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Cytotoxic and peptidase inhibitory activities of selected non-hepatotoxic cyclic peptides from cyanobacteria

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Abstract

Toxic cyanobacterial blooms are a rich source of metabolites having a variety of biological activities. Two main groups of cyclic peptides, depsipeptides and ureido linkage-containing peptides, reportedly inhibit serine peptidases. We characterised their inhibitory properties against selected peptidases and investigated their influence on cell viability. The depsipeptide planktopeptin BL1125 is a strong linear competitive tight-binding inhibitor of leukocyte (K_i=2.9 nM) and pancreatic (K_i=7.2 nM) elastase and also of chymotrypsin (K_i=6.1 nM). Anabaenopeptins B and F show no inhibition against chymotrypsin, but inhibit both elastases. The tested cyclic peptides do not inhibit trypsin, urokinase, kallikrein 1 or cysteine peptidases. All three tested cyanopeptides show no short-term cytotoxicity in concentrations of up to 10 μM, but impair the metabolic activity of normal human astrocytes after prolonged exposure (48–96 h), whereas glioblastoma cells, tumour cells of the same type, are resistant. Strong inhibition and relative selectivity of the tested cyanopeptides suggests that they are potential candidates for application in inflammatory diseases and possibly some types of cancers.

Keywords: anabaenopeptin; cyanopeptides; cytotoxicity; planktopeptin; *Planktothrix rubescens*; protease inhibition.

Introduction

Cyanobacteria constitute a large, diverse and widely distributed group of prokaryotes, occupying a wide variety of habitats (Fogg et al., 1973). Mass occurrences of cyanobacteria, called cyanobacterial blooms, are ubiquitous in eutrophic lakes, ponds and rivers. Toxic cyanobacterial blooms are common events worldwide as well as in Slovenia (Sedmak and Kosi, 1997) and can harm animal and human health (Carmichael et al., 2001). Cyanobacteria are a rich, readily available source because of their fast growth and the ability to form dense, almost monospecific aggregations. They produce not only toxins but also other metabolites with strong biological activities. Cyclic peptides are the best known and most abundant structural type, but other metabolites, such as linear peptides, guanidines, phosphonates, purines and macrolides, are also synthesised by cyanobacteria. Non-hepatotoxic cyclic peptides belonging to two main groups, depsipeptides and peptides with the ureido linkage, possess serine peptidase inhibitory activity (Namikoshi and Rinehart, 1996), usually characterised by IC_{50} values. Depsipeptides contain a unique amino acid unit, 3-amino-6-hydroxy-2-piperidon (Ahp), and generally exhibit proteolytic inhibitory activity (Namikoshi and Rinehart, 1996; Okano et al., 1999). Anabaenopeptins (AnPs) belong to the group of peptides with the ureido linkage (Harada et al., 1995; Fujii et al., 1996, 2002) and exhibit relaxing activity to norepinephrine-induced contraction of rat aortic preparations (Harada et al., 1995; Harada, 2004). Although AnPs were suggested to inhibit peptidases (Repka et al., 2004), there are no clear experimental results to confirm this.

Both hepatotoxic and non-hepatotoxic cyanopeptides influence the morphology and physiology of phytoplankton species with a strong negative effect on biodiversity (Sedmak and Kosi, 1998a,b; Sedmak and Eleršek, 2006; Sedmak et al., 2008a). *Planktothrix rubescens* (DC. ex Gomont) is a hepatotoxic, bloom forming cyanobacterial species, in which, beside microcystins, considerable amounts of peptidase inhibitory cyclic peptides are produced (Grach-Pogrebinsky et al., 2003, 2004; Sedmak et al., 2008b). Along with well known compounds, such as AnPs A, B and F, new depsipeptides, planktopeptins (PP BL) have been isolated with strong elastase and chymotrypsin inhibitory activity (Grach-Pogrebinsky et al., 2003).

Here, we report on three cyclic cyanopeptide representatives isolated from a *P. rubescens* bloom – one depsipeptide planktopeptin BL1125 (PP BL1125) and two very common ureido linkage possessing cyclic peptides, AnP B and AnP F (Figure 1). We studied these with the aim of (a) thoroughly characterising their inhibitory activities against the broad spectrum of peptidases that are physiologically relevant for inflammatory diseases and cancer development, and (b) investigating their influence on the metabolism of one normal (normal human astrocytes, NHA) and two tumour cell lines [human glioblastoma (U87) and human large cell lung carcinoma (LCLC 103H)].

