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Research Article

Small, novel proteins from the mistletoe *Phoradendron tomentosum* exhibit highly selective cytotoxicity to human breast cancer cells

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Abstract. Four novel proteins (phoratoxins C–F) have been isolated from the North American mistletoe *Phoradendron tomentosum*. The amino acid sequences of these phoratoxins were determined unambiguously using a combination of Edman degradation and trypsin enzymatic digestion, and by electrospray ionization tandem mass spectrometry sequencing. Phoratoxins C, E and F consist of 46 amino acid residues; and phoratoxin D of 41. All proteins had six cysteines, similar to the earlier described phoratoxins A and B, which are thionins. The cytotoxicity of each protein was evaluated in a human cell line panel that represented several cytotoxic drug-resistance mechanisms. For the half-maximal inhibitory concentrations (IC_{50} values) of the different cell lines in the panel, correlation with those of standard drugs was low. The most potent cytotoxic phoratoxin C was further tested on primary cultures of human tumor cells from patients. The solid tumor samples from breast cancer cells were 18 times more sensitive to phoratoxin C than the tested hematological tumor samples.

Key words. Breast cancer; cytotoxicity drug screening; mistletoe proteins; *Phoradendron tomentosum*; phoratoxins; thionins; tumor cell lines.

Introduction

Antimicrobial peptides are key elements in the innate host defence against infections. The mammalian, insect and plant defensins are one type of these antimicrobial peptides, 3–5 kDa in size, basic and rich in cysteine residues. Another group of small, cysteine-rich, highly basic proteins that are thought to play a role in the protection of plants against microbial infections are the thionins. Thionins are toxic to bacteria, fungi, yeast and various mammalian cell types [1, 2].

The small proteins from European mistletoe, *Viscum album* L., called viscotoxins, belong to the group of thionins, and were first isolated and sequenced by Samuelsson and co-workers [3, 4]. American mistletoe (*Phoradendron*) produces related toxins called phoratoxins or ligatoxins [5–7]. From Californian mistletoe, *Phoradendron tomentosum* (DC) Engelm. subsp. *macrophyllum* (Cockerell) Wiens [8–10], two phoratoxin variants, A and B, have hitherto been isolated and sequenced. Phoratoxins and viscotoxins are related to the family of α- and β-

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thionins, a group of highly basic, cysteine-rich proteins consisting of 45–47 amino acids found in the endosperm of several Gramineae. Those proteins have three or four disulphide bridges [2], contributing to their thermal stability [11, 12], which retain their biological activity after heating [13]. Antimicrobial activity has been demonstrated for several thionins [14–19], supporting the proposal that thionins are defence proteins [15]. Other evidence for a defence role stems from the observation that the expression of several thionins is inducible by phytopathogenic fungi [14, 20–23]. Furthermore, high expression levels of viscotoxin A3 complementary DNA (cDNA) in transgenic *Arabidopsis thaliana* have been shown to enhance resistance against the pathogen *Plasmidiophora brassicae* [24].

Viscotoxins are toxic to mammals [25] and other biological systems [26, 27]. The toxicity of viscotoxins and that of other thionins is associated with increased permeability of plasma membrane, leading to depolarization and Ca-influx, and, potentially, to cell death. These proteins have also been shown to generate reactive oxygen species (ROS) [28], and to induce expression of a mitochondrial membrane protein called Apo2.7 [29], a marker for the detection of apoptotic cells [30].

Since 1990, tens of thousands of substances have been tested in large scale for anticancer activity in the U.S. National Cancer Institute (NCI) cell line panel, consisting of 60 different human tumor cell lines, representing all major forms of human malignancies. Results reveal characteristic profiles of in vitro sensitivities for cytotoxic agents, with different modes of action [31]. Using the same general principles for data evaluation, Dhar and coworkers (1996) [32] have shown that by using a panel of only 10 human cell lines representing defined types of cytotoxic drug resistance, mechanisms of anticancer drugs can be predicted.

In the present study we have isolated and sequenced four novel phoratoxins (named phoratoxin C–F) and the previously known phoratoxin B. Using the cell-line-panel approach described above, we obtained cytotoxic activity profiles for these five phoratoxins.

Materials and methods

Plant material

Leaves from *Phoradendron tomentosum* subsp. *macrophyllum* (Cockerell) Wiens growing on *Populus fremontii* S. Wats were collected on the Sacramento River near Ria Vista, California, United States. The leaves were treated as described by Samuelsson and Ekblad in 1967 [8].

Isolation procedure

The plant material from *P. tomentosum* L. was homogenized and extracted in 2% acetic acid. The concentrated extract was evaporated in vacuo, lyophilized, and stored at –20 °C [6, 8]. The dried extract was diluted with distilled water, and the pH was set to 5.0. The solution was diluted with water until the conductivity was slightly lower than the acetate buffer. The solution was passed through a cellulose phosphate column and washed with 20 column-volumes of 0.1 M NaOAc (pH 5.0). The adsorbed substances were eluted with 0.1 M NaOAc containing 0.8 M NaCl (pH 5.0). Fractions were collected and concentrated in vacuo. To remove the salt, the fraction was purified on a Sephadex G-25 column. The high molecular fraction was collected, evaporated in vacuo, applied to a cation-exchange column (SP-Sephadex C25), and eluted with a linear gradient from 0.05 M sodium phosphate (pH 5.05) to 0.3 M sodium phosphate (pH 6.0). Fractions I–V were collected, desalted and lyophilized [10].

