Peptides with *in vitro* anti-tumor activity from the venom of the Eastern green mamba, *Dendroaspis angusticeps* (Elapidae)

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**ABSTRACT**

Two structurally related (48.6\% amino acid sequence identity) peptides with cytotoxic activity against human non-small cell lung adenocarcinoma A549 cells were purified from the venom of the Eastern green mamba *Dendroaspis angusticeps* using reversed phase HPLC. The peptides were identified as members of the three-finger superfamily of snake toxins by mass fingerprinting of tryptic digests. The more potent peptide (LC\textsubscript{50} against A549 cells = 56±4\,µg/ml) was identical to the previously described toxin C13S1C1 and the less active peptide (LC\textsubscript{50} against A549 cells = 106±5\,µg/ml) was identical to toxin F-VIII. Toxin C13S1C1 was also cytotoxic against breast adenocarcinoma MDA-MB-231 cells (LC\textsubscript{50} = 62±2\,µg/ml) and colorectal adenocarcinoma HT-29 cells (LC\textsubscript{50} = 110±4\,µg/ml). Although the peptide was appreciably less hemolytic activity against human erythrocytes (LC\textsubscript{50} >600\,µg/ml), it was cytotoxic to human umbilical vein endothelial HUVEC cells (57±3\,µg/ml) indicating no differential activity against cell lines derived from neoplastic tissues. Toxin F-VIII was not cytotoxic to MDA-MB-231, HT-29 cells, and HUVEC cells at concentrations up to 300\,µg/ml and was not hemolytic at concentrations up to 1\,mg/ml. Neither peptide inhibited growth of reference strains of *Escherichia coli* or *Staphylococcus aureus* (MIC values >200\,µg/ml).

**KEYWORDS:** *Dendroaspis*, cytotoxicity, anti-cancer activity, toxin C13S1C1, toxin F-VIII

**INTRODUCTION:** *Dendroaspis*, cytotoxicity, anti-cancer activity, toxin C13S1C1, toxin F-VIII

Venoms from both vertebrate and invertebrate species represent a huge and essentially unexplored reservoir of bioactive components (Calvete, 2009) and recent advances in methodology, permitting characterization of these components from small quantities of material, has meant that venoms are becoming increasingly important in natural products-based drug discovery. The venom of the eastern African arboreal snake, the Eastern green mamba *Dendroaspis angusticeps* (Elapidae) has proved to be a rich source of peptides that have been used as molecular tools to study the physiology and pathophysiology of receptors and ion channels. These include toxins with high affinity and specificity for muscarinic acetylcholine receptor subtypes (Karls son et al, 2000) and for \(\alpha_1\)- and \(\alpha_2\)-adrenoreceptors (Maïga et al, 2012), as well as dendrotoxins that selectively block voltage-dependent potassium channels of the Kv1 subfamily (Harvey and Robertson, 2004) and calcicludins that block L-type calcium channels (Schweitz et al, 1994). In addition, green mamba venom contains a wide range of other biologically active compounds such as fasciculins that potently inhibit acetylcholinesterase (Rodriguez-Ithurralde et al, 1983), and a natriuretic peptide (DNP) with greater potency and increased stability compared with ANP, BNP, and CNP from mammals (Vink et al, 2012).
There is a constant need for new types of anti-cancer agents particularly in cases where the tumor is not responsive to conventional pharmaceutical therapy due to the development of drug resistance (Lord and Ashworth, 2013). Because of their non-specific and destructive mechanism of action, a number of naturally occurring cytotoxic host-defense peptides in frog skin secretions have been shown to have therapeutic potential for development into anti-cancer agents (Conlon et al, 2014). The presence in venoms of peptides and proteins with cytotoxic activity against tumor cells has been described for a wide range of snake species, particularly those belonging to the Viperidae family [reviewed in (Jain and Kumar, 2012; Conlon et al, 2013)]. There have been fewer studies in vivo involving peptides from elapid snakes but dendrotoxin-κ from black mamba venom (Jang et al, 2011) and α-cobratoxin from the venom of the Thai cobra Naja kaouthia (Grozio et al, 2008) have been shown to suppress the growth of tumors induced by human non-small cell lung adenocarcinoma A549 cells in nude mice.