Results

Preliminary screening for peptide inhibitory activity

Inhibitory activities of PP BL1125, AnP B and AnP F were screened spectrophotometrically or spectrofluorimetri-

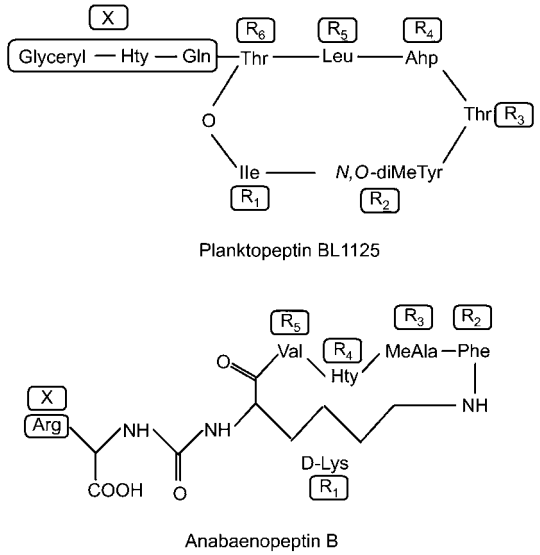

Figure 1 Schematic structures of non-hepatotoxic cyclic peptides from the cyanobacteria *Planktothrix rubescens*.
PP BL1125 is a 19-membered depsipeptide with a 3-amino-6-hydroxy-2-piperidon (Ahp) unit (redrawn following Grach-Pogrebinsky et al., 2003). PP BL1061: X=Glyceryl-Leu-Gln. Scyptolin A: X=But-Ala-Thr, position R_1=cmTyr (3′-chloro-N-methyl-Tyr), position R_2=Val. Anabaenopeptin is an ureido linkage possessing cyclic cyanopeptide (redrawn following Fujii et al., 2000). AnP B: position R_5=Val, AnP F: position R_5=*allo*-Ile. Oscillamide Y: X=Tyr, position R_5=*allo*-Ile. X indicates commonly variable positions.

cally with cyclic peptides added in excess. Human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE) were inhibited by all three cyanopeptides. PP BL1125 strongly inhibited chymotrypsin, whereas neither AnP was active. Trypsin, kallikrein 1 and urokinase and cysteine cathepsins B, K, L and S were also not inhibited by the cyanopeptides.

Kinetic measurements

The mechanism of HLE inhibition by PP BL1125 was investigated using the specific velocity plot [Baici, 1981; Eq. (1)]. The primary plot produced a series of lines running towards the bottom right of the plot and intersecting near abscissa values of 1, which is consistent with the behaviour of pure competitive inhibitors (Figure 2).

The inhibition profiles of HLE and PPE by the inhibitors are shown in Figure 3 and the corresponding K_i values are given in each plot. PP BL1125 bound tightly to both elastases, and therefore experimental points were fitted with the model function for linear competitive tight-binding inhibition [Eq. (2)] and the calculated K_i values were in the nM range. PP BL1125 also bound tightly to chymotrypsin [K_i value (6.1±1.1) nM, plot not shown], which was not inhibited by AnPs.

The AnPs inhibited HLE and PPE, but not chymotrypsin, and were much weaker inhibitors than PP BL1125 (Figure 3). The inhibition profiles of HLE were fitted with the equation for linear competitive inhibition [Eq. (3)] and the calculated K_i values were in the 0.1–1 μM range. The inhibition profiles of PPE had sigmoid shapes, which were best fitted with a model function describing the binding of two inhibitor molecules to the enzyme [Eq. (4)]. The sigmoid shapes of reaction rate versus inhibitor concentration shown in Figure 3E,F points to a deviation from conventional competitive inhibition. Binding of an additional peptide substrate molecule besides the canonical active-site binding is a documented phenomenon for porcine pancreatic elastase. This results in non-Michaelian kinetics and substrate activation (Bieth and Wermuth, 1973; Bieth et al., 1989). The peptidic nature of our inhibitors supports the notion that, besides binding to the active site of porcine pancreatic elastase, a second inhibitor molecule can bind to an additional site and produce sigmoid inhibition profiles. The K_i values for the binding of the first inhibitor molecule ($K_{i,1}$) were in the 1–2 μM range, whereas the K_i values for the second inhibitor molecule ($K_{i,2}$) were approximately 50-fold higher.