Preparative high-performance liquid chromatography (HPLC) was done using a Shimadzu LC10 system, equipped with a Shimadzu SPD-Mavp diode array detector. The ultraviolet (UV) absorbance was simultaneously recorded at 215 and 280 nm. Fractions were collected manually. The proteins in fraction IV (phoratoxins C and D) were isolated by repeatedly injecting portions of up to 1 mg of fraction IV on a 250 × 10 (i.d.) mm Rainin Dynamax column (C_{18} 5 µm, 300 Å), eluted by a mobile phase of 30% acetonitrile containing 0.2% trifluoroacetic acid (TFA) at a flow rate of 1.4 ml/min.

The proteins from fraction V were isolated at a flow rate of 0.8 ml/min using a gradient of 25–45% organic modifier (acetonitrile/isopropanol, 6/4) in 0.1% TFA. Phoratoxins B and E were first eluted in close proximity ~37% organic modifier, and then phoratoxin F was eluted at 38% of the organic modifier.

Reduction, alkylation and protein sequencing

The proteins were reduced, and the cysteines were alkylated with 4-vinylpyridine as previously described [33]. The reduced and alkylated proteins were desalted on a 30 × 2.1 (i.d.) mm Brownlee Aquapore column (7 µm, 300 Å) connected to an ÄKTA basic system (Amersham Pharmacia Biotech, Uppsala, Sweden) eluted at a flow rate of 50 µl/min with a 30-min linear acetonitrile gradient (10–60%) containing 0.1% TFA. The thus alkylated proteins were sequenced on a peptide sequencer (model ABI 476A; Applied Biosystems, Foster City, CA).

Mass spectrometry

Electrospray ionization tandem mass spectra were recorded with a QTOF mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray interface. The resulting data were analyzed using MassLynx software (Micromass, Manchester, UK). The spectra were averaged, baseline subtracted, smoothed and centered, or transformed using the Micromass Maximum Entropy 3 program (MaxEnt3) on the averaged spectra. The samples

were desalted essentially as described by Wilm and coworkers (1996) [34]. The spectra were recalibrated using internal trypsin fragments when possible, and the instrument was otherwise expected to be below 100 ppm in error based on measurements before and after each experiment.

Quantitative amino acid analysis

The proteins were hydrolyzed for 24 h at 110 °C with 6 N HCl containing 2 mg/ml phenol, and the hydrolysates were analyzed with an LKB model 4151 Alpha Plus amino acid analyzer using ninhydrin detection. Quantitative amino acid analysis was performed at the Amino Acid Analysis Centre, Department of Biochemistry, Uppsala University.

Cell line panel

The cells were grown in RPMI-1640 medium (HyClone) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 µg/ml streptomycin and 60 µg/ml penicillin (Sigma, St Louis, MO). Cultures were passaged twice weekly. For experimental purposes cells were harvested in log phase. The cell density was 5000/well for NCI-H69 and 20,000/well for all the other cell lines. The purified phoratoxins were tested in a standard panel consisting of 10 cell lines at six different concentrations, obtained by fivefold serial dilution, using 2 µM as the maximum concentration. After 72 h of treatment, the percentage of living cells was determined. The cell line panel, maintained as described earlier [32], represents a set of cell lines with defined mechanisms of resistance. Table 1 lists each cell line, and its origin and mechanism of resistance.

Patient samples

A total of 24 patient-tumor samples from different diagnostic groups were used to determine the activity of phoratoxin C. The primary human tumor cells were sampled from patients with the purpose to test the sensitivity profile in order to predict the most adequate therapy for the individual patient. The 15 solid and 9 hematological tumors were used to determine the concentration-response relationship for phoratoxin C. The diagnostic groups based on origin were acute lymphocytic leukemia, acute myelocytic leukemia, chronic lymphocytic leukemia, breast carcinoma, ovarian carcinoma, neuroblastoma, colon carcinoma, adenocarcinoma of the lung, abdominal mucous adenocarcinoma, non-Hodgkin's lymphoma and solid childhood tumor. The overall percentage of previously untreated patients was 54%. Four samples of normal peripheral-blood mononuclear cells (PBMCs) from healthy blood donors were compared with samples from the five patients with chronic lymphocytic leukemia.

The tumor samples were obtained by bone marrow/peripheral blood sampling, routine surgery or diagnostic biopsy. The local ethics committee at the Uppsala University Hospital approved the procedures. The leukemic cells and PBMCs were isolated from the peripheral blood by 1.077 g/ml Ficoll-Paque (Amersham Pharmacia-Biotech, Uppsala, Sweden) density gradient centrifugation [35]. Tumor tissue from solid tumor samples was minced into small pieces before the tumor cells were isolated using collagenase dispersion followed by Percoll (Amersham-Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation [36]. Cell viability was determined using the trypan blue exclusion test. The cell density was 10,000/well for solid tumors and 100,000/well for hematological tumor cells.

Table 1. The cell lines, their origins and their mechanisms of resistance.

