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Grassypeptolides As Natural Inhibitors of Dipeptidyl Peptidase 8 and T-Cell Activation

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Abstract

Natural products made by marine cyanobacteria are often highly modified peptides and depsipeptides that have the potential to act as inhibitors for proteases. In the interest of finding novel protease inhibition activity and selectivity grassypeptolide A (**1**) was screened against a panel of proteases and found to selectively inhibit DPP8 over DPP4. Grassypeptolides were also found to inhibit IL-2 production and proliferation in activated T-cells, consistent with a putative role of DPP8 in the immune system. These effects were also observed in Jurkat cells, and DPP activity in Jurkat cell cytosol was shown to be inhibited by grassypeptolides. *In silico* docking suggests two possible binding modes of grassypeptolides – both at the active site of DPP8 and at one of the entrances to the internal cavity. Collectively these results suggest that grassypeptolides may be useful tool compounds in the study of DPP8 function.

Keywords

grassypeptolides; natural product; protease inhibition; immune agents; dipeptidyl peptidase 8

Although originally thought to fulfil only degradative and catabolic functions, proteases are now recognized to be involved in regulation of diverse biological processes, from blood

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coagulation to immune function.^[1] Because of this, they are potentially attractive drug targets in many therapeutic areas.^[2] However, because of the ubiquitous nature of protease regulation, non-specific inhibitors could suffer from serious off-target effects. For example, the clinical development of matrix metalloprotease (MMP) inhibitors for treatment of cancer largely failed due to non-specific inhibition.^[2] Several MMPs had not been discovered at the time of the development of these agents, and there was an inadequate understanding of the importance of MMPs in pro-survival pathways.^[2] Molecular tool compounds that have differing selectivities amongst a protease family may help delineate the function of cryptic members, and this is especially needed for enzymes of unknown function that are related to drug targets. Natural products are a potential source of such tool compounds. As products of evolution, they often exhibit bioactivity against specific protein targets, and in fact account for the majority of pharmacophores of drugs in use today.^[3] Importantly, natural products generally have highly complex and densely functionalized structures that differ significantly from the results of human rational drug design.

In our own efforts, we have identified several natural products that inhibit proteases. Free-living marine cyanobacteria have proved to be a prolific source of modified peptides, which are presumably produced as a form of chemical defence.^[4] We have found two groups of natural protease inhibitors, lyngbyastatins 8–10^[5] and symplostatin 5^[6] that potently inhibit the serine protease elastase, and the grassystatins,^[7] which preferentially inhibit the aspartic protease cathepsin E. We have also designed synthetic analogues of tasiamide B^[8,9] that inhibit another aspartic protease, BACE1, which is implicated in Alzheimer's disease. Another group of compounds that we have isolated are grassypeptolides A–C (**1–3**, Scheme 1), bis-thiazoline containing cyclic depsipeptides from samples of *Lyngbya confervoides*.^[10,11] Previously, **1–3** were found to be cytotoxic to a variety of transformed human cancer cell lines, with **3** being the most potent. Our total synthesis of grassypeptolide A (**1**) confirmed the structure.^[12] Synthetic material was used to demonstrate that **1** downregulates cyclin D and upregulates p27 and p21 to modulate cell cycle distribution, and that the compound increases PARP cleavage and decreases BCL-2 and BCL-xL expression, correlating with the pro-apoptotic activity of **1**.^[12] Additional grassypeptolides were discovered by other groups,^[13,14] all of which show some degree of toxicity, and analogs with 28S configuration such as **3** show the greatest potency.

Prompted by findings for similar cyclic peptides,^[15,16] we investigated the metal binding of **1** and **3** and found that they bind to Cu²⁺ and Zn²⁺ ions.^[11] Both of these metals are known to play crucial roles in the mechanism of certain enzymes, for example the MMPs and CuZn-superoxide dismutase. In the present work, we therefore decided to screen grassypeptolide A (**1**, the most abundant natural product of the series) against a representative panel of proteases (Figure 1). However, except for a weak effect on MMP13, we did not see inhibition of any metalloprotease in the panel. Instead, the strongest hits were the cysteine protease cathepsin L, the serine protease activated protein C and the dipeptidyl peptidase DPP8, which were inhibited to 6%, 14% and 23% residual activity, respectively, compared to solvent control at a screening concentration of 20 μ M.