Inhibition of elastinolysis by PP BL1125

The inhibitory properties of PP BL1125 in the presence of a natural substrate were determined against the elas-

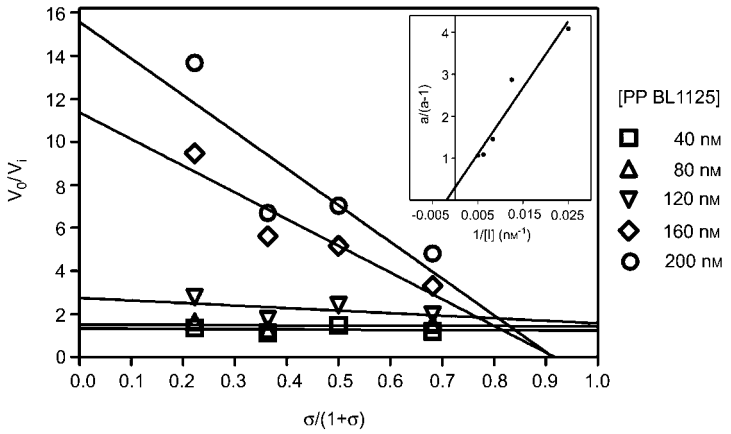

Figure 2 Inhibition of HLE by PP BL1125 analysed with the specific velocity plot.
Data were recorded at five different inhibitor concentrations (40 nM, 80 nM, 120 nM, 160 nM and 200 nM) and four different reporter substrate concentrations (5 μM, 10 μM, 15 μM and 30 μM N-MeOSuc-AAPV-pNA) and then plotted as ν_0/ν_i vs. $\sigma/(1+\sigma)$ as described by Baici (1981). Data were collected spectrophotometrically at 405 nm. The replot of $a/(a-1)$ vs. $1/[I]$, where a denotes the intersection point with the ordinate axis of a line corresponding to inhibitor concentration I, is shown in the insert.

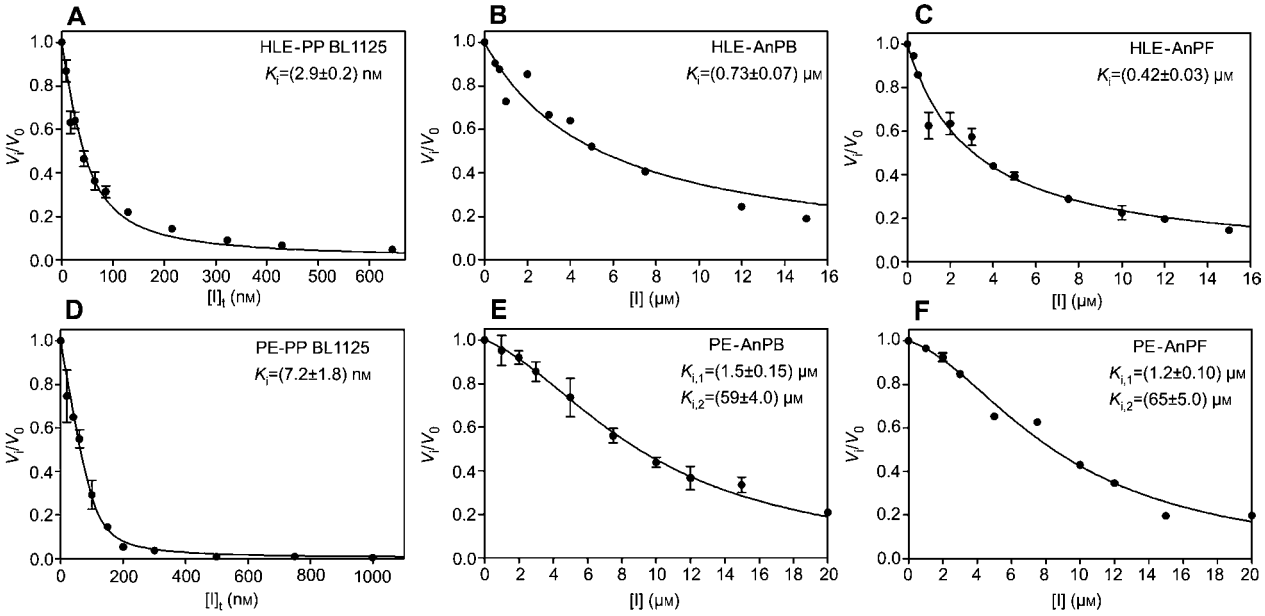

Figure 3 Inhibition profiles of HLE and PPE by PP BL1125, AnP B and AnP F.
Experimental data for PP BL were fitted with Eq. (2) (linear competitive tight-binding inhibition), the inhibition of HLE by AnPs was fitted with Eq. (3) (linear competitive inhibition) and the inhibition of PPE by AnPs was fitted with Eq. (4) (binding of two inhibitor molecules to two sites on the enzyme). Data were collected spectrophotometrically at 405 nm using the substrates N-MeOSuc-AAPV-pNA for HLE and N-Suc-AAA-pNA for PPE, respectively.

tinolytic activity of HLE. Elastolytic activity was reduced by ~60% at an inhibitor/enzyme molar ratio of 1:1 and by ~90% at a molar ratio 5:1 (Figure 4). Pre-incubation of the enzyme with elastin did not affect the inhibitory activity of PP BL1125.