Cell line	Origin	Mechanism of resistance [32]
RPMI 8226-S	myeloma	parental
RPMI 8226-LR5	myeloma	GSH-associated
RPMI 8226-dox40	myeloma	P-gp associated
U-937 GTB	mistiocytic lymphoma	parental
U-937 Vcr	mistiocytic lymphoma	tubulin-associated
NCI-H69	small Cell Lung Cancer	parental
NCI-H69 AR	small Cell Lung Cancer	MRP-associated
CCRF-CEM	leukemia	parental
CEM-VM-1	leukemia	topo II-associated
ACHN	renal	primary resistant

topo II, topoisomerase II; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein); GSH, glutathione.

Measurement and data analysis of cytotoxic activity

The phoratoxins were tested for cytotoxicity using the non-clonogenic 72-h fluorometric microculture cytotoxicity assay (FMCA) [37, 38]. The FMCA is based on measurement of fluorescence generated by hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes, as previously described. Each purified protein was tested in triplicates of six different concentrations, obtained by fivefold serial dilution, starting from a maximum concentration of 2 µM (purified proteins). Experiments with cell lines were repeated 2–3 times. For a successful analysis, criteria for quality included a fluorescence signal in the control wells of more than 10 times the mean blank value, and a mean coefficient of variation (CV) in the control and blank wells of less than 30%.

V-shaped 96-well microtitre plates (Nunc, Roskilde, Denmark) were prepared with 20 µl per well of test solution at 10 times the desired concentration, with the aid of a programmable pipetting robot (Pro/Pette, Perkin Elmer, Norwalk, CT, USA). The final volume in each well was always 200 µl. Each plate contained three substances, using triplicate wells for each concentration and compound, six control wells, six blank wells and three wells for each positive (0.1% Triton X-100) and negative (PBS) control. For later use, the plates were stored frozen at –70 °C for up to 3 months [35].

To measure cell survival, a survival index (SI) was defined as the ratio of fluorescence in experimental wells and that in control wells (with blank values subtracted) multiplied by 100 to reflect a percentage. The half-maximal inhibitory concentration (IC_{50} value), calculated for each compound, was defined as the concentration of the compound at SI equal to 50%. The resistance factor (RF) value was also calculated for each compound, defined as the ratio of the IC_{50} value in the resistant subline and the IC_{50} value of its (sensitive) parental cell line. The pairs of resistant/parental cell lines used for RF calculations of P-glycoprotein (P-gp), glutathione (GSH), multidrug resistance protein (MRP), topoisomerase II (topo II) and tubulin-associated resistance were RPMI 8226Dox40/RPMI 8226S, RPMI 8226LR-5/RPMI 8226S, H69AR/NCI-H69, CEM-VM-1/CCRF-CEM and U-937-Vcr /U-937-GTB, respectively [32].

A procedure similar to the COMPARE analysis described by Paull and co-workers (1989) [39] (using Pearson's correlation coefficients) was used for comparing compounds and rank-ordering them for their similarity to a 'mean' profile [40]. In addition, linear correlation of log IC_{50} values for two compounds, x and y, was determined using Excel (Microsoft).

Results

Isolation and identification of proteins from *Phoradendron tomentosum*

From the chromatographic fraction IV of an acetic acid extract of *P. tomentosum* (see 'Materials and methods') two proteins were isolated using reversed phase (RP)-chromatography and named phoratoxins C and D. The molecular weights of phoratoxins C and D were experimentally determined by mass spectrometry to be 4879.3 MH^+ and 4311.2 MH^+.

Fraction V from *P. tomentosum* was also further purified by RP chromatography. Two novel proteins named phoratoxins E and F and the previously described phoratoxin B [10] were isolated from this fraction, and the molecular weights were determined to be 4893.0 MH^+ (phoratoxin B), 4879.2 MH^+ (phoratoxin E) and 4873.2 MH^+ (phoratoxin F).

Amino acid analysis

The results from the amino acid analyses of the isolated proteins are shown in table 2. The data are presented as percent molar composition for each residue, as well as the number of residues obtained from sequencing.

Table 2. The amino acid composition and molecular weights of the phoratoxin. For each protein, the residues from amino acid analysis are listed to the left, and the residues from sequencing, to the right.

Amino acid	Phoratoxin B		Phoratoxin C		Phoratoxin D		Phoratoxin E		Phoratoxin F	
Asp/Asn (D/N)	4.0	4 (2 N)	3.2	3 (2 N)	3.1	3 (2 N)	4.18	4 (2 N)	4.4	4 (2 N)
Thr (T)	4.8	5	6.0	6	5.0	5	5.09	5	5.1	5
Ser (S)	4.9	5	5.0	5	4.2	4	5.2	5	5.2	5
Pro (P)	1.9	2	1.8	2	2.2	2	2.1	2	2.0	2
Gly (G)	5.9	6	6.3	6	5.3	5	6.4	6	5.0	5
Ala (A)	2.0	2	2.1	2	2.0	2	2.1	2	2.9	3
Cys (C)	4.8[a]	6[b]	5.8[a]	6[b]	5.2[a]	6[b]	5.2[a]	6[b]	5.2[a]	6[b]
Val (V)	–		–		–		1.1	1	–	
Ile (I)	3.5	4	3.7	4	3.6	4	2.7	3	3.8	4
Leu (L)	1.0	1	1.0	1	1.0	1	1.2	1	2.0	2
Tyr (Y)	1.0	1	1.0	1	0.9	1	1.0	1	1.0	1
Phe (F)	1.0	1	1.0	1	1.0	1	1.0	1	–	
His (H)	1.0	1	1.0	1			1.0	1	1.0	1
Lys (K)	3.9	4	4.1	4	4.2	4	4.3	4	4.2	4
Arg (R)	2.9	3	3.0	3	3.0	3	3.1	3	3.0	3
Trp (W)	1.0[c]	1	1.0[c]	1			1.0[c]	1	1.0[c]	1
No. aa's		46		46		41		46		46
Mass, native (MH^+ measured)		4893.0		4879.3		4311.2		4879.2		4873.2
Mass, native (calculated)		4892.3		4878.3		4310.1		4878.3		4872.3
Mass, alkylated (MH^+ measured)[d]		5235.4		5221.1		4653.3		5221.6		5215.8
Mass, alkylated (calculated)[e]		5234.5		5220.5		4652.2		5220.5		5214.5

