Physiol. Res. 52: 367-372, 2003

Quaternary Benzo[c]phenanthridine Alkaloids as Inhibitors of Dipeptidyl Peptidase IV-Like Activity Bearing Enzymes in Human Blood Plasma and Glioma Cell Lines

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Received May 13, 2002
Accepted August 12, 2002

Summary

Quaternary benzo[c]phenanthridine alkaloids (QBA), fagaronine (FA), sanguinarine (SA), chelerythrine (CHE) and the QBA extract from *Macleya cordata* (EX) exerted differential inhibitory effect on the hydrolytic activity of particular dipeptidyl peptidase (DPP)-like enzyme isolated from human blood plasma and from human and rat glioma cell lines. The low-MW form of DPP-IV-like enzyme activity, corresponding most probably to DPP-8, observed only in glioma cells but not in human plasma, was inhibited preferentially by SA, CHE and EX, and only slightly by FA. The alkaloid inhibitory effect was concentration-dependent in the range 25-150 μM and directly pH-related. In addition, a subtle but consistent inhibition of the intermediate-MW form of DPP-IV-like enzyme activity, ascribed to DPP-IV/CD26, observed only in human plasma and of the attractin (high-MW form of DPP-IV-like enzyme activity, expressed in U87 glioma cells) by the studied alkaloids was observed. We conclude that some of the QBA biological effects could be determined by tissue and cell type specific dipeptidyl peptidase IV-like molecules expression pattern.

Key words

Fagaronine • Sanguinarine • Chelerythrine • Macleya cordata • Dipeptidyl peptidase IV • Attractin

Introduction

Quaternary benzo[c]phenanthridine alkaloids (QBA) whose most studied representatives are sanguinarine, chelerythrine and fagaronine (Fig. 1) display a wide spectrum of non-specific biological activities (Faddejeva and Belyaeva 1997) and affect basic molecular targets common to mammalian cells. Fagaronine was studied intensely for its anti-tumor activity (Suffness and Cordell 1985). Extracts from *Macleya cordata* and *Sanguinaria canadensis*, containing sanguinarine and chelerythrine, are active components of oral hygiene products with an antiplaque effect. SANGROVIT®, a farm animal weight stimulant, contains *Sanguinaria canadensis* rhizomes as an active component. To obtain a deeper insight into QBA biological activity, we should focus not only on their "direct" targets in the cell but also on possibly more complex pathways of their action. The interaction with enzymes involved in numerous physiological functions is an example of such an "indirect" QBA effect. Recently, we described inhibition of aminopeptidase-N and

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dipeptidyl peptidase IV (DPP-IV) activity by QBA in C6 glioma cells (Šedo *et al*. 2002). We have now been focusing more on heterogeneous DPP-IV-like activity bearing enzymes to investigate their inhibition by QBA as one possible pathway that could help to explain the diverse QBA biological activities.

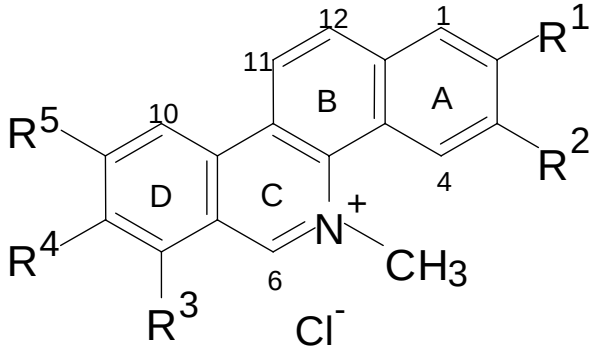

Fig. 1. *Alkaloid structures*
SANGUINARINE:
$R^1+R^2 = OCH_2O$, $R^3+R^4 = OCH_2O$, $R^5 = H$
CHELERYTHRINE:
$R^1+R^2 = OCH_2O$, $R^3 = R^4 = OCH_3$, $R^5 = H$
FAGARONINE:
$R^1 = OH$, $R^2 = OCH_3$, $R^3 = H$, $R^4 = R^5 = OCH_3$