Cytotoxicity determination (MTT assay)

The MTT assay detects the reduction of MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenase, to the blue formazan (Mosmann, 1983), which reflects the normal functioning of mitochondrial and cell viability (Lau et al., 2004). It is widely used for analysing cytotoxicity, cell viability and proliferation of living cells (Mosmann, 1983). We used MTT to investigate the influence of PP BL1125, AnP B and AnP F on the growth characteristic of selected cell lines.

After prolonged exposure, all the cyclic peptides, in a concentration range from 0.1 to 10 μM, had a significant influence on the metabolic activity of normal NHA cells. PP BL1125 decreased the metabolic activity of NHA cells significantly ($p<0.05$) after 72 h at the highest concentration (10 μM) and after 96 h exposure at all three concentrations. AnPs caused a significant decrease of metabolic activity after 48 h exposure. Besides the strong influence after 72 and 96 h, AnP B significantly decreased metabolic activity of normal NHA cells after 48 h at the maximal concentration (10 μM). AnP F showed the strongest influence on cell metabolism. Decreased metabolic activity was observed after 48 h at all three concentrations. No cytotoxic effects of PP BL1125, AnP B or AnP F were observed within the first 24 h (Figure 5). Under the same experimental conditions, cyclic peptides did not affect the metabolism of tumour cell lines U87 and LCLC 103H (data not shown).

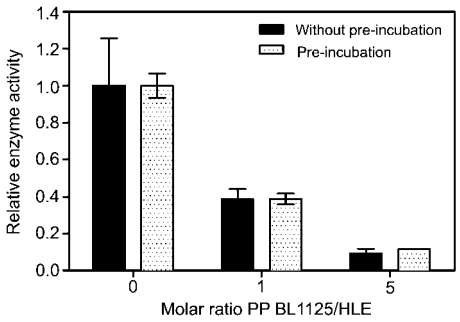

Figure 4 The influence of enzyme-substrate pre-incubation on the inhibitory activity of PP BL1125.
Human leukocyte elastase inhibition with PP BL1125 was measured at different molar ratios using pre-incubation of natural elastin as substrate.

Discussion

PP BL1125 has been confirmed as being among the most potent Ahp containing elastase and chymotrypsin inhibitors. We found out that PP BL1125 is highly selective for the serine peptidases PPE, HLE and chymotrypsin, whereas other serine peptidases, trypsin and trypsin-like enzymes, urokinase and kallikrein 1 as well as cysteine peptidases cathepsins B, K, L and S were not inhibited. The inhibition constant (K_i) values for both elastases and chymotrypsin are in the nM range. PP BL1125 is a linear competitive tight-binding inhibitor of HLE and is effective in inhibiting the cleavage not only of synthetic substrate but also of elastin of natural provenance. This is in contrast to other natural macromolecular