[a] With a separate sample, following oxidation with performic acid, half-cystine was determined as cysteic acid.
[b] Cysteine was determined as (pyridylethyl)cysteine, following alkylation with 4-vinylpyridine.
[c] Tryptophan was determined photometrically.
[d] Measured mass of protein alkylated by iodoacetamide in excess.
[e] Calculated mass of fully C-carbamidomethylated protein.

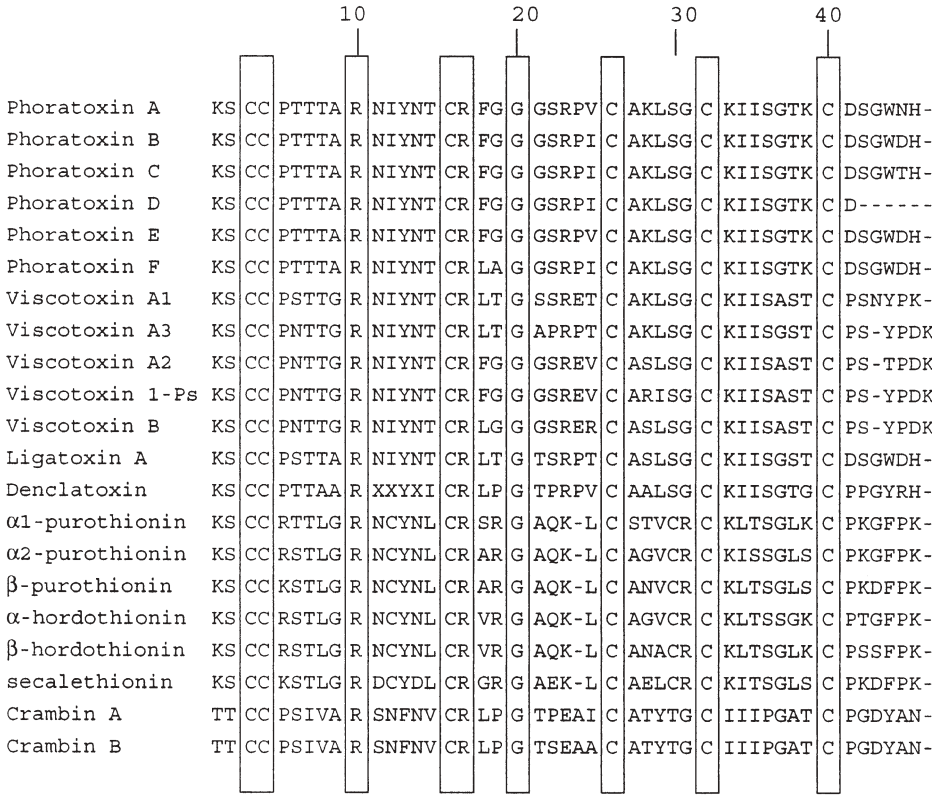

Figure 1. Alignment of phoratoxins with other thionins. Alignment of amino acid sequences of the members of the four types of thionins currently available in the literature, including the EMBL and SWISS-PROT databases. The one-letter code for amino acids is used, and the sequences were derived from phoratoxin A and B [9, 10]; viscotoxin A1, A2, A3, B and 1-Ps [58–61]; ligatoxin [7]; denclatoxin [62]; α1-, α2- and β-purothionin [63–66]; α- and β-hordothionin [67]; secalethionin [68]; crambin A and B [69, 70].

Sequencing of proteins from *P. tomentosum*

The native proteins (3 nmol) were subjected to reduction and alkylation with 4-vinylpyridine. The reaction products were sequenced by automated Edman degradation and the amino acid sequences confirmed by tandem mass spectrometry (MS/MS) sequencing. Cysteine was determined as carbamidomethyl cysteine after alkylation with iodoacetamide. The MH^+ of the carbamidomethylated proteins (table 2) exceeded that of the native proteins by 343.2 Da. This corresponds to the mass of six carbamidomethylated cysteines (57.03 Da for each derivatized cysteine), which indicates the presence of six cysteine residues in the native proteins. The sequences of the isolated proteins are shown in figure 1; and table 3 contains comparisons between molecular weights of all theoretical trypsin digests of the proteins and the observed experimental MS fragments (MH^+).

