

# Cyclotides: A Novel Type of Cytotoxic Agents

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## Abstract

**Cytotoxic activities of three naturally occurring macrocyclic peptides (cyclotides) isolated from the two violets, *Viola arvensis* Murr. and *Viola odorata* L., were investigated. A nonclonogenic fluorometric microculture assay was used to examine cytotoxicity in a panel of 10 human tumor cell lines representing defined types of cytotoxic drug resistance. Additionally, primary cultures of tumor cells from patients, and for comparison normal lymphocytes, were used to quantify cytotoxic activity. All three cyclotides, varv A, varv F, and cycloviolacin O2, exhibited strong cytotoxic activities, which varied in a dose-dependent manner. Cycloviolacin O2 was the most potent in all cell lines (IC<sub>50</sub> 0.1–0.3 μM), followed by varv A (IC<sub>50</sub> 2.7–6.35 μM) and varv F (IC<sub>50</sub> 2.6–7.4 μM), respectively. Activity profiles of the cyclotides differed significantly from those of antitumor drugs in clinical use, which may indicate a new mode of action. This, together with the exceptional chemical and biological stability of cyclotides, makes them interesting in particular for their potential as pharmacological tools and possibly as leads to antitumor agents.**

## Introduction

In a previous study, extracts of plants were fractionated and screened for cytotoxic activity,<sup>3</sup> which led to the fractionated extract from *Viola arvensis* Murr. (Violaceae) becoming a prime target for additional studies. The extract's biological activity was concentrated in two peptide-containing fractions, from which varv A and varv F, the two most abundant peptides, were isolated (1, 2). Both peptides belong to a family of macrocyclic cystine-knotted peptides, referred to as cyclotides (3), shown previously to exhibit hemolytic, antimicrobial, and antiviral properties (3–5).

Cyclotides are small globular microproteins with a unique head-to-tail cyclized backbone, which is stabilized by three disulfide bonds (3), as shown in Fig. 1. The number and positions of cysteine residues are conserved throughout the family, forming the cyclic cystine-knot motif (5) that acts as a highly stable and versatile scaffold on which the more variable loops are arranged.

Over 40 members of this rapidly growing family of peptides are described (6) and have been divided into two cyclotide subfamilies known as bracelet and Moebius (3), depending on structural conformation. Because both cyclotides varv A and varv F belong to the Moebius subfamily, also included was a cyclotide of the bracelet subfamily, isolated from *Viola odorata*, cycloviolacin O2 (3).

Our study aimed to investigate the cytotoxicity of cyclotides from the genus *Viola* and to provide a preliminary description of its modes of action. For this purpose, we used an experimental design analogous to the one published by Dhar *et al.* (7). In this design, the three cyclotides were tested for cytotoxic activity by a panel of 10 human tumor cell lines, representing selected types of drug resistance.

## Materials and Methods

**Cyclotides.** Fractionation of *Viola arvensis* for the initial screening was done according to Claeson *et al.* (1). Using adsorption chromatography on Sephadex LH-20, the active compounds were concentrated in two fractions, from which varv A and varv F were isolated using reversed-phase chromatography (2). The third cyclotide, cycloviolacin O2 described by Craik *et al.* (3), was isolated from a butanol-soluble fraction of *Viola odorata* by a combination of high-performance cation exchange and reversed-phase chromatography and then was identified using nanospray mass spectrometry. Purified cyclotides were dissolved in 10% ethanol for the cytotoxicity assay.

**Reagents.** A prepared stock solution of fluorescein diacetate (10 mg/ml in DMSO) was kept at –20°C under light-free conditions. Cell growth medium was prepared from RPMI 1640 stock supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 μg/ml streptomycin, and 60 μg/ml penicillin. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

**Human Tumor Cell Lines.** The cell line panel consisted of four sensitive parental cell lines (ccrf-cem, nci-h69, rpmi-8226/s, and u-937gtb), five drug-resistant sublines (ccrf-cem/vm-1, nci-h69ar, rpmi-8226/dox40, rpmi-8226/lr-5, and u-937vcr), and one cell line with primary resistance, achn. The cell lines and the drug resistance phenotypes are described in Table 1. The maintenance and sources of the cell lines have been described by Dhar *et al.* (7).