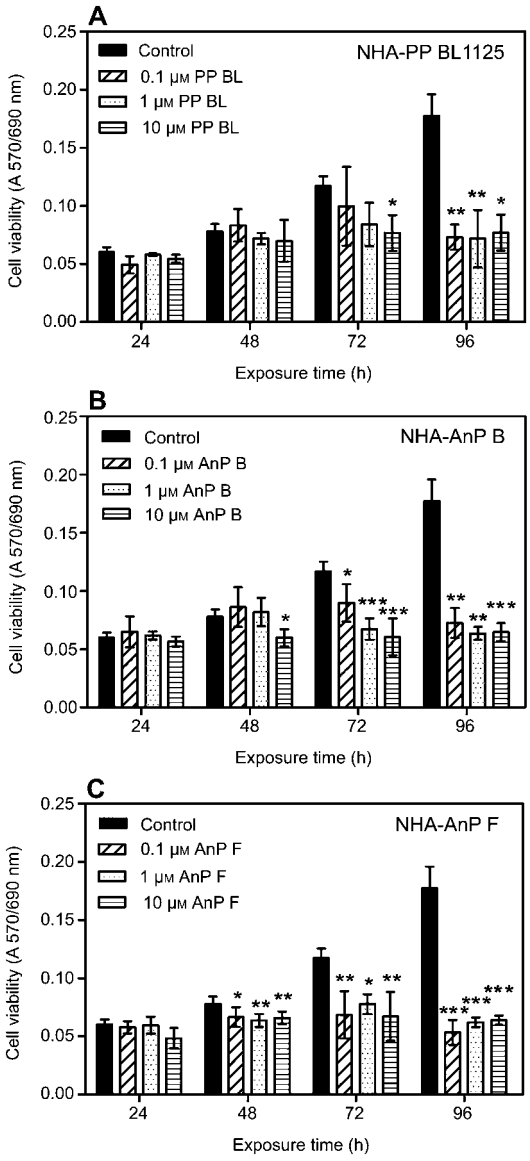

Figure 5 The influence of PP BL1125 (A), AnP B (B) and AnP F (C) on the metabolic activity of normal human astrocytes (NHA).

The influence was tested using the MTT assay (24, 48, 72 and 96 h). Results are presented as mean absorbance (A 570/690 nm) obtained from five measurements of a particular experiment. The statistical significances of the differences between controls and treated cells at a particular exposure time were tested with one-way analysis of variance followed by the Bonferroni multiple comparison test. The asterisks denote statistical significance (at $p<0.05$).

inhibitors, which do not reduce elastinolytic activity, due to their large molecular mass and the tight bond of the enzyme to the substrate (Baici, 1990; Novinec et al., 2007). PP BL1125, being only 1125 Da, effectively inhibited the elastinolytic activity even when the enzyme has been allowed to form a tight bond complex with elastin. The virtually identical inhibition constants for all three enzymes indicate that PP BL1125 does not discriminate between them. Related compounds, such as scyptolin A, have been shown to bind directly into the active centre of the target peptidase in a substrate-like manner (Matern et al., 2003). This is consistent with the inhibitory mechanism observed for PP BL1125 as well as its indiscriminate inhibition of different serine peptidases with chymotrypsin-like specificity, while showing no inhibitory activity against trypsin-like enzymes. The only cyclic depsipeptide produced in cyanobacteria that exhibits a comparable degree of chymotrypsin inhibition is the phosphate containing micropeptin T-20 (Okano et al., 1999). All other members of the oscillapeptin group inhibit chymotrypsin only at significantly higher concentrations (Grach-Pogrebinsky et al., 2003), whereas some micropeptins do not inhibit chymotrypsin at all (Reshef and Carmeli, 2001).

Considering the structure-function relationship of cyanodepsipeptides, lipophilic amino acids appear to be relevant in selecting for inhibition of chymotrypsin and related serine peptidases. Furthermore, the flexible side chain composition of PP BL1125 and PP BL1061 show relative selectivity for elastase and chymotrypsin (Grach-Pogrebinsky et al., 2003). The crystal structure of PPE in complex with the inhibitor scyptolin A revealed how the cyanodepsipeptide inhibitor uses its four N-terminal amino acid residues to bind specifically to PPE. The rigid ring moiety prevents hydrolysis by excluding the water from the active centre (Matern et al., 2003). PP BL1125 is an analogue of scyptolin A and the superposition shows nearly identical conformations of the 19-membered ring. Moreover, two of the four amino acid residues that scyptolin A uses to bind at S sub-sites of PPE are identical in PP BL1125 (Matern et al., 2003). Similarly to PP BL1125 nostopeptins A and B, both possessing the Leu-Ahp motif, are also potent elastase and chymotrypsin inhibitors (Okino et al., 1997). The very absence of the Leu-Ahp motif in AnPs might be the reason for their inability of chymotrypsin inhibition.

The first biological activity that was attributed to AnPs was weak relaxation of norepinephrine induced constriction of rat aortic preparations. No animal toxicity and no inhibitory activity of protein phosphatases was detected (Harada et al., 1995). Also, peptidase inhibition has not been reported, with the exception of strong inhibition of carboxypeptidase A with AnP G, AnP H and AnP T, but not with AnP B, AnP E and AnP F (Itou et al., 1999). The inhibition of a serine peptidase by AnP B and AnP F was first demonstrated in our laboratories (Sedmak et al., 2008b), and here we confirm and characterise the inhibitory activity of both AnPs against HLE and PPE. A possible contamination of the AnP preparations with the highly active PP BL1125 is excluded, as AnPs show no chymotrypsin inhibition activity at all. Moreover, the same results were obtained with AnP preparations, using two different methods of isolation (Grach-Pogrebinsky et al., 2003; Sedmak et al., 2008b). We therefore conclude that the AnPs known so far, including the closely structurally related oscillamide Y, cannot inhibit chymotrypsin and trypsin-like enzymes (Marsh and Bradley, 1997; Fujii et al., 2000), but are able to inhibit elastase. AnPs B and F are both linear competitive inhibitors of HLE, while binding of two inhibitor molecules to the enzyme was observed in the case of PPE, suggesting two binding sites. Similarly to scyptolins and PP BLs, AnPs might also prevent the hydrolytic attack by covering the active centre with the rigid ring structure (Matern et al., 2003). The shape and size together with the rigid ring structure

given by the presence of D-Lys make AnPs elastase inhibitors.