Sequencing by means of Edman degradation of the alkylated phoratoxin C yielded an N-terminal sequence of 36 residues, KSC*C*P TTTAR NIYNT C*RFGG GSRPI C*AKLS GC*KII S, where C* stands for the pyridylethylated cysteine. To gain information on the C-terminal part of the molecule, the pyridylethylated protein was subjected to digestion by trypsin (table 3) and analyzed

Table 3. Comparison between theoretical trypsin digest and the observed experimental MH^+.

Fragment[a]	Phoratoxin B		Phoratoxin C		Phoratoxin D		Phoratoxin E		Phoratoxin F	
18–28	1092.6	1093.6	1092.6	1093.6	1092.6	1093.6	1078.6	1079.6	1072.6	1073.6
2–10	939.4	940.4	939.4	940.3	939.4	940.3	939.4	940.4	939.4	940.3
11–17	883.4	884.5	883.4	884.4	883.4	884.4	883.4	884.4	883.4	884.4
40–46	819.3	820.3	805.3	806.3	805.3	–	819.3	820.3	819.3	820.3
34–39	618.4	619.4	618.4	619.4	618.4	619.4	618.4	619.4	618.4	619.4
29–33	507.3	508.3	507.3	508.3	507.3	508.3	507.3	508.3	507.3	508.3

Masses of theoretical trypsin digests of phoratoxin B–F compared with experimentally observed MS fragments. For each protein, the calculated masses are listed to the left; and the measured masses (MH^+), to the right. [a] The tryptic fragments, in order of decreasing molecular weights.

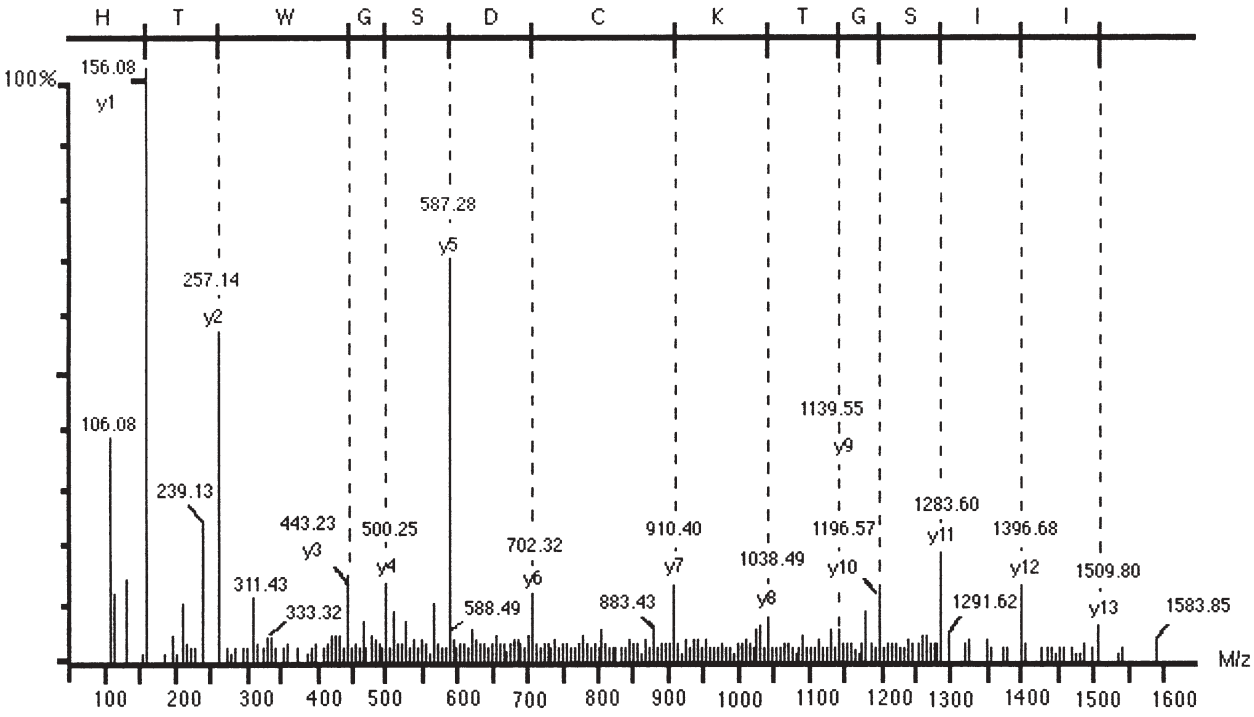

Figure 2. The partial C-terminal sequence of phoratoxin C. The C-terminal MS/MS spectrum of the $[M+4H]^{4+}$ ion at *m/z* 1378.5 of the native protein, phoratoxin C. The Y series ions Y_1 through Y_{13} are consistent with the sequence IISGTKCDSGWTH.

by MS/MS. One of the tryptic fragments gave the partial C-terminal sequence C*DSGWTH at *m/z* 455.7 $[M+2H]^{2+}$, and the native protein gave the partial C-terminal sequence IISGTKC*DSGWTH at *m/z* 1378.5 $[M+4H]^{4+}$, by MS/MS analysis (fig. 2).

Phoratoxin D yielded an N-terminal sequence of 39 residues, KSC*C*PTTAR NIYNT C*RFGG GSRPI C*AKLS GC*KII SGTK, by Edman degradation. The native protein fragment gave the partial C-terminal sequence KIISGTKC*D at *m/z* 1052.25 $[M+4H]^{4+}$, by MS/MS analysis.

