Phytochemistry 152 (2018) 61–70

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

PepSAVI-MS reveals anticancer and antifungal cycloviolacins in *Viola odorata*

Nicole C. Parsley [a], Christine L. Kirkpatrick [a], Christopher M. Crittenden [b], Javad Ghassemi Rad [c], David W. Hoskin [c, d, e], Jennifer S. Brodbelt [b], Leslie M. Hicks [a, *]

[a] Department of Chemistry, University of North Carolina at Chapel Hill, NC, USA
[b] Department of Chemistry, University of Texas at Austin, TX, USA
[c] Department of Pathology, Dalhousie University, Nova Scotia, Canada
[d] Department of Microbiology and Immunology, Dalhousie University, Nova Scotia, Canada
[e] Department of Surgery, Dalhousie University, Nova Scotia, Canada

ARTICLE INFO

Article history:
Received 9 February 2018
Received in revised form
12 April 2018
Accepted 24 April 2018

Keywords:
Viola odorata, Violaceae
Bioactive peptides
Antimicrobial peptides
Cyclotides
Mass spectometry

ABSTRACT

Widespread resistance to antimicrobial and cancer therapeutics is evolving in every country worldwide and has a direct impact on global health, agriculture and the economy. The specificity and selectivity of bioactive peptide natural products present a possible stopgap measure to address the ongoing deficit of new therapeutic compounds. PepSAVI-MS (**S**tatistically-guided bio**A**ctive **Pep**tides prioritized **VI**a **M**ass **S**pectrometry) is an adaptable method for the analysis of natural product libraries to rapidly identify bioactive peptides. This pipeline was validated via screening of the cyclotide-rich botanical species *Viola odorata* and identification of the known antimicrobial and anticancer cyclotide cycloviolacin O2. Herein we present and validate novel bioactivities of the anthelmintic *V. odorata* cyclotide, cycloviolacin O8 (cyO8), including micromolar anticancer activity against PC-3 prostate, MDA-MB-231 breast, and OVCAR-3 ovarian cancer cell lines and antifungal activity against the agricultural pathogen *Fusarium graminearum*. A reduction/alkylation strategy in tandem with PepSAVI-MS analysis also revealed several previously uncharacterized putatively bioactive cyclotides. Downstream implementation of ultraviolet photodissociation (UVPD) tandem mass spectrometry is demonstrated for cyO8 as a method to address traditionally difficult-to-sequence cyclotide species. This work emphasizes the therapeutic and agricultural potential of natural product bioactive peptides and the necessity of developing robust analytical tools to deconvolute nature's complexity.

1. Introduction

Antimicrobial peptides (AMPs) have guided the coevolution of countless biological species over hundreds of millions of years, the arsenal of naturally-occurring molecular weapons growing in potency and complexity as organisms compete for resources and real-estate. Conserved across all domains of life, AMPs are ancient defense molecules with broad functions (Ajesh and Sreejith, 2009; Pasupuleti et al., 2012; Peschel and Sahl, 2006; Tassanakajon et al., 2015). In multicellular organisms, highly diverse ribosomally-synthesized AMPs are fundamental to the innate immune response, driving off invading bacterial, fungal, and viral pathogens, whereas prokaryotes may wield narrow-spectrum bactericidal AMPs to procure their place in ecological niches (Ganz, 2003; Kommineni et al., 2015; Loo et al., 2017; Mahlapuu et al., 2016). Acting by physical disruption of target membranes via an induced or permanent amphipathic motif (Sato and Feix, 2006; Zhang and Gallo, 2016), the mechanism of action (MOA) employed by most known AMPs has historically prevented the development of widespread AMP resistance in nature, requiring major remodeling of basic membrane structure (Peters et al., 2010). Antimicrobial peptides often exhibit broad spectrum activity across diverse phyla; however, AMPs can be highly specialized, targeting specific and unique membrane constituents (Herbel and Wink, 2016; Lee and Kim, 2015). Unlike neutrally-charged mammalian cells, both cancer and microbial cells rich with anionic molecules can be selectively targeted by AMPs with cell death caused by either