Strong binding and selectivity classify PP BL1125 and AnPs as valuable tools in the studies of inflammation and inflammatory cancers. Depsipeptides have even been suggested as anticancer agents (Ballard et al., 2002) and it was therefore crucial to test their cytotoxicity on normal and tumour cells. We observed a significant influence on the metabolism of NHAs after prolonged exposure to cyanopeptides, the strongest being with AnP F, followed by AnP B, although they are much weaker peptidase inhibitors than PP BL1125. These results strongly suggest that there is no relation between peptidase inhibition and growth inhibition. The mechanism could be related to a cytostatic activity, similar to that of another cyclic depsipeptide dolastatin 13, which has already been described as a strong cytostatic agent (Petit et al., 1989). The growth inhibition could be due to the protein phosphatase inhibition already demonstrated for cyanodepsipeptides and for cyclic peptides of the AnP family (Sano et al., 2001; Repka et al., 2004). This urges reconsideration of the term 'non-toxic' cyclic peptides, and we propose to designate them non-hepatotoxic cyclic peptides.

Furthermore, we were interested if the cyanopeptides influence cell metabolism in cancer cells of the same histological origin, as this would make the peptides potential anticancer candidates. However, human astrocytic glioblastoma cells (U87) and the malignant lung carcinoma cell line (LCLC 103H) were more resistant to cyclic peptide cytotoxicity up to a final concentration of 10 μM compared to normal astrocytes (data not shown). This is similar to the observations on mice melanoma cell line (B16), which was unaffected by the presence of all the cyanopeptides in final concentrations as high as 100 μM (Sedmak et al., 2008b). This is not unexpected, as cancer lines are generally more resistant to cytostatic and cytotoxic agents.

It is conceivable that cyanobacteria produce the unusual peptide metabolites to regulate their life cycle and to defend their ecological niche, respectively. Therefore, the natural role of non-hepatotoxic cyanopeptides might first of all be the regulation of cyanobacterial adaptive responses (for a review see Mann, 2000). Adaptation to the ever changing environment is the fundament of cyanobacterial evolutionary success and ATP-dependent Clp serine peptidases play a key role in this process. Non-hepatotoxic cyanopeptides might be those that help the regulation of housekeeping enzymes in producing cyanobacteria (Clarke, 1999; Stanne et al., 2007) and at the same time exert negative selective pressure on other organisms by interfering with their normal cell division, growth and viability. Therefore, further research will be directed to reveal possible cyanopeptides inhibition of Clp serine peptidases.

Conclusions

The study of PP BL1125, AnP B and AnP F inhibitory activity is the first thorough characterisation of peptidase inhibitors from cyanobacteria. The depsipeptide PP BL1125 is one of the most potent elastase and chymotrypsin inhibitors within the cyanopeptide family. AnPs B and F are highly selective elastase inhibitors, with no activity towards chymotrypsin and trypsin. Representatives of both cyanopeptide groups of the cyclic peptides possessing the ureido group, the AnPs and the depsipeptide PP BL1125, are all cytotoxic to normal cell lines after prolonged exposure, presumably due to their cytostatic activity.

Materials and methods

Inhibitors

Cyanopeptides PP BL1125, AnP B and AnP F were isolated from bloom forming cyanobacteria *P. rubescens* using reverse phase extraction, as described previously (Sedmak et al., 2008b). Stock solutions (1 mM) in 1% methanol were prepared for each cyanopeptide. For comparison, pure non-hepatotoxic cyclic peptides derived from the methanol/water extraction method were also used (Grach-Pogrebinsky et al., 2003).

Cell lines

Human glioblastoma cells U87 (Cambrex, East Rutherford, NJ, USA) were grown and maintained in monolayer culture in minimal essential medium (PAA, Paching, Austria), supplemented with 10% foetal bovine serum (FBS, Euroclone, Siziano, Italy), 1% non-essential amino acids (Sigma, St. Louis, MO, USA), 1% penicillin/streptomycin (Euroclone) and 4 mM L-glutamine (L-Gln, Euroclone). Normal human astrocytes (NHA, ATTC, Wesel, Germany) were grown and maintained in monolayer culture in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-Gln. Human large cell lung carcinoma LCLC 103H (German Cancer Research Centre, Heidelberg, Germany) were grown and maintained in monolayer culture in RPMI-1640 medium (ATTC), supplemented with 10% FBS and 1% penicillin/streptomycin.