The pyridylethylated phoratoxin E yielded an N-terminal sequence of 34 residues, KSC*C*P TTTAR NIYNT C*RFGG GSRPV C*AKLS GC*KI, by Edman degradation. The native protein fragments gave the sequences ISGC*K at *m/z* 1378.5 $[M+4H]^{4+}$ and, from the C-terminal sequence, gave DSGWDH at *m/z* 1378.2 $[M+4H]^{4+}$, by MS/MS analysis.

Edman degradation of phoratoxin F yielded 34 residues from the N-terminal end: KSC*C*P TTTAR NIYNT C*RLAGG GSR PIC*AK LSGC*KI. The tryptic fragments gave the sequence LSGC*KIISGTK at *m/z* 612.35 $[M+2H]^{2+}$, and MS/MS analysis of the native protein gave the C-terminal sequence DSGWDH at *m/z* 1101.8 $[M+4H]^{4+}$.

The previously known phoratoxin B was also isolated and sequenced. The pyridylethylated phoratoxin B yielded an N-terminal sequence of 39 residues: KSC*C*P TTTAR NIYNT C*RFGG GSRPI C*AKLS GC*KII SGTK. One of the tryptic fragments gave the C-terminal sequence C*DGWDH at *m/z* 462.7 $[M+2H]^{2+}$.

Alignment of phoratoxins with other thionins

For several members of the thionin group, amino acid sequences were aligned (fig. 1). The sequences are currently available in the literature, including the EMBL and SWISS-PROT databases. The primary structures of the phoratoxins share a high degree of homology with the viscotoxins and the α- and β-thionins.

Cytotoxic activity in the cell line panel

The five proteins isolated from *P. tomentosum* induced a concentration-dependent decrease in cell viability. The IC_{50} values of these isolated mistletoe toxins (table 4) ranged from 0.04 to 0.83 µM, with phoratoxin C (mean IC_{50} = 0.16 µM) being the most potent toxin, and phoratoxin F (mean IC_{50} = 0.4 µM) being the least potent. In the small-cell lung-cancer cell lines NCI-H69 and NCI-H69AR, the phoratoxins were equipotent. The proteins showed high potency towards the cell line NCI-H69, whereas the potency for MRP-expressing subline NCI-H69AR was much lower.

Correlation analysis of the phoratoxins

The isolated proteins were tested in the cell line panel and compared with respect to their cytotoxicity (table 5, part

Table 4. IC_{50} values (μM) in the cell line panel for the isolated proteins.

Cell line	PTX B	PTX C	PTX D	PTX E	PTX F
RPMI 8226-S	0.35	0.18	0.27	0.43	0.45
RPMI 8226- LR5	0.30	0.18	0.27	0.38	0.36
RPMI 8226-dox 40	0.37	0.13	0.23	0.43	0.51
U-937 GTB	0.21	0.09	0.15	0.35	0.31
U-937 Vcr	0.18	0.10	0.14	0.29	0.23
NCI-H69	0.038	0.040	0.045	0.038	0.038
NCI-H 69AR	0.23	0.23	0.24	0.26	0.30
CCRF-CEM	0.33	0.20	0.38	0.45	0.50
CEM-VM-1	0.32	0.15	0.22	0.34	0.42
ACHN	0.59	0.34	0.64	0.58	0.83
Mean	0.29	0.16	0.26	0.36	0.40

Each experiment was repeated two to three times. Before estimating the IC_{50}, all individual survival indices (%, i.e. for each drug and concentration) were pooled and averaged to get the most accurate dose-response curve. Hence the IC_{50} is determined from one dose-response curve based on two to three individual experiments. PTX, phoratoxin.

Table 5. Correlation analysis of the phoratoxins.

	R				
	PTX B	PTX C	PTX D	PTX E	PTX F
a					
PTX B	1.00	0.88	0.94	0.97	0.99
PTX C	0.88	1.00	0.96	0.82	0.87
PTX D	0.94	0.96	1.00	0.89	0.94
PTX E	0.97	0.82	0.89	1.00	0.98
PTX F	0.99	0.87	0.94	0.98	1.00
b					
Doxorubicin	0.11	0.34	0.28	–0.02	0.09
Vincristine	–0.26	0.03	–0.10	–0.41	–0.29
Cytarabine	–0.07	0.10	0.01	–0.18	–0.12
Melphalan	–0.01	0.18	0.05	–0.07	–0.05
Topotecan	0.00	0.32	0.15	–0.14	–0.04

Correlation coefficients (*R*) obtained in cell line panel, used for analyzing relationships of the log IC_{50} values of the phoratoxins (*a*) among themselves and (*b*) with standard drugs. PTX, phoratoxin.

a). The correlation (*R*) between the isolated proteins was high (0.82–0.99). The highest correlation (0.99) was between phoratoxins B and F. The standard drugs in our database, of currently more than 140 standard and experimental chemotherapeutic drugs [32], are defined representatives for well-established mechanistic classes of chemotherapeutic drugs used in the clinic. The correlation of the toxins with standard drugs (table 5, part b) was low ($R < 0.35$). Table 6 presents in rank order a list of the 10 highest correlations of cytotoxic standard drugs and experimental agents with known targets previously tested in the cell line panel. Surprisingly, digitoxin had overall the highest correlation to phoratoxins.