The aim of the present study was to analyse a venom sample from an elapid snake, the green mamba D. angusticeps for the presence of components with anti-tumor activity using A549 cells, breast adenocarcinoma MDA-MB-231 cells, and colorectal adenocarcinoma HT-29 cells. The cytotoxic activity against tumor cells was compared with hemolytic activity against human erythrocytes, cytoxic activity against human umbilical vein endothelial HUVEC cells, and antimicrobial activity using reference strains of Gram-negative Escherichia coli and Gram-positive Staphylococcus aureus bacteria.

MATERIALS AND METHODS

Cytotoxicity assays

Human non-small cell lung adenocarcinoma A549 cells were maintained at 37°C in RPMI 1640 medium containing 2mM L-glutamine and supplemented with 10% (v/v) fetal calf serum (FCS, Biowest, Nouaille, France), and antibiotics (penicillin 50U/ml; streptomycin 50µg/ml) (Attoub et al, 2013). Human breast adenocarcinoma MDA-MB-231 cells and human colorectal adenocarcinoma HT-29 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with antibiotics (penicillin 50U/ml; streptomycin 50µg/ml) and 10% (v/v) FCS (Attoub et al, 2013). EndoGRO human umbilical vein endothelial cells (HUVECs) were maintained in EndoGRO MV-VEGF Complete Media Kit (Millipore, Temecula, CA, USA) (Conlon et al, 2013). In all experiments, cell viability was higher than 99% using trypan blue dye exclusion. Cells were seeded in 96-well plates at a density of 5×10⁴ cells/well. After 24hrs, cells were treated for 24hrs with increasing concentrations of purified toxin F-VIII (1 - 300µg/ml) and toxin C13S1C1 (1–100µg/ml) in triplicate. The effect of the peptides on cell viability was determined by measurement of ATP concentrations using a CellTiter-Glo Luminescent Cell Viability assay (Promega Corporation, Madison, WI, USA). Luminescent signals were measured using a GLOMAX Lumimeter system. The LC₅₀ value, calculated by non-linear regression analysis using commercially available software (SPSS version 17.0; SPSS Inc, Chicago, IL, USA), was taken as the mean concentration of peptide producing 50% cell death in three independent experiments.

Hemolysis assay

The hemolytic activities of the purified peptides in the concentration range of 75–600µg/ml for toxin C13S1C1 and in the range 125–1000µg/ml for toxin F-VIII against washed human erythrocytes (2×10⁶ cells) from a healthy donor were determined as previously described (Conlon et al, 2013).

Antimicrobial assays

The abilities of freeze-dried aliquots (100µl) of chromatographic effluent (Figure 1) to inhibit the growth of reference strains of E. coli (ATCC 25726) and S. aureus (ATCC 25923) were determined in duplicate using 96-well microtiter cell-culture plates. After reconstitution in Mueller-Hinton broth (50ml), the fractions were incubated with an inoculum (50ml of 10⁹ colony forming units/ml) from a log-phase culture of the bacteria for 18hrs at 37°C in a humidified atmosphere of air. After incubation, the absorbance of each well was determined at 630nm using a microtiter plate reader. Minimum inhibitory concentrations (MICs) of the purified toxins against S. aureus and E. coli were measured in the concentration range of 6.25–200µg/ml by a standard double dilution method (Clinical Laboratory Standards Institute, 2008).

Purification of the cytotoxic peptides

Venom from D. angusticeps was purchased in lyophilized form from a commercial supplier (Jonathan Leakey Ltd, Nakuru, Kenya). The venom sample (432mg) was redissolved in 0.1% (v/v) trifluoroacetic acid(TFA)/water (4ml) and injected onto a (2.2cm×25cm) Vydac 218TP1022 (C-18) reversed-phase HPLC column (Grace, Deerfield, IL, USA) equilibrated with 0.1% (v/v) TFA/water at a flow rate of 6ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10min and to 63% (v/v) over 60min using linear gradients. Absorbance was monitored at 214nm and 280nm, and fractions (1min) were collected. Freeze-dried aliquots (100ml) of the fractions were reconstituted in RPMI 1640 medium (100ml) and their abilities to produce cytolysis of lung adenocarcinoma A549 cells were determined as described in the cytotoxicity assays section. Fractions containing peptides with cytotoxic activity were successively chromatographed on a (1cm×25cm) Vydac 214TP510 (C-4) column and a (1cm×25cm) Vydac 208TP510 (C-8) column at a flow rate of 2ml/min. The concentration of acetonitrile in the eluting solvent was raised from 21% to 42% over 60min for the purification of toxin F-VIII, and from 21% to 56% for the purification of toxin C13S1C1.