All cell lines were plated on T-75 Cell Culture Flask (Corning, Inc., Corning, NY, USA) and incubated in a standard CO_2 incubator at 37°C and in 5% CO_2 atmosphere (Kambič, Semič, Slovenia). At 80% confluence, cells were trypsinised (0.25% trypsin, 0.2% EDTA), stained with Trypan blue and counted with a haemocytometer.

Enzyme assays

Assays with HLE (Elastin Product Company, Inc., Owensville, MO, USA) were performed in 0.05 M sodium phosphate buffer, pH 7.4, using the chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (N-MeOSuc-AAPV-pNA) (Elastin Product Company), while assays with PPE (Serva, Heidelberg, Germany) were performed in 0.05 M sodium phosphate buffer, pH 8.5, using the chromogenic substrate N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (N-Suc-AAA-pNA) (Sigma). Both elastases were first dissolved in 0.02 M sodium acetate buffer, pH 5.0. Chymotrypsin (Serva) was assayed in 0.05 M sodium phosphate buffer, pH 7.8, using the chromogenic substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) (Bachem, Bubendorf, Switzerland). Trypsin was assayed in 0.05 M sodium phosphate buffer, pH 7.4, using the chromogenic substrate benzyloxycarbonyl-Phe-Arg-*p*-nitroanilide (Z-FR-pNA) (Bachem). Final enzyme concentrations used in our experiments were 43 nM for HLE, 100 nM for PPE, 42 nM for chymotrypsin and 400 nM for trypsin. Kinetic measurements of HLE, PPE, chymotrypsin and trypsin were carried out with a Cary 50 Bio UV-VIS spectrophotometer (Varian, Palo Alto, CA, USA) at 25°C.

Urokinase (Leo Pharmaceutical Products, Ballerup, Denmark) and kallikrein from porcine pancreas (kallikrein 1, Sigma) were assayed in 0.05 M sodium phosphate buffer, pH 7.4, using the fluorogenic substrate H-Pro-Phe-Arg-7-amino-4-methylcoumarine (H-PFR-AMC) (Bachem). Final enzyme concentrations in the assays were 10 PU [Ploug unit, unit of urokinase activity equivalent to approximately 1.4 CTA (Committee on Thrombolytic Agents)] for urokinase and 0.25 nM for kallikrein 1.

Recombinant cathepsins B, K, L and S were produced in *Escherichia coli* according to previously published procedures (Dolinar et al., 1995; Kopitar et al., 1996; D'Alessio et al., 1999). Assays with cathepsins B, K, L in S were performed in 0.05 M sodium phosphate buffer, pH 6.5, supplemented with 2.5 mM dithiothreitol (DTT) and 2.5 mM EDTA, using the fluorogenic substrate benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarine (Z-FR-AMC) (Bachem). Final enzyme concentrations were 0.2 nM for cathepsin B, 0.5 nM for cathepsin K, 0.1 nM for cathepsin L and 2.5 nM for cathepsin S. Kinetic measurements were carried out with an LS50B fluorescence spectrophotometer (Perkin Elmer, Waltham, MA, USA) at 25°C.

To test the inhibitory activities of three cyanopeptides, mixtures of appropriate substrate and buffer were prepared and cyanopeptides were added in excess. The reactions were started by addition of the enzyme and progress was monitored continuously for 3 min spectrophotometrically at 405 nm for HLE, PPE, chymotrypsin, trypsin and spectrofluorometrically at excitation wavelength (λ_{ex}) 370 nm and emission wavelength (λ_{em}) 460 nm for urokinase, kallikrein 1 and cathepsins B, K, L and S. When inhibition was observed, additional experiments to determine the kinetic constants were performed.