Resistance factors in the mechanism-based cell line evaluation

The RF values were calculated for the P-gp, topo II, MRP, GSH and tubulin resistance mechanisms (table 7). The isolated phoratoxins showed the highest RF values (ranging from 5.3 to 7.8) for the MRP-associated mechanism. The overall low RF values for the other resistance mechanisms examined (RF < 3) indicate minimal dependence on these mechanisms.

Cytotoxic activity on primary tumor cells from patients

The activity of phoratoxin C was further characterized in 24 human tumor samples. The IC_{50} values of phoratoxin C (table 8) ranged from 87 nM to 2.1 μM. Interestingly, phoratoxin C had the highest activity in the four breast carcinoma samples, with an IC_{50} value of 87 nM. Phoratoxin C was generally more toxic to the solid tumor samples relative to hematological tumor samples, shown in table 8 by the ratio H/S of the mean IC_{50} value for the hematological tumors and the mean for the solid tumors (H/S = 3.5). Phoratoxin C was pronouncedly selectively toxic to the solid tumor samples from breast carcinoma (BC) relative to the hematological (CLL) tumor samples

Table 6. Ranking of the 10 highest correlation coefficients (R) among all drugs and investigational agents tested in the cell line panel.

Rank	PTX B	R	PTX C	R	PTX D	R	PTX E	R	PTX F	R
1	digitoxin[a]	0.84	digitoxin[a]	0.71	digitoxin[a]	0.81	digitoxin[a]	0.82	digitoxin[a]	0.85
2	spirogermanium	0.62	lanatoside C[a]	0.54	lanatoside C[a]	0.64	spirogermanium	0.56	spirogermanium	0.59
3	lanatoside C[a]	0.58	spirogermanium	0.51	spirogermanium	0.64	lanatoside C[a]	0.46	lanatoside C[a]	0.57
4	digoxin[a]	0.43	5-aza-2-cytidine	0.50	5-aza-2-cytidine	0.44	digoxin[a]	0.40	digoxin[a]	0.43
5	5-aza-2-cytidine	0.27	mitoxantrone	0.48	mitoxantrone	0.40	*m*-L-sarcolysin	0.13	5-aza-2-cytidine	0.27
6	mitoxantrone	0.25	idarubicin	0.45	etoposide	0.40	5-aza-2-cytidine	0.13	mitoxantrone	0.22
7	*m*-L-sarcolysin	0.24	etoposide	0.45	idarubicin	0.38	mitoxantrone	0.07	zinostatin	0.20
8	zinostatin	0.23	*m*-L-sarcolysin	0.43	digoxin[a]	0.34	zinostatin	0.06	*m*-L-sarcolysin	0.19
9	etoposide	0.19	podophyllotoxin	0.38	*m*-L-sarcolysin	0.31	L-alanosine	0.05	etoposide	0.18
10	idarubicin	0.18	estramustin	0.37	zinostatin	0.29	melphalan	0.05	idarubicin	0.16

[a] C.f. [40]. PTX, phoratoxin.

Table 7. Mechanistic resistance factors (RF) for the isolated phoratoxins.

	RF[a]				
	PTX B	PTX C	PTX D	PTX E	PTX F
P-pg	1.1	0.7	0.8	1.0	1.1
GSH-associated	0.8	1.0	1.0	0.9	0.8
Topo II-associated	1.0	1.3	1.7	1.3	1.2
Tubulin	0.8	1.1	1.0	0.8	0.7
MRP	6.1	5.7	5.3	7.0	7.8

[a] Resistance factor = (IC_{50} resistant cell line)/(IC_{50} parental cell line). PTX, phoratoxin; P-gp, P-glycoprotein; GSH, glutathione; topo II, topoisomerase II; MRP, multidrug-resistance associated protein.

Table 8. Comparison of the IC_{50} values (μM) of phoratoxin C for tumor cells from patients.

Patient samples	IC_{50} (μM)	n	n pretreated samples
PBMC	0.7	4	
Hematological			
ALL	0.7	1	1
AML	1.2	3	3
CLL	1.6	5	1
Mean (hematological)	1.4		
Solid			
Abdominal mucous-producing adenocarcinoma	0.1	1	1
Adenocarcinoma	0.3	1	1
Breast carcinoma	0.087	4	2
Colon carcinoma	1.4	1	
Neuroblastoma	0.3	1	
Non-Hodgkins lymphoma	2.1	1	1
Ovarian carcinoma	0.3	5	1
Solid childhood tumor	0.1	1	
Mean (solid)	0.4		
Ratios			
PBMC/BC	8.0		
CLL/BC	18.4		
CLL/Ovca	5.3		
PBMC/CLL	0.4		
$Hematological_{total}/Solid_{total}$	3.5		

CLL, chronic lumphocytic leukaemia; AML, acute myelocytic leukaemia; ALL, acute lymphocytic leukaemia; BC, breast carcinoma; Ovca, ovarian carcinoma; PBMC, peripheral blood mononuclear cells.

(CLL/BC = 18.4), and selectively toxic to the breast cancer cells (PBMC/BC = 8.0) relative to normal PBMCs.