Identification of the cytotoxic peptides

The molecular masses of the purified peptides were determined by LC-MS using a nano-Acquity UltraPerformance LC (UPLC) with a BEH130 C18 (100µm×100mm, 1.7µm particle size) column in-line with a Waters SYNAPT G2 High Definition Mass Spectrometry System. The flow rate was set to 0.6µl/min and the column was eluted with a linear gradient of 0.1% (v/v) formic acid in water (solution A) and 0.1% (v/v) formic acid in acetonitrile (solution B),
isocratically 1% B for 1min, followed by 1–12% B for 1min, 12–40% B for 15min, and 40–85% B for 2min.

The purified cytotoxic peptides were initially analyzed by SDS-PAGE on 15% (w/v) polyacrylamide gels under reducing and non-reducing conditions and the protein bands were excised from Coomassie Brilliant Blue-stained gels and subjected to automated reduction, alkylation, and in-gel digestion with sequencing grade porcine pancreatic trypsin using a Progest™ digestor (Genomic Solutions, Ann Arbor, MI, USA). The tryptic peptide mixtures in 15ml of 5% (v/v) acetonitrile containing 0.1% (v/v) formic acid were separated by nano-Acquity UltraPerformance LC using the same chromatographic conditions as described above for the purified parent molecules. Doubly and triply charged ions were selected for collision-induced dissociation (CID) MS/MS. Fragmentation spectra were interpreted manually (de novo sequencing) or processed in a Waters Corporation’s ProteinLynx Global SERVER 2013 version 2.5.2. with Expression version 2.0 against the 81 Dendroaspis venom sequences retrieved from UniProtKB/Swiss-Prot (UniProt release 2014_02) and the non-redundant NCBI database (GenBank Release 200.0). MS/MS mass tolerance was set to ±0.6Da. Carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively.

RESULTS

Purification of the cytotoxic peptides

The elution profile on a preparative Vydac C-18 column of the venom from D. angusticeps is shown in Figure 1. Under the conditions of assay, the prominent peaks designated 1 and 2 contained material with cytotoxic activity (>98% cell death during a 24 h incubation) against human lung adenocarcinoma A549 cells and were purified further. The dashed line shows the concentration of acetonitrile in the eluting solvent.

Identification of the cytotoxic peptides

The identities of the cytotoxic peptides were determined by CID-MS/MS mass spectrometric analysis of fragments generated by in-gel tryptic digestion. In the case of the peak 1 peptide, the following fragments were identified: MICYSHK, TPQPSATITCEEKTCYKK, TPQPSATITCEEKTCYK, TPQPSATITCEEKTCY, SVRLPAIVAGR, GCGCPSKEMLVAIHCCR, and EMLVAIHCCR. The component was unambiguously identified as toxin F-VIII (UniProtKB/Swiss-Prot: P01404) and the complete primary structure is shown in Figure 3. The C-terminal fragment SDKCNE was not identified, presumably because it was cleaved to SDK and CNE. The average molecular mass ([M+H]+) of the purified toxin F-VIII is consistent with its proposed structure: \( M_{obs} \) 6597.8, \( M_{calc} \) 6597.8. \( M_{obs} \) refers to the observed molecular mass in Daltons and \( M_{calc} \) refers to the molecular mass calculated from the proposed sequences shown in Figure 3.

In the case of the peak 2 peptide, the following fragments were identified: ICYSHKLLQAK, TTKTCEENSCYKR, SLPKIPLIIIGR, GCGCPLTLPLFLR, IPIIIRGCGCPLTLPLFLR, and IKCCTSDKCN. The component was unambiguously identified as toxin C13S1C1 (UniProtKB/Swiss-Prot: P18329) and the complete primary structure is shown in Figure 3. The average molecular mass ([M+H]+) of the purified peptide is also consistent with its proposed structure: \( M_{obs} \) 6653.0, \( M_{calc} \) 6652.6.
Cytotoxic activities of the peptides