* Corresponding author. 125 South Road, Kenan Laboratories, C045, Chapel Hill, NC 27599, USA.
E-mail address: lmhicks@unc.edu (L.M. Hicks).

https://doi.org/10.1016/j.phytochem.2018.04.014
0031-9422/

membranolytic or intracellular mechanisms (Brogden, 2005; Guzman-Rodriguez et al., 2015; Lee and Kim, 2015; Mahlapuu et al., 2016; Rotem and Mor, 2009). With the recent surge of clinical multidrug-resistant pathogens and cancers, AMPs have become increasingly attractive therapeutic candidates boasting novel and diverse MOAs (Bahar and Ren, 2013; Craik et al., 2017; Mahlapuu et al., 2016).

Bioactive peptides exist in all organisms, however the abundance and diversity of these molecules is vastly underexplored as their identification in a sea of macromolecules and small molecule metabolites presents significant analytical challenges. With the implementation of high resolving power mass spectrometry, sensitive bioactivity assays, tailored and streamlined automated data processing and statistical analyses, identification of AMPs from complex natural extracts is viable via the PepSAVI-MS pipeline (Kirkpatrick et al., 2017, 2018). Following this pipeline, peptide libraries are screened for growth inhibition in adaptable, high-throughput bioactivity assays and the relative abundances of peptidyl species in each fraction are determined via mass spectrometry. Statistical analysis via an elastic net regression model ranks the most probable peptidyl species responsible for the observed bioactivity. PepSAVI-MS does not require multiple iterations of fractionation as in bioassay-guided fractionation (Britton et al., 2018), nor knowledge of existing gene clusters as in genome mining (Scheffler et al., 2013). PepSAVI-MS uniquely allows for simultaneous contributions from multiple molecular species contributing to synergistic interactions. The components of peptide libraries can be tailored readily to contain constitutive or inducible AMPs by modifying experimental conditions. While a necessary and unique benefit of PepSAVI-MS, the ability to account for synergistic effects as well as contributions from multiple peptidyl species greatly increases the number of putative bioactive targets. As such, strategies to prioritize target species or focus the analysis on a specific protein class of interest (e.g. cyclotides as presented herein) will further streamline the process of rapid bioactive peptide discovery.

Cyclotides are a class of cyclic, disulfide-rich, plant-derived peptides found primarily in the Violaceae and Rubiaceae families that boast a diverse range of innate bioactivities, from antibacterial to anticancer, anti-HIV, molluscicidal and insecticidal (Burman et al., 2010; Craik et al., 1999; Fensterseifer et al., 2015; Gerlach et al., 2010; Plan et al., 2008; Pranting et al., 2010; Wang et al., 2008) indicating a highly stable, evolvable scaffold. Approximately 400 unique cyclotide sequences have been documented; however, up to 150,000 are estimated to exist in nature (Burman et al., 2015; Gruber et al., 2008; Hellinger et al., 2015; Narayani et al., 2017). The extraordinary stability conferred by the highly complex cyclic cysteine knot (CCK) motif found in all known cyclotides makes the conserved three-dimensional structure of this peptide class an ideal scaffold for protein engineering (Camarero, 2017; White and Craik, 2016; Wong et al., 2012). Naturally-occurring and modified cyclotides have already found application in both medicine and commercial agriculture. Grafting therapeutic peptides into cyclotide scaffolds alleviates the issue of peptide instability *in vivo*, and has the potential to deliver a variety of highly specific and efficacious peptide-based therapeutics via oral administration and to intracellular targets (Henriques et al., 2015; Wong et al., 2012). Meanwhile, Sero-X, a potent bioinsecticide derived from the cyclotide-containing extract of *Clitorea ternatea*, was approved for use on cotton plants in Australia in early 2017. Highly effective at controlling insect pests, Sero-X does not harm beneficial insects such as bees and ladybugs, and exemplifies the next level of safe and effective biocontrol that can be achieved with peptide-based treatments (APVMA, 2016).