Discussion

This study reinforces and extends previous work [5, 6, 9, 25, 41–46] on proteins from *P. tomentosum*. Here we have described the isolation and characterization of four new proteins and the previously known protein phoratoxin B. Each phoratoxin was sequenced by a combination of N-terminal Edman degradation and C-terminal tandem MS sequence analyses of Cys-alkylated protein, native protein and of tryptic fragments of the Cys-alkylated protein. The results of the sequencing together with the results of quantitative amino acid analyses of the proteins, after complete hydrolysis in 6 N HCl, unambiguously determined the primary structures of the phoratoxins B–F as shown in figure 1. Phoratoxins B–F were all basic, containing six cysteine residues; and all, except phoratoxin D, were shown to have 46 amino acids. Phoratoxin D has 41 amino acids and is most likely a naturally truncated protein. We have no indication that phoratoxin D should be an artifact. Several thionins have been sequenced, and appear to be highly homologous at the amino acid level. The primary structures of some of these thionins are presented in figure 1, which also includes the newly isolated phoratoxins. The primary structures of the phoratoxins and the viscotoxins share a high degree of similarity with the known plant α- and β-thionins.

All the phoratoxins tested showed cytotoxic activity. In the cell line panel, activity varied between different cells. The highest potency was seen in NCI H69, a small-cell

lung cancer cell line. When the activity patterns of the phoratoxins were compared with those of standard agents, using correlation analysis of log IC_{50} values, low correlation coefficients were evident. These results clearly suggest an alternative mechanism of action, different from the reference compounds [32]. Interestingly, when a database of drug response patterns of more than 100 additional investigational agents was searched for similarities with the phoratoxins, digitoxin [40] was found to exhibit the highest correlation coefficients. Notably, in this respect, the unusual stimulatory effects of phoratoxins (data not shown) on granulocyte exocytosis are found as well for the cardiac glycoside digitoxin [47]. The similar mistletoe proteins, viscotoxins, also affect the exocytotic process of the granulocytes by stimulating of the granulocytes to release reactive oxygen intermediates [28]. Further, thionins from *Viscum album* have also been shown to increase permeability of plasma membrane leading to depolarization and Ca^{2+} influx, potentially mediating mitochondrial damage and eventually leading to cell death [28]. Treatment of cells with thionins also causes leakage of ions across the cell membrane [48, 49]. Divalent metal ions inhibit thionin toxicity, with 2–5 mM Ca^{2+} being sufficient to completely protect cells [48]. Correlation is good between cytotoxicity and ability to form ion channels, and Hughes and co-workers (2000) [50] propose that the primary mode of action for the toxicity of thionins is due to their ability to form ion channels in cell membranes, and that the passage of ions through the channel probably involves Tyr-13. However, the exact molecular mechanism for the cytotoxic effects of the compounds investigated here remains to be determined.

Our in vitro studies indicate a possible role of MRP in the cytotoxic potency of the mistletoe proteins from *P. tomentosum*. When the cell line NCI-H69AR, selected for doxorubicin resistance, was compared with its parental cell line, the resistance factor was moderately high for all the phoratoxins, suggesting involvement of an MRP-associated resistance mechanism. Phoratoxins had high resistance factors (RF = 5.7) for the MRP-associated mechanism. MRP confers resistance to a broad range of natural compounds, and mediates the ATP-dependent membrane transport of glutathione S conjugates of chemotherapeutic drugs [51]. Like P-gp, MRP is expressed not only in resistant tumor cells, but also in normal human tissues. Many studies on cells overexpressing MRP or P-gp have shown a concentration of the drug inside cytoplasmic vesicles followed by an exocytotic process. From a putative therapeutic standpoint, the phoratoxins do not appear to be sensitive to other common resistance mechanisms, including P-gp-, topo II-, and GSH-mediated drug resistance.

When phoratoxin C was further tested on primary cultures of human tumor cells from patients, substantial toxicity activity was observed at below micromolar concentrations in several samples, including those of solid tumor origin. In fact, some of the solid tumor samples were more sensitive to phoratoxin C than the hematological tumor samples (CLL/BC = 18). In our FMCA, established chemotherapeutic agents are generally more active against hematological tumors than against solid tumors [52–55]. For example, the defined response rate for vinorelbine was found to be 93% in CLL, but only 27% in ovarian cancer [56]. Later, FMCA-detected toxicity against ovarian carcinoma cells was shown to be a better predictor of clinical activity than toxicity against CLL or cell lines [53]. Most standard and investigational agents show a ratio well below unity. For example, one FMCA-based study, with 18 clinically used cytotoxic drugs, showed that for the ratio of responders among solid and hematological tumors in vitro, only cisplatinum reached a ratio greater than 1 (~1.3) [57]. Furthermore, this estimate was shown to positively correlate with solid tumor activity, and the high ratios observed for phoratoxin C are notable. If this antitumor activity will translate in vivo, these compounds may serve as a lead prototype for developing a new class of anticancer agents with improved activity against solid tumor malignancies.

In summary, this study shows that a series of novel proteins isolated from the North American mistletoe has high antitumor activity in vitro. The mechanism of action appears unique, and the proteins demonstrate a pharmacological profile with unusually high antitumor activity against solid tumors. Further investigations will be required to determine the clinical potential of this class of compounds.

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