Toxin C13S1C1 showed cytotoxic activity against human non-small cell lung adenocarcinoma A549 cells, breast adenocarcinoma MDA-MB-231 cells, colorectal adenocarcinoma HT-29 cells, and human umbilical vein endothelial HUVEC cells. The LC_{50} values are shown in Table 1. The effect of increasing concentrations of the peptide on cell viability is shown in Figure 4. A549 cells (LC_{50} = 56µg/ml; approx. 8 µM) and HUVEC cells (LC_{50} = 57µg/ml; approx. 8µM) were the most sensitive to the cytotoxic action of the peptide and HT-29 cells the most resistant (LC_{50} = 110µg/ml; approximately 16µM). The amount of available pure peptide was insufficient to determine the LC_{50} value for toxin C13S1C1 against human erythrocytes but the peptide produced only 21% hemolysis after a 1hr incubation with a 600µg/ml concentration. Toxin C13S1C1 did not inhibit the growth of *E. coli* and *S. aureus* at concentrations up to 200µg/ml. In contrast, toxin F-VIII was active only against A549 cells (LC_{50} = 106µg/ml; approximately 15µM) and did not produce significant cell death in either MDA-MB-231 cells or HT-29 cells at concentrations up to 300µg/ml during a 24hr incubation (Table 1). The peptide showed no hemolytic activity against human erythrocytes and no antimicrobial activity against *E. coli* and *S. aureus* at concentrations up to 1mg/ml.

**DISCUSSION**

The present study has shown that, under the conditions of assay, toxin C13S1C1 is the component with the greatest cytotoxicity against three human tumor cell lines in the venom of the green mamba *D. angusticeps*. However, its therapeutic potential as an anti-tumor agent is restricted by appreciable cytotoxic activity against non-neoplastic HUVEC cells. A second component, toxin F-VIII was cytotoxic only against lung adenocarcinoma A549 cells. These peptides were first identified on the basis of relatively low toxicity in mice (Joubert and Taljaard, 1980) and ability to block voltage-dependent potassium channels (Viljoen and Botes, 1974). As shown in Figure 3, toxin C13S1C1 and toxin F-VIII are structurally related (48.6% amino acid sequence identity)
Table 1. Cytotoxicities of toxin C13S1C1 and toxin F-VIII against human non-small cell lung adenocarcinoma A549 cells, breast adenocarcinoma MDA-MB-231 cells, colorectal adenocarcinoma HT-29 cells, human umbilical vein endothelial HUVEC cells, and red blood cells (RBC)

|               | A549   | MDA-MB-231 | HT-29  | HUVEC  | RBC  |
|---------------|--------|------------|--------|--------|------|
| Toxin C13S1C1| 56±4   | 62±2       | 110±4  | 57±3   | >600 |
| Toxin F-VIII  | 106±5  | >300       | >300   | >300   | >1000|

Data show LC_{50} values (µg/ml) ±SEM.

Figure 4. Effects of (A) toxin C13S1C1 and (E) toxin F-VIII on the viability of non-small cell lung adenocarcinoma A549 cells; (B) toxin C13S1C1 and (F) toxin F-VIII on the viability of breast adenocarcinoma MDA-MB-231 cells; (C) toxin C13S1C1 and (G) toxin F-VIII on the viability of colorectal adenocarcinoma HT-29 cells, and (D) toxin C13S1C1 and (H) toxin F-VIII on the viability of human umbilical vein endothelial HUVEC cells. All experiments were repeated at least three times. Columns: mean; bars: SEM.
identity) and are members of the three-finger toxin (3FTx) superfamily (Kini and Doley, 2010). Peptides in this family are characterized by three antiparallel β-sheet loops that are stabilized by four, or less commonly five, disulfide bridges. 3FTx peptides are not confined to the venoms of elapid snakes (mambas, cobras, and kraits) but have also been identified in the venoms of colubrids (Pawlak et al, 2009), hydrophoids (Fahari et al, 2007) and vipers (Doley et al, 2008).

Several components in venoms from Viperidae have been shown to possess potent anti-bacterial and anti-fungal activity although the precise biological significance of this observation is unclear [reviewed in (Samy et al, 2011; Conlon et al, 2013)]. Among snakes belonging to the Elapidae, L-amino acid oxidases from the Central Asian cobra Naja naja oxi ana venom (Samel et al, 2008) and from the king cobra Ophiophagus hannah (Lee et al, 2011) show broad-spectrum antibacterial activity whereas omwaprin from the venom of inland taipan Oxyuranus microlepidotus is active only against Gram-positive bacteria (Wang et al, 2008). However, neither toxin F-VIII nor toxin C135IC1 showed growth inhibitory activity against reference strains of the Gram-negative E. coli and the Gram-positive S. aureus at concentrations up to 200µg/ml.

CONCLUSION

Toxin C135IC1 shows broad-spectrum anti-tumor activity combined with low cytotoxicity to red blood cells. However, its therapeutic potential for development into an anti-cancer agent is low because of its lack of selectivity towards cell lines derived from neoplastic tissues.

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COMPETING INTERESTS

None declared.

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