{max} (log ε): 221 (3.19), 236 (3.17), 273 (3.58), 301 (3.48), 311 (3.48), 329 (3.36). IR, MS and NMR spectra were consistent with the structures of the above alkaloids.

Chemicals. Dulbecco's modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were from Gibco, Paisley, Scotland. Human serum albumin (USA), globulin free (approx. 99%), Exbio (Olomouc, Czech Republic) and bovine serum albumin (BSA) fraction V (>97%) from Merck, (Darmstadt, Germany). Other chemicals and enzyme substrates were from Sigma (St Louis, USA).

CZE determination of binding constants. Conditional binding constants of **1** and **2** with albumins were calculated from electrophoretic mobility data (Barták *et al.*, 2000) measured by the capillary zone electrophoresis method (CZE) (Rundlett and Amstrong, 1997), 13.5 mM phosphate-Tris buffer pH 7.40 was used.

Cells. The C6 rat glioma cell line (Benda *et al.*, 1968) was obtained from the European Collection of Cell Cultures, Wiltshire, UK. Cells were cultured in Nunc tissue plastic (Roskilde, Denmark) at 37 °C in DMEM supplemented with 10% FCS under a humidified (90%) atmosphere of 5% CO_2/95% air.

Enzyme assays. The APN and DPP-IV activities were measured photometrically with substrates Ala-pNA and Gly-Pro-pNA, as described previously (Nagatsu *et al.*, 1976). The incubation mixture (total volume 250 μL) contained 200 μL of 66 mM Tris-HCl buffer pH 8.0 with or without tested alkaloids (final concentration 25 and 50 μM; it was not possible to test higher alkaloid concentrations due to their limited solubility) or sanguiritrin (final concentration 9 and 18 μg/mL) with or without BSA and 50 μL of cell suspension of about 2×10^6 cells in phosphate buffered saline (137 mM NaCl, 2.68 mM KCl, 4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.40) or 50 μL of FCS. Preincubation was carried out at 37 °C for 15 min. Then the reaction was initiated by adding 50 μL of substrate solution, to reach a final concentration of 1.4 mM. After 30 min incubation in a water-bath at 37 °C with continual agitation of test tubes, the reaction was stopped by adding 1 mL of buffer pH 4.2. The controls included all the reagents in the assay, with the enzyme added after the reaction was stopped. The absorbance of liberated 4-nitroaniline was measured spectrophotometrically at 405 nm in the supernatant after centrifugation (6000 × **g**, room temperature, 10 min) using blank samples containing appropriate concentrations of each alkaloid. All assays were done in duplicate. For calibration curves, DMSO solutions of 4-nitroaniline were used. The enzyme activity was calculated in nkat/mL of the reaction mixture and expressed as residual activity (%). The fluorescent kinetic method for the measurement of protease activities using aminomethylcoumarin labelled substrates was not applicable to our experiments because of the quenching of relevant excitation wavelengths by alkaloids in the reaction mixture.

Statistical analysis. The data are expressed as means of duplicate measurements ± SEM.

RESULTS AND DISCUSSION

Chelerythrine (**1**), sanguinarine (**2**) and sanguiritrin were strong inhibitors of APN activity in C6 cells (Fig. 2A). The efficacy of these alkaloids and sanguiritrin was comparable to that of APN inhibitors Bestatin (Ubenimex), ((2S,3R-3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine) and Amastatin ((2S,3R-3-amino-2-hydroxy-5methylhexanoyl)-Val-Val-Asp-OH. HCl). Under the experimental conditions used, **1** and **2** occurred in the pseudobase (alkanolamine) form (Dostál *et al.*, 1996), which is the actual reactive species forming a complex with the enzyme, due to the formation of a labile covalent bond. On the other hand, fagaronine (**3**), a quaternary cation at pH 7.40, did not inhibit at either concentration tested. Interestingly, however high the activity of APN was measurable in FCS, we did not observe an inhibition by any alkaloid tested (data not shown). Thus, APN activity was studied in the cell system, in the presence or absence of BSA. Albumin significantly diminished the inhibitory effects of **1, 2** and **3** against APN activity (Fig. 2A). Such effects were dose dependent with 50–500 μg/mL concentrations of BSA (data not shown). Higher BSA concentrations in the enzyme assay were impossible to reach due to sample precipitation. In order to quantify the intensity of the QBA interaction with albumins, the