**Primary Human Cells.** Ovarian carcinoma cells were obtained using surgical procedures. The ovarian tissue was minced into small pieces, after which tumor cells were isolated by collagenase dispersion followed by Percoll (Amersham Pharmacia-Biotech, Uppsala, Sweden) density gradi-

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Table 1 Human tumor cell line panel representing a defined set of drug resistance types<sup>a</sup>

Cell lines, parental:resistant	Origin	Selecting agent	Resistance type
RPMI-8226/s:RPMI-8226/Dox40	Myeloma	Doxorubicin	P-gp mediated
RPMI-8226/s:RPMI-8226/LR-5	Myeloma	Melphalan	GSH associated
CCRF-CEM:CCRF-CEM/VM-1	T-cell leukaemia	Teniposide	Topo II associated
NCI-H69:NCI-H69/AR	Small cell lung cancer	Doxorubicin	MRP mediated
U-937GTB:U-937Vcr	Histiocytic lymphoma	Vincristine	Tubulin associated
ACHN	Renal adenocarcinoma	Vincristine	Primary MDR

<sup>a</sup> P-gp, P-glycoprotein-classical MDR; GSH, glutathione; Topo II, topoisomerase II-atypical MDR; MRP, MDR-associated protein; MDR, multidrug resistance.

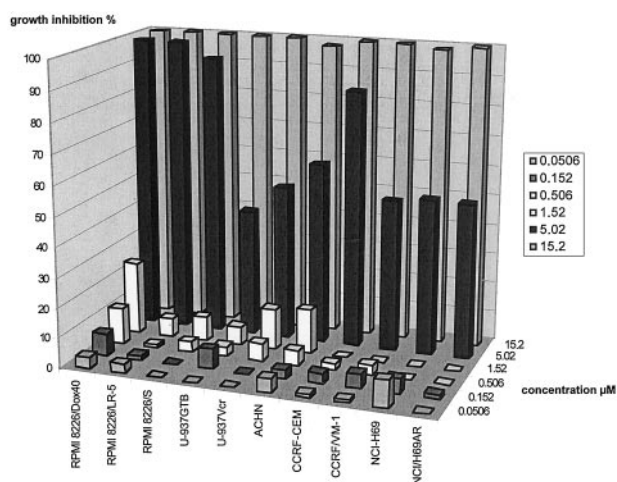


Fig. 2. Cytotoxicity of varv A at six concentrations for a panel of 10 human tumor cell lines.

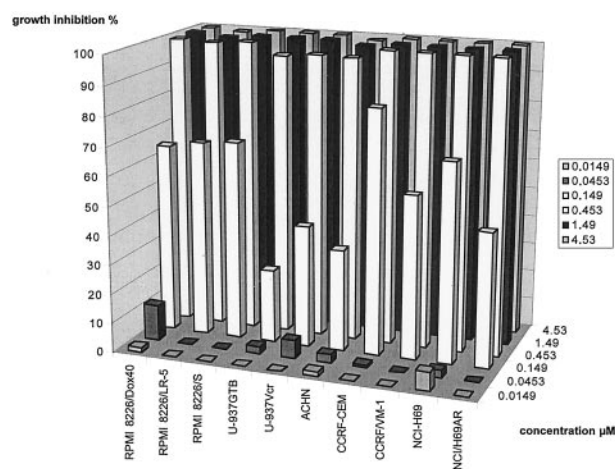


Fig. 4. Cytotoxicity of cyclotriacin O2 at six concentrations for a panel of 10 human tumor cell lines.

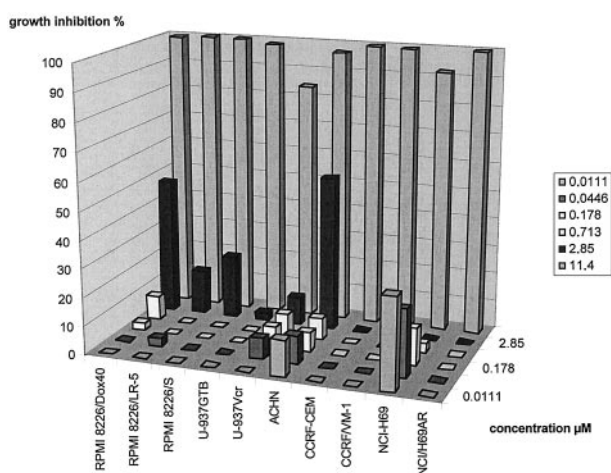


Fig. 3. Cytotoxicity of varv F at six concentrations for a panel of 10 human tumor cell lines.