Dipeptidyl peptidase IV (DPP-IV, EC 3.4.14.5) was originally believed to be the only membrane-bound enzyme specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. Many biologically active peptides contain an evolutionary conserved proline residue as a proteolysis-processing regulatory element and, therefore, proline-specific proteases could be seen as their important "check-points" (Vanhoof *et al*. 1995). Thus, proteolytical activation and inactivation of such peptides was originally expected to be the main physiological function of DPP-IV. Subsequently, three general mechanisms of DPP-IV activity have been postulated: (i) Limited proteolysis, i.e. highly specific processing of biologically active peptides, leading to their functional activation or inactivation (Mentlein 1999). This mechanism has been shown to play a role in immune and endocrine system regulations (Fleischer 1994, Vanhoof *et al*. 1995), diabetes mellitus pathogenesis (Holst and Deacon 1998) and HIV infection (Vanhoof *et al*. 1995); (ii) Cell-cell, cell-extracellular matrix and cell-virus contacts; DPP-IV was described to be a collagen- and fibronectin-binding protein (Cheng *et al*. 1998), a co-receptor for HIV-1 (Fleischer 1994), and a homing factor for organ-specific metastasizing of breast tumors (Cheng *et al*. 1998); (iii) Signal transduction; DPP-IV/CD26 is considered as a co-receptor transmitting specific signals through the plasma membrane (Gaetaniello *et al*. 1998).

However, other molecules, even structurally non-homologous with the DPP-IV but bearing corresponding enzyme activity, have been identified recently, namely fibroblast activation protein α, dipeptidyl peptidase IV-β, N-acetylated α-linked acidic dipeptidase, quiescent cell proline dipeptidase/dipeptidyl peptidase II, attractin, and dipeptidyl peptidase 8 (for review see Šedo and Malík 2001). Comparing the structure and relatedness of molecules associated functionally or structurally with dipeptidyl peptidase IV led to a grouping classified as "DPP-IV activity and/or structure homologues" (DASH). DASH were shown to participate in a broad array of complex processes such as cell proliferation and differentiation (Reinhold *et al*. 2000), neoplastic transformation (Iwata and Morimoto 1999) and apoptosis (Gaetaniello *et al*. 1998). Specific inhibitors of such enzymes are assumed to be of therapeutic significance in the treatment of HIV infection, diabetes mellitus and as an immunosuppressant in transplantation surgery and autoimmune diseases, including multiple sclerosis (Reinhold *et al*. 2000).

It is being reported here that fagaronine, sanguinarine, chelerythrine and the alkaloid extract from *Macleya cordata* exert different inhibitory effects on DASH molecules isolated from human blood plasma and human and rat glioma cell lines.

Methods

Alkaloids

QBA extract from *Macleya cordata* (Papaveraceae) (EX) was purchased from CAMAS Technologies, Inc. (Broomfield, USA). The extract contains chelerythrine (CHE) and sanguinarine (SA) in the ratio 1:3. Chelerythrine and sanguinarine were isolated using CC on alumina (Dostál *et al.* 1992). Chelerythrine in 95 % purity, m.p. 200-204 °C (cf. 202-203 °C, Southon and Buckingham 1989) and sanguinarine in 98.1 % purity, m.p. 279-282 °C (cf. 277-280 °C, Southon and Buckingham 1989) were attained and their purity was determined by HPLC. Fagaronine (FA) was synthesized by Šmidrkal (1988), mp 203-206 °C (Southon and Buckingham 1989). IR, UV, MS and NMR spectra were consistent with the structures of the above mentioned alkaloids.

Chemicals

Chromogenic substrate 7-(glycyl-L-prolylamido)-p-nitroanilid (GP-pNA) was from Bachem (Bubbendorf, Switzerland). All other chemicals were purchased from Sigma (Prague, Czech Republic).