Mass spectrometry has been used to successfully identify many cyclotides, with the standard approach including pre- and post-reduction and alkylation with iodoacetamide to reveal any peaks with a mass shift of 348.16 Da (Gruber et al., 2008) and linearization using endoproteinase Glu-C via a single conserved glutamic acid residue (Poth et al., 2011), or other enzymes (Chan et al., 2013), enabling efficient fragmentation to obtain primary protein sequence, possibly in tandem with transcriptome-mining (Koehbach et al., 2013). While successful, sample loss from the numerous sample preparation steps hinders the sequence elucidation of less abundant peptidyl species. Likely, the challenges associated with purifying structurally similar cyclotides and cyclotide sequencing are why hundreds of putative cyclotide masses have been identified via reduction/alkylation methods, but only a fraction of these masses have been sequenced (Burman et al., 2015; Narayani et al., 2017). A top-down approach in which cyclotides are analyzed undigested would ameliorate sample loss issues (Mohimani et al., 2011), but has not been demonstrated for cyclotides. While the commonly used collision induced dissociation (CID) is a practical method for short, linear peptides or enzymatically-digested proteins, CID often provides insufficient fragmentation of intact cyclic peptides. Ultraviolet photodissociation (UVPD) is an emerging technique that yields highly complex but rich fragmentation data, generating several possible types of fragmentation events (e.g. *a*, *b*, *c*, *x*, *y*, and *z*) (Supplemental Fig. 1) (Shaw et al., 2013). UVPD has been used to fragment circular (stapled) peptides (Crittenden et al., 2016), but no cyclotides have been sequenced with UVPD previously.

The botanical species *Viola odorata* L. (Violaceae) abundantly produces >30 known, unique cyclotide sequences with potent and diverse bioactivities (Ireland et al., 2006), and may harbor up to 166 cyclotide species as indicated by mass shift analysis (Narayani et al., 2017). A *V. odorata* peptide library was screened against breast, prostate, and ovarian cancer cell lines for validation of the PepSAVI-MS pipeline (Kirkpatrick et al., 2017). Constituents of this complex botanical extract demonstrated substantial cancer cell cytotoxicity in all assays, particularly across cyclotide-containing library fractions. While known and novel cycloviolacin O2 (cyO2) activity was verified, our results indicated additional cyclotide species responsible for the activity observed in the cancer cell panel. Further analysis presented herein revealed another cyclotide, cycloviolacin O8 (cyO8), as a putative anticancer peptide (Fig. 1). Isolated cyO8 demonstrated micromolar bioactivities against MDA-MB-231 breast, PC-3 prostate, and OVCAR-3 ovarian cancer cell lines. Additionally, the *V. odorata* library was shown to exhibit robust activity against the filamentous fungus *Fusarium graminearum*, responsible for the devastating disease Fusarium Head Blight (FHB); however, the bioactive constituents were not identified (Kirkpatrick et al., 2017). Herein, PepSAVI-MS revealed the *F. graminearum* antifungal activity of cyO8, which was confirmed with isolated peptide. While cyO8 has known anthelmintic activity against nematode larvae (Colgrave et al., 2008), this work expands the reported bioactivities to anticancer and antifungal for this cyclotide. Additionally, a simple reduction/alkylation strategy was implemented to further refine molecular targets revealed with PepSAVI-MS, identifying several putative anticancer and antifungal cyclotides that require further molecular characterization. As these masses are low in abundance, traditional MS-based sequencing methods are not sufficient and alternative sequence elucidation techniques are required. Using cyO8 as a representative species, we show the complexity of cyclic-peptide 193 nm UVPD fragmentation and attempt to discern common cyclotide fragmentation patterns to inform application to future *de novo* sequencing algorithms.