We investigated the three hits further by determining the IC₅₀ of inhibition for grassypeptolides A–C (**1–3**, see Table 1). For all compounds the IC₅₀ values fell into the

order DPP8 < Cat L < APC. Importantly, all three compounds showed selectivity for DPP8 over DPP4, ranging from 9.9 fold (**2**) to 38.2 fold (**3**). The selectivity is less than that of some isoindoline *N*-capped amino acid-derived DPP8/9 inhibitors,^[17–19]. The most potent of these synthetic inhibitors has an IC₅₀ of 8.8 nM against DPP8 and a selectivity of over 11,000 for DPP8 vs. DPP4,^[19] but the grassypeptolides represent a novel structural class of DPP inhibitors. We previously found **3** to be more cytotoxic than either **1** or **2**. Since **3** has similar inhibitory potency against DPP8 versus **1** and **2**, this activity is likely not responsible for toxicity in the tested cancer cell lines.

DPP8 is closely related to DPP4, a validated target in the treatment of type 2 diabetes.^[20] A handful of DPP4 inhibitors are currently marketed for this indication, and several more are in development.^[20] Inhibition of DPP4 leads to an increase in one of its substrates, GLP-1, which in turn stimulates insulin production and inhibits glucagon release, thus lowering blood glucose.^[20] Both DPP8 and DPP9 are widely expressed in different tissues,^[21] but the functions of these two enzymes have not been delineated. Some evidence implicates roles in the immune system for both DPP8 and DPP9. For example, both enzymes are upregulated in a rat model of asthma, while expression levels of DPP4 are unchanged.^[22] DPP9 has also been implicated in the general turnover of proline-containing peptides, potentially exerting control over which cytosolic peptides are presented as antigens by MHC class I complexes.^[23] The first known natural substrate of DPP9 was the antigenic peptide RU1_{34–42} – in this case action of DPP9 reduces presentation of the mature epitope.^[23] There remains little direct evidence of DPP8/9 involvement in immune system function, and recent work implicates DPP8/9 in other processes, such as epidermal growth factor signaling^[24] and energy homeostasis.^[25] Thus additional selective inhibitors that could be used as tool compounds may be useful for further study of these enzymes.

Because DPP8 has been implicated in immune system functions, we investigated the effect of grassypeptolide A (**1**) on the activation of T-cells by exogenous anti-CD3 and anti-CD28. These antibodies simulate co-stimulation of the T-cell receptor (which includes CD3 as a subunit) and CD28, leading to T-cell activation. We found that **1** was able to reduce IL-2 production, one of the hallmarks of T-cell activation (see Figure 2A). We also found a reduction in the stimulated T-cell proliferation seen in response to anti-CD3/CD28 (see Figure 2B). Previously, similar results were also observed for human T-cells *in vitro* with another selective DPP8/9 inhibitor,^[26] potentially implicating DPP8 or 9 in this effect. We went on to corroborate this finding by investigating the effect of grassypeptolides B (**2**) and C (**3**) in the transformed Jurkat cell line,^[27] which has T-cell characteristics and produces IL-2 upon appropriate stimulation. We found that both compounds were able to reduce the production of IL-2 in response to dual stimulation with phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA, see Figure 3A).^[28] In this cell line we observed a less dramatic effect on proliferation (Figure 3B), indicating that reduction in IL-2 production is not a simple function of cell number.

We sought to determine whether DPP activity was indeed being compromised in Jurkat cells by treatment with **1**. We therefore prepared subcellular fractions of cells corresponding to cell membranes and cytosol, and tested the effect of **1** on the cleavage of a DPP substrate by enzymes in these fractions. DPP4 is a membrane-bound protein, whereas DPP8 and 9 are

found in the cytosol.^[29] Our results, shown in Figure 4, are consistent with a selective inhibition of DPP8/9 over DPP4, as we found **1** inhibited DPP-like activity to a greater extent in the cytosol fraction versus the membrane fraction. Concurrently, the nonselective control inhibitor P32/98 showed similar potency in both cell fractions. The potency of **1** in this assay is much less than exhibited in live Jurkat cells (Figure 3). This may reflect additional non-inhibited enzymes carrying out DPP-like activity in crude lysates, inefficient inhibition of cleavage with an artificial substrate (vide infra) or it may indicate that the reduction of IL-2 production could be mediated by another mechanism.

As **1** is a novel structural class of DPP inhibitor, we sought to rationalize its inhibitory activity on DPP8 by carrying out *in silico* molecular docking of **1** into a previously reported homology model of DPP8.^[30] Like related enzyme structures, the DPP8 homology model has a large internal cavity where the active site resides. This cavity is accessible from two openings – the main opening that is proximal to the catalytic triad (Ser739, His849, Asp817), and the narrower opening formed by the 8-bladed β -propeller domain at the opposite end of the protein. When we used the entire internal surface of DPP8 as the search space for molecular docking, it was found that **1** docked to two distinct regions (see Figure 5). Interestingly, docking to the inside surface of the propeller opening gave lower calculated binding energies compared to the other side close to the catalytic triad (–10.5 vs. –9.5 kcal/mol, respectively).