Cell cultures

The C6 rat glioma cell line (glioma grade I) was from the European Collection of Cell Cultures (Wiltshire, UK). Human U373 (glioma grade III) and U87 (glioblastoma multiforme grade IV) cell lines were obtained from ATCC (Rockville, USA). The cells (passage numbers between 10 and 30) were cultured in 10 cm diameter plastic Petri dishes (NUNC, Roskilde, Denmark) at 37 °C in DMEM, supplemented with 10 % FCS, under a humidified (>90 %) atmosphere of 5 % CO_2/95 % air from 3 (C6 cells) to 5 (U373 and U87 cells) days to reach confluence of about 75 % (Mareš *et al.* 2001). Cell viability evaluated by trypan blue exclusion was always >90 %. Cells were counted using a Coulter Counter Z2 (Krefeld, Germany).

Plasma collection

Blood was collected from the cubital vein of a human donors into a heparinized sterile syringe and then centrifuged at 500 x *g* for 10 min at room temperature. The supernatant (plasma) was diluted with an equilibrating buffer to the final protein concentration of 5 mg/ml and processed by gel filtration.

Gel filtration

Gel filtration was performed as described (Malík *et al.* 2001) on Sephracryl G-300 (Pharmacia, Uppsala, Sweden). Cell suspensions were lysed in an equilibrating buffer (100 mM NaCl, 10 mM NaH_2PO_4, 40 mM Na_2HPO_4, 0.02 % sodium azide, 0.1 % Triton X-100, pH 7.4) and ultracentrifuged (200 000 x *g*; 45 min; 4 °C). The supernatant (volume approximately 4 ml; protein concentration 2-3 mg/ml) or blood plasma samples (diluted to reach protein concentration 4-5 mg/ml) were loaded onto a chromatographic column (1.5 x 95 cm) and then eluted with the equilibrating buffer. 1.5 ml fractions were collected in 15 min intervals. The void volume of the chromatographic system was assessed by Dextran Blue elution.

Enzyme activity and inhibition assay

The DPP-IV-like enzyme activities were measured by the photometric method with substrate GP-pNA as described elsewhere (Šedo and Malík 2002) with some modifications. The assay was carried out at room temperature in 96 well plates. Each well contained a mixture of 50 µl of the sample, 2-6 µl of the particular alkaloid stock-solution (to reach the appropriate final concentration) and 150 µl of 0.1 M phosphate buffer of appropriate pH (4.0-8.0). The reaction was started by addition of 2 µl of the stock 2.6 mM GP-pNA solution. pNA release from the substrate was monitored spectrophotometrically (MultiScan MF, Lab Systems) at 405 nm for 1 h at 5 min intervals. All samples were measured in triplicate against appropriate blank samples.

Statistical analysis

The data are expressed as mean of triplicate measurements ± S.D.

Results

As we described recently, there are two DPP-IV-enzyme activity bearing molecules in glioma cell lines studied: (a) a "low-MW form" of about 320 kDa expressed in all cell lines and (b) attractin, a 570-kDa form expressed only in human U87 cell line (Malík *et al.* 2001). In the human plasma, we found a predominant peak of DPP-IV-like enzyme activity of about 440 kDa ("intermediate-MW form") and a minor high-MW peak of about 600 kDa (Fig. 2). The pH profiles of all these enzyme forms were almost identical, reaching maximal activity at pH 8.

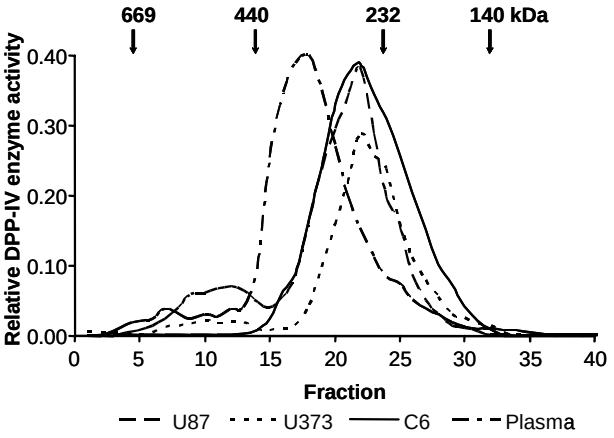

Fig. 2. *Distribution of GP-pNA-cleaving activity from glioma cell lines and human blood plasma after Sephacryl G-300 gel filtration. The arrows indicate elution of molecular weight markers (kDa). Fraction No. 1 represents the first sample after the void volume elution.*