have been based on their resemblance to other antimicrobial peptides and their mechanisms (4, 5, 11). One such family of polypeptides, the defensins, also share some structural properties with the cyclotides, such as size, and the organization in  $\beta$ -sheets reinforced by three disulfide bridges. Defensins are known to be distributed widely in plants, as well

Table 2 Cyclotide activity as IC<sub>50</sub> for the peptides in the cell line panel ( $\mu\text{M}$ )<sup>a</sup>

Cell line	Varv A	Varv F	Cyclotriacin O2
RPMI-8226/s	3.24	5.90	0.12
RPMI-8226/Dox40	2.73	3.14	0.12
RPMI-8226/LR-5	3.19	6.31	0.12
U-937GTB	6.35	7.07	0.26
U-937Vcr	4.84	7.45	0.20
ACHN	4.19	2.63	0.22
CCRF-CEM	3.56	7.13	0.11
CCRF-CEM/VM-1	4.97	7.15	0.14
NCI-H69	4.88	7.49	0.12
NCI-H69AR	4.89	7.12	0.26

<sup>a</sup> As the estimated IC<sub>50</sub> value is below the tested concentrations, the lowest tested concentration is used.

as in animals, where they play an important part of the innate immune system (12, 13); significantly, they have antitumor effects (14, 15) and several characteristics coinciding with observations made on the cyclotides in this study. Bateman *et al.* (15) described the effects of human neutrophil defensin HNP 1 on a number of cancer cell lines *in vitro*. Like the cyclotides, most cells were killed at concentrations between 1 and 8  $\mu\text{M}$ , and the dose-response curve showed a similar, very sharp profile.

HNP 1 has also been shown to lyse solid tumor cells from human neuroblastoma, at similar concentrations (1.7–17  $\mu\text{M}$ ;

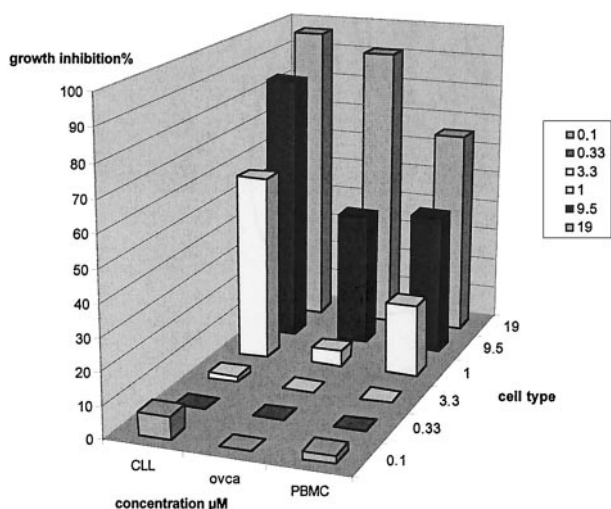


Fig. 5. Cytotoxicity of varv A at six concentrations in primary cultures of human tumor cells.

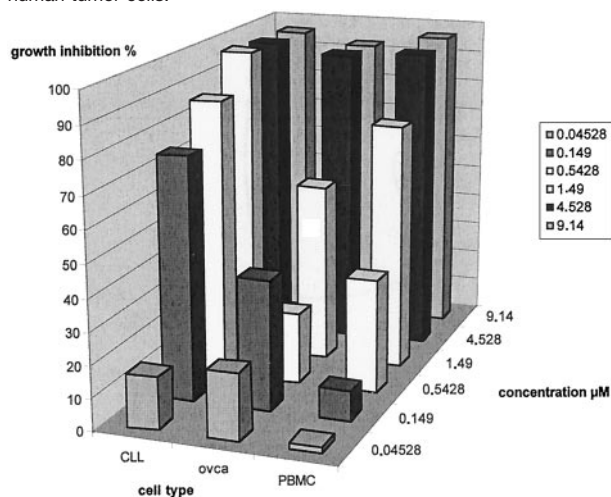


Fig. 6. Cytotoxicity of cycloviolacin O2 at six concentrations in primary cultures of human tumor cells.