The docking pose of **1** close to the active site (pose 1) does not bring the compound into contact with the proline binding site, nor the P2 site previously proposed to be important for other classes of inhibitors.^[31] This pose is possibly stabilized by π – π interactions between the *N*-Me-Phe unit of **1** and the P1 pocket of the hydrolase domain, containing many aromatic residues (Phe651, Phe671, His736, Trp738 and Tyr860, also known as the T-stacking conserved aromatic motif,^[30] see Figure 5). Interestingly, DPP4 possesses an extra aromatic residue in this pocket – Tyr752 (Glu861 in the DPP8 homology model, see Figure S1). If pose 1 is placed in an aligned X-ray structure of DPP4 (PDB code 2BGR),^[32] it can be seen that a steric clash would occur between the *N*-Me-Phe and Tyr752. Compound **1** does dock into DPP4 at this site, but the *N*-Me-Phe is not bound so deeply into the aromatic pocket (see Figure S1). Potentially, the deeper binding of the *N*-Me-Phe side chain in the pocket could perturb the relative positions of the beta sheet and alpha helix that are attached to the active site Ser739 and His849, respectively.

Likewise, at the propeller opening site (pose 2), there are several aromatic residues that could form π – π interactions to **1** (His156, Phe437, Phe440, Phe567, see Figure 5). In DPP4 these are replaced by Ser106, Glu361, His363 and Lys463, respectively, and thus **1** docks into DPP4 at this site with a different orientation, where there is a likely edge-to-face π – π interaction with Trp215 (see Figure S1). Binding at this site would effectively block the propeller opening in both DPP4 and DPP8, and therefore might disproportionately affect cleavage of substrates that use this entrance. Since the substrate used in our enzymatic inhibition experiments (H-GP-AMC) is fairly small, inhibition by blocking the propeller opening may not have been detected. In light of the docking results, it may be that the mode of inhibition may simply be steric hindrance of the substrate around the active site, along with prevention of its entrance through the propeller opening. Both of these may be more

effective inhibition strategies against larger substrates, and this could potentially explain the fairly high IC₅₀ values when measured with a small substrate (H-GP-AMC), compared to the effect on T-cells. Some candidate substrates for DPP8 have been identified through proteomics,^[25] and all of them are proteins containing more than 200 amino acids. These larger substrates may require the propeller opening, or may be less able to displace **1** from the active site compared to a smaller substrate.

We have shown that the grassypeptolides inhibit DPP8, and molecular docking suggests that **1** may bind to the inner cavity of the enzyme at two distinct sites. Thus, the compound may prove to be a valuable tool in studying the relative importance of the propeller opening for different substrates when they are identified.

Experimental Section

Isolation of grassypeptolides

The grassypeptolides were isolated from samples of *L. confervoides* as previously described.^[11] After natural sources of grassypeptolide A were exhausted, material obtained through total synthesis^[12] was used.

Protease inhibition screen

The initial protease screen was carried out as previously described,^[7] by Reaction Biology Corp. (Malvern, PA, using 20 μ M **1**). Follow up experiments to determine the IC₅₀ values of **1–3** used the same conditions as for the screen but with a range of concentrations for the test compounds, each in duplicate.

T-Cell IL-2 and proliferation assay

Human CD3+ T-cells were purified from peripheral blood mononuclear cells of a healthy adult volunteer, using MACS Pan T-cell Isolation Kit (Miltenyi Biotech). A 96-well plate was coated with murine anti-CD3 (OKT3, 0.5 μ g/mL) and anti-CD28 (4B10, 5 μ g/mL) as previously described.^[33] T-cells were added to the pre-coated plate (1×10^5 cells/well), and incubated in serum-free AIM-V medium in the presence of different concentrations of grassypeptolide A (**1**, added as a solution in DMSO) for 48 h. Concentrations of IL-2 in the supernatants were then quantified using sandwich ELISA. To quantify proliferation, cells were grown under the same conditions for 48 h, after which time MTT dye was added to wells in order to measure relative cell number.