As shown in Figure 3, the low-MW form of DPP-IV-like enzyme activity was inhibited preferentially by SA, CHE and EX, and only slightly by FA. Inhibition of this form was similar in all cell lines tested. The alkaloid inhibitory effect was concentration-dependent in the ranges 25-150 μM and directly pH-related, being absent below pH 6.0 (data not shown). Higher concentrations of alkaloids were impossible to achieve due to their limited solubility. In addition, subtle but consistent inhibition of the intermediate-MW form and attractin by all alkaloids was observed in all experiments (Fig. 3).

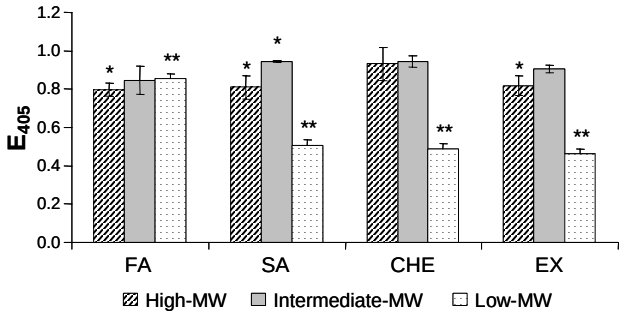

Fig. 3. *Effect of fagaronine (FA), sanguinarine (SA), chelerythrine (CHE) and Macleya cordata extract (EX) on particular DPP-IV-like enzyme activity forms. Striped bars: high-MW form/attractin, dotted bars: intermediate-MW form/DPP-IV, gray bars: low-MW form/DPP-8. Alkaloids were used in final concentration of 50 μM. Data (mean ± SD, n=15) are presented as relative enzyme activity (1=control without inhibitor). Statistical significance of differences: *p<0.05, **p<0.001.*

Discussion

Chelerythrine activates caspase-3 and thus acts as an inductor of apoptosis (Sweeney *et al*. 2000). Generally, cellular targets of protease effectors comprise a number of proteins participating in the activation/inhibition of the enzyme responsible for the observed biological activity. Thus CHE, listed as a potent protein kinase C inhibitor, does not inhibit the enzyme in question (Lee *et al*. 1998), but its inhibitory activity consists in the activation of p38 and c-Jun N-terminal kinases (Yu *et al*. 2000). Nevertheless, chelerythrine was described to induce rapid and synchronous progression of polymorphonuclear leukocytes into the apoptotic process *via* a protein kinase C-independent mechanism (Sweeney *et al*. 2000).

As we showed (Šedo *et al*. 2002), QBA are potent inhibitors of DPP-IV-like enzyme activity in glioma cells. In fact, the total cellular DPP-IV-like enzyme activity represents the sum of hydrolytic activities of several separate molecular species, so called DASH (Šedo and Malík 2001). Cell and organ specific DASH repertoire plays a critical role in the regulation of cell proliferation, differentiation, apoptosis and energy metabolism. Thus, we decided to study the effect of QBA on subseparated DPP-IV-like enzyme activity bearing molecules, to investigate the complexity of a possible mechanism of the effect of QBA alkaloids.

Considering MW, pH optima, and inhibitory parameters of particular DPP-IV-like enzyme activity fractions in our experiments we assume that the high-MW, intermediate-MW, and low-MW forms represent attractin (Malík *et al*. 2001), DPP-IV/CD26 (Duke-Cohan *et al*. 1996), and DPP-8 (Abbott *et al*. 2000), respectively.