Kinetic analysis of inhibition mechanisms

The mechanism of HLE inhibition by PP BL1125 was analysed with the specific velocity plot (1) according to Baici (1981). Mixtures of the enzyme HLE and different concentrations of cyanopeptide PP BL1125 (up to 200 nM) were prepared and incubated for 15 min. Reactions were started by addition of different concentrations of substrate (up to 150 nM) in 0.05 M sodium phosphate buffer, pH 7.4 and the progress monitored continuously for 3 min at 405 nm. Reaction rates were plotted as ν_0/ν_i versus $\sigma/(1+\sigma)$, according to the specific velocity equation:

$$\frac{\nu_0}{\nu_i} = \frac{[I]\left(\frac{1}{\alpha K_i} - \frac{1}{K_i}\right)}{1+\beta\frac{[I]}{\alpha K_i}}\,\frac{\sigma}{1+\sigma} + \frac{1+\frac{[I]}{K_i}}{1+\beta\frac{[I]}{\alpha K_i}}, \qquad (1)$$

where ν_i and ν_0 are reaction rates in the presence and absence of inhibitor, σ equals $[S]/K_m$ and α and β are dimensionless coefficients. The inhibition mechanism was analysed as originally described by Baici (1981).

All inhibition constants were determined by measuring enzyme activity in the presence of increasing concentrations of inhibitor at a fixed substrate concentration. Final enzyme concentrations in the reaction mixtures were 43 nM HLE, 100 nM PPE and 42 nM chymotrypsin. The model functions described below were then fitted to the experimental data by non-linear regression analysis using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). In the case of PP BL1125, the model function describing linear competitive tight-binding inhibition is as follows:

$$\nu_i = \frac{1}{2}\,\frac{\nu_0}{[E]_t}\left([E]_t - [I]_t - K_i(1+\sigma) + \sqrt{\left([I]_t - [E]_t - K_i(1+\sigma)\right)^2 + 4[E]_t K_i(1+\sigma)}\right), \qquad (2)$$

where $[E]_t$ and $[I]_t$ are total concentrations of enzyme and inhibitor in the reaction mixture (Henderson, 1972).

The inhibition of HLE by AnPs was fitted with the function describing linear competitive inhibition:

$$\nu_i = \frac{\nu_0(1+\sigma)}{1+\sigma+\frac{[I]}{K_i}}. \qquad (3)$$

The inhibition of PPE by AnPs was fitted with the model describing the binding of two inhibitor molecules to two sites on the enzyme (Segel, 1975):

$$\nu_i = \frac{\nu_0(1+\sigma)}{1+\sigma+\frac{2[I]}{K_i}+\frac{[I]^2}{\alpha K_i^2}}. \qquad (4)$$

Inhibition of elastinolysis

The inhibition of elastinolytic activity with PP BL1125 was measured using the modified procedure by Schwabe (1973). Elastin from bovine neck ligament (Elastin Product Company) was suspended in 50 mM sodium phosphate buffer pH 7.4 at a total concentration of 10 mg/ml. Reactions were started by addition of the enzyme HLE (34 μM). PP BL1125 was added to the mixture, either prior to the addition of the enzyme or following incubation of the enzyme with substrate (30 min). The inhibitor was added in ratios PP BL1125/HLE=1 and PP BL1125/HLE=5. Mixtures were incubated in a TS-100 Thermo shaker at 37°C for 90 min and the reactions then stopped by adding 12 μl 100% (w/v) trichloroacetic acid. Samples were incubated for 15 min on ice. The precipitated peptides were removed by centrifugation (15 min, T 4°C, 16 000 *g*) and 100 μl of each clear supernatant were added to 3 ml 0.2 M sodium borate buffer, pH 8.5, and then combined with 1 ml of a fluorescamine solution (15 mg/100 ml in acetone) under vigorous stirring. The fluorescence of the labelled peptides was measured at λ_{ex}=390 nm and λ_{em}=480 nm.

Cytotoxicity determination (MTT assay)

The MTT assay was performed in 96-well plates according to Mosmann (1983). The cells were grown as described above and harvested from a cell culture flask, counted and 3000 cells were seeded in 200 μl of appropriate medium per well of 96-well flat-bottom Costar culture plates (Corning). Cells were permitted to adhere for 5 h, and treated with three different concentrations (0.1, 1 and 10 μM) of individual cyclic peptides (PP BL1125, AnP B and AnP F) for 21, 45, 69 and 93 h. Cells that were not exposed to the cyclic peptides were used as control. Following incubation in CO_2 incubator, 20 μl of 5 mg/ml MTT were added to each well. Plates were incubated in CO_2 incubator for 3 h. The medium was removed and 200 μl DMSO/well was added to dissolve the crystals, resulting in a dark-blue solution. The amount of formazan, equivalent to the cell number, was quantified spectrophotometrically at λ 570 vs. 690 nm, using a microplate reader (Tecan, Männedorf, Austria).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows. In the MTT test, mean values of the

parallels and standard deviations of absorbance were calculated for each test and the significance level was set at $p<0.05$. A one-way analysis of variance was used to assess significant differences of biochemical data among groups and the Bonferroni multiple comparison test to compare data between groups.

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