Jurkat IL-2 and viability assay

Jurkat cells were grown in 96-well plates (100,000 cells/well), in RPMI-1640 medium in the presence of PMA (80 nM, Sigma-Aldrich, added as a solution in DMSO), PHA (10 μ g/mL, Sigma-Aldrich, added as a solution in PBS), and varying concentrations of **2** or **3** (added as 100 $\times$ freshly prepared stock solutions in EtOH), following the procedure of Fischer et al.^[28] The DMSO content of the culture wells was kept below 0.25% as it was found to be toxic to Jurkat cells at higher levels. After 24 h incubation at 37 °C in a humidified atmosphere containing CO₂ (5%), the IL-2 content of the supernatants was quantified using a hIL-2 alphaLISA kit and EnVision detector (Perkin Elmer). To quantify viability, cells were

grown under the same conditions, and then developed with MTT dye according to the manufacturer's protocol (Promega).

Jurkat cell endogenous DPP activity assay

Jurkat cells were grown and subjected to subcellular fractionation as described by Rockstroh *et al.*^[34] Various concentrations of compound or solvent control, subcellular fractions and substrate were preincubated for 10 min at 37 °C before the absorbance at 405 nm was monitored for 5 min with a microplate reader.^[35] The slope was used to quantify enzyme activity. The amount of protein in the cytosolic/membrane fraction used for each reaction was 500 µg, obtained from 5×10^6 cells. DPP8/9 and DPP4 activity were determined separately in cytosolic and membrane fractions with the substrate Gly-Pro-pNA (0.5 mM in 0.05 M Tris buffer, pH 8.3, containing 10 mM EDTA and 14 µg/mL aprotinin). Both Gly-Pro-pNA and P32/98 (positive control inhibitor) were obtained from Enzo (Farmingdale, NY).

Molecular docking

A previously determined crystal structure of compound **1**^[10] was docked into DPP4 and DPP8 using Autodock Vina 1.1.1,^[36] with an exhaustiveness value of 100, and with a search box encompassing the entire interior of the respective protein. A previously published homology model of DPP8 was used,^[30] as no crystal structure is available. The crystal structure 2BGR was used for docking to DPP4,^[32] as this structure was the basis of the DPP8 homology model. The two proteins were aligned to each other in PyMOL (Schrödinger, LLC) prior to docking in order to aid comparison between the docking poses of each. Each docking run produced 100 binding modes, which were examined in ascending order of calculated binding energy. The binding poses shown in Figure 5 are the lowest binding energy structures of the two binding sites found in the total set of binding modes.

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Figure 1. Protease panel treated with grassypeptolide A (**1**), 20 μM. Values represent % residual enzyme activity compared to solvent control and are additionally represented by a continuous color scale (0% red, 100% green).

Figure 2.

A) Effect of grassypeptolide A (**1**) on IL-2 production by T-cells in response to anti-CD3/CD28. B) Effect of grassypeptolide A (**1**) on T-cell proliferation in response to anti-CD3/CD28.

Figure 3.

A) Effect of grassypeptolides B (**2**) and C (**3**) on IL-2 production by Jurkat cells in response to PMA and PHA. B) Effect of grassypeptolides B (**2**) and C (**3**) on viability in Jurkat cells stimulated with PMA and PHA.

Figure 4. Effect of grassypeptolide A (**1**) on DPP activity in A) Jurkat cell cytosol and B) Jurkat cell membrane fractions.

Figure 5.

Two docked poses of grassypeptolide A (**1**) generated by *in silico* molecular docking into a homology model of DPP8. A) Overview of the two binding sites identified. B) Closeup of pose 1 (close to active site), showing the catalytic triad and aromatic residues close to the *N*-Me-Phe unit of **1**. C) Closeup of pose 2 (inside surface of propeller opening), showing aromatic residues close to the phenyl rings or other π bonds of **1**.

- 1** R = Et, 28R
2 R = Me, 28R
3 R = Et, 28S

Scheme 1.
Structures of grassypeptolides A–C (1–3)

Table 1

IC₅₀ values of grassypeptolides against cathepsin L, dipeptidyl peptidases and activated protein C.

Compound	Cat L	DPP4	DPP8	APC
Grassypeptolide A (1)	14.0 μ M	347 μ M	11.5 μ M	79.0 μ M
Grassypeptolide B (2)	21.3 μ M	164 μ M	16.6 μ M	66.6 μ M
Grassypeptolide C (3)	20.4 μ M	245 μ M	6.41 μ M	62.0 μ M
Control ^[a]	238 pM	155 nM	1.45 μ M	1.40 μ M

^[a] For cathepsin L, the positive control inhibitor was E64, for DPP4 and DPP8, the control inhibitor was P32/98, and for APC the positive control was gabexate mesylate.