The most potent inhibition by QBA was observed in DPP-8, resembling the low-MW form of DPP-IV-like enzyme activity. DPP-8 is a ubiquitous soluble non-glycosylated serine protease, localized in the cytoplasmic (non-lysosomal) compartment, acting preferably at neutral pH (Abbott *et al*. 2000). Based on the structural similarity with DPP-IV, DPP-8 was proposed to be involved in T cell activation and immune function (Abbott *et al*. 2000). Functional studies dealing with DPP-8 have not been published yet. Thus, biological impact of its inhibition by QBA remains fully speculative.

Attractin was originally described to be an immunoregulatory protein, expressed and secreted by activated T-cells, mediating their costimulation to recall antigen-driven proliferation (Duke-Cohan *et al*. 1998). Later studies demonstrated its presence in other organ systems, where it is expected to participate in the regulation of pleiotropic phenotypic features including tumor susceptibility, pigmentation and body weight (Gunn *et al*. 1999). Some of these functions may at least be dependent on attractin enzyme activity. Thus, QBA mediated attractin inhibition could result in a broad array of functional effects, depending on the organ system affected.

There is contradictory evidence whether attractin (Duke-Cohan *et al*. 1998) or DPP-IV/CD26 (Durinx *et al*. 2000) represents the main source of serum DPP-IV-like enzyme activity. We observed two fractions of DPP-IV-like enzyme activity in human plasma, which supports the hypothesis concerning the heterogeneity of DPP-IV there. Indeed, it is possible to speculate that their proportion could be dependent on either individual or specific

conditions. Decreases in serum DPP-IV-like enzyme activity were observed to be inversely related to increasing severity of depression in patients exposed to immunochemotherapy (Maes *et al*. 1997). Interestingly, serum DPP-IV-like activity is also significantly decreased in patients with food intake disorders. Yet, it is worth mentioning that glucagon-like peptide 1 and 2 are DPP-IV substrates and, therefore, QBA-mediated inhibition of DPP-IV-like enzyme activity can influence intestinal motility and function as well as modulate the effect of both hormones upon the total body weight (Hildebrandt *et al*. 2001).

Additionally, we have also found DPP-IV-like enzyme activity fraction with parameters (acidic pH optima, low molecular weight, substrate preference) resembling quiescent cell proline peptidase/dipeptidyl peptidase II in all glioma cell lines, but not in human plasma (unpublished results). Inhibition of quiescent cell proline peptidase (QPP) is believed to be a trigger of a specific apoptotic pathway in quiescent lymphocytes (Chiravuri *et al*. 1999). Unfortunately, we were unable to detect any inhibition of the above-mentioned DPP-IV-like enzyme activity by QBA. This could be due to QBA inactivity below pH 6, i.e. conditions optimal for QPP enzyme activity. Nonetheless, QPP could be considered as a possible QBA target, at least in some cell systems.

Inhibitors of DPP-IV-like activity bearing molecules are believed to be of significant therapeutic impact in the treatment of HIV infection, diabetes mellitus, and as an immunosuppressant in the transplantation surgery and autoimmune diseases, including multiple sclerosis. Even though valuable attempts have been made, there is still a lack of commercially available specific substrates and inhibitors of individual DPP-IV-like enzyme activity bearing molecules. On the other hand, from a functional point of view, an inhibitor itself does not need to be ultimately specific for a particular DASH molecule; its "specificity" could be provided by cell/immediate environment specific expression pattern of these enzymes.

To conclude, we propose that, among numerous others, some of QBA biological effects could be mediated by their interaction with the heterogeneous group of DASH. Moreover, a specific DASH expression pattern determines the quality of such alkaloid effects, which can be seemingly paradoxical in certain tissues and cell systems.

Acknowledgements

This work was supported by the Grant Agency of Charles University grant 58/1999/C, the First Medical Faculty of Charles University Research Project No. 206019-2 – "Oncology", by Grant Agency of the Czech Republic, Project No. 303/01/P085, by the Ministry of Education MSM 151100003.

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Reprint requests

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