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%, and 88%, DPP IV by 38%, 62%, and 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.

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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 enduring 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-
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 (Sevcik 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°C–203°C; Southon and Buckingham, 1989), UV (EtOH) λmax (log e): 226 (4.39), 230 (4.42), 343 (sh, 4.34), 352 (4.35), 431 (3.70), and (2) in 98% purity, MP 279°C–282°C (lit. 277°C–280°C; Southon and Buckingham, 1989), UV (EtOH) λmax (log e): 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 Šmidral (Šmidral, 1988), MP 203°C–206°C (lit. 202°C, Southon and Buckingham, 1989), UV (EtOH) λmax (log e): 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% CO2/95% 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 2×106 cells in phosphate buffered saline (137 mM NaCl, 2.68 mM KCl, 4 mM Na2HPO4, 1.76 mM KH2PO4, 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-5-methylhexanoyl)-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
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

| 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  |

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

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