Ref. 14). This is also one of the most intriguing results obtained in this study. The cyclotides killed solid cell lines (NCI-H69AR), as well as the ones known to be more sensitive (NCI-H69) with little or no difference in  $IC_{50}$ s. Defensins are known to disrupt cell membranes by forming membrane pores, acting as ion channels through the lipid bilayer (12, 16, 17). This ability is associated with their amphipathic structure, displaying distinct hydrophobic and hydrophilic surfaces. These structural properties are also shared by the cyclotides, and Tam *et al.* (4) found that the cyclotides, having more pronounced clustering of charge and hydrophobic residues, were the more potent against microbes. Moreover, in analogy, cycloviolacin O2, showing the most pronounced amphipathic sequence of the three cyclotides studied here, also proved to be the most potent (Fig. 1).

The initial interaction between cyclotides and microbial cell membranes is salt dependent, suggesting electrostatic interactions as the major driving force (4). This is a well-known

Table 3  $IC_{50}$ s in patient samples ( $\mu$ M)

Cell	Varv A	Cycloviolacin O2
Chronic lymphocytic leukemia-CLL	1.34	0.10
Ovarian carcinoma-OVCA	11.03	1.32
Healthy lymphocytes-PBMC <sup>a</sup>	12.13	0.87

<sup>a</sup> PBMC, peripheral blood mononuclear cell.

Table 4 Correlation coefficients for activity profiles (cyclotide profiles versus currently used anticancer drug profiles)<sup>a</sup>

Drug	Group	Varv A	Varv F	Cycloviolacin O2
Doxorubicin	Topo II inhibitor	0.15	-0.19	0.23
Vincristine	Tubulin-active	0.24	0.09	0.20
Cytarabine	Antimetabolite	-0.11	-0.52	0.14
Melphalan	Alkylating agent	-0.04	-0.45	0.17
Topotecan	Topo I inhibitor	0.19	-0.24	0.23

<sup>a</sup> Topo II, topoisomerase II; Topo I, topoisomerase I.

fact for the defensins, whose positive charge is proposed to regulate selectivity for bacterial membranes rich in negatively charged lipids, relative to the more neutral eukaryotic cells. Recently, Huang (18) described the lipid composition of the cell's membranes as another, equally important regulatory factor for the action of lytic and antimicrobial peptides. They showed that, in low concentrations, peptides tend to bind to the head group region of the membrane lipids in a functionally inactive state. As the peptide concentration increases above a certain threshold value, depending on the composition of lipids in the cell membrane, the peptides form the pore state lethal to the cell.

Thus, peptide selectivity may be considered as a function of differences in lipid composition of different cell membranes. The cocktail of cyclotides produced by a single plant species (2) might be a reflection of this, in which a common and well-defined scaffold is used to display variable loops targeted for a specific type of cell. The exceptional stability of the cyclotides also makes them well suited to fulfill such a task, properties that already have drawn attention to them as molecular frameworks for drug design (5).

Cyclic backbones now emerge as a widespread structural stabilizer for biologically active peptides. Cytotoxic peptides showing this distinctive character have been found in other organisms, with cyclosporin as the most well-known example. A more recent example is a larger, 21 amino acid cyclic peptide isolated from *Escherichia coli*, which interestingly, showed similarities in the three-dimensional structure with the cyclotide scaffold (19). The hitherto only known example of mammal origin is the rhesus  $\theta$  defensin 1, an 18 amino acid antimicrobial cyclic peptide isolated from rhesus macaque (*Macaca mulatta*) leukocytes. This particular polypeptide is formed by head-to-tail ligation of two truncated  $\alpha$ -defensins (20).

Here we investigated the cytotoxic properties of three cyclotides isolated from the Violaceae plant family and demonstrated that their activity profiles differ from anticancer drugs presently in use. Differences are also found between

activity profiles of, on one hand, varv A and varv F, and on the other hand, cycloviolacin O2. The reason for this is still unknown but merits additional studies. Comparison of the described activity with the structurally closely related defensin family indicates a shared mode of action, that of cytotoxicity mediated by generation of ion channels formed in cell membranes.

We have also shown that the studied cyclotides vary in potency and selectivity, which in combination with our knowledge of the vastness of the library, makes their promising potential as antitumor agents particularly interesting and in need of further exploration.

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