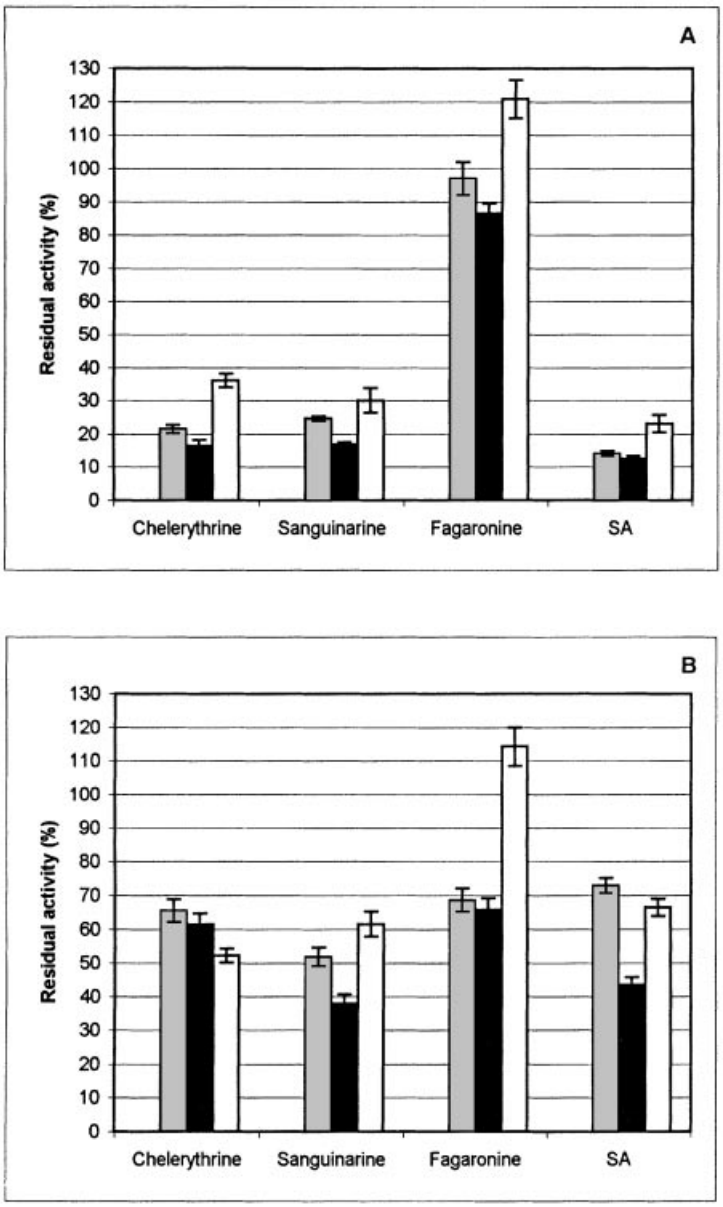

Figure 2. Effect of chelerythrine (**1**), sanguinarine (**2**), fagaronine (**3**) and sanguiritrin (**SA**) on the C6 rat glial cell APN (panel A) and DPP-IV (panel B) enzyme activities. Grey bars and black bars: 25 μM and 50 μM of the alkaloid, 9 and 18 μg/mL of sanguiritrin respectively, white bars: combination of 50 μM (18 μg/mL) of alkaloid (sanguiritrin) with 500 μg/mL of BSA in the reaction mixture.

conditional binding constants of alkaloids **1** and **2** were measured electrophoretically (Table 1). Both alkaloids exhibited strong interaction with BSA and HSA at pH 7.40. The stabilities of complexes 1 and 2 with both albumins are not different. The investigation of **1** and **2** forms participating in interactions with albumins showed the pseudobases as the interacting species. The inhibitory effect of sanguiritrin on APN activity is similar to that of alkaloids **1** and **2**. As for the second enzyme system tested, DPP-IV, we failed to obtain unambiguous results (Fig. 2B). Substances **1, 2** and sanguiritrin inhibited in a similar way to the specific DPP-IV inhibitors Diprotin A (H-Ile-Pro-Ile-OH) and B (H-Val-Pro-Leu-OH) (data not shown). In this case albumin significantly diminished the inhibitory activity of **2** and sanguiritrin but not that of **1**. Fagaronine (**3**) inhibited DDP-IV less efficiently but the effect of albumin addition was the most marked among the tested alkaloids. We were not able to determine fagaronine (**3**) binding constants due to the absence of its electrophoretic mobility at pH 7.40. The ionization of hydroxyl substituent at C2 of **3** may be considered

Table 1. Conditional binding constants of chelerythrine (1) and sanguinarine (2) with albumins

	(log K*[a])	
Target	Chelerythrine	Sanguinarine
Bovine serum albumin	4.39 ± 0.01	4.37 ± 0.04
Human serum albumin	4.72 ± 0.02	4.74 ± 0.01

[a] Measured in 13.5 mM phosphate-Tris buffer, pH 7.4, I = 0.03 M, 25°C.

responsible for its zwitterion form, and, consequently, for the absence of an electrophoretic charge. We deduce that for DPP-IV inhibition, the QBA form is not the most important factor since comparable inhibition was achieved with both alkaloids **1** and **2** occurring as pseudobases and with **3** in the form of a quaternary cation. The unexpected reversal effect of albumin addition on the DPP-IV activity was observed for the inhibition by **1**. We assume that QBA inhibition of DPP-IV is complex and we are not able to explain it unambiguously at present. Marked, but nonspecific, effects of QBA on the functions of structurally different biopolymers support the views of the authors who consider questionable the long-term use of natural remedies containing QBA (Damm *et al*., 1999). Our results presented here raise questions on the safety of QBA containing plant extracts as well. Due to their reactivity, QBA can be included among compounds with mutagenic and/or carcinogenic characteristics. Further studies related to the QBA toxicity and to the mechanism of their action in different types of mammalian cell models are underway in this laboratory.

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports, projects number J14/98: 151100003, J13/98: 444400004 and by the Grant Agency of Charles University, project number 58/1999/C.

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