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RESEARCH HIGHLIGHT

The CT20 peptide: more than a piece of bax

Michael W. Lee[1], Rania Bassiouni[2], Ashley Iketani[2], Orielyz Florez[3], J. Manuel Perez[3], Annette R. Khaled[2]

[1]*Department of Medical Education and College of Medicine, University of Central Florida, Orlando, FL 32827*
[2]*The Burnett School of Biomedical Science, College of Medicine, University of Central Florida, Orlando, FL 32827*
[3]*Nanoscience Technology Center, University of Central Florida, Orlando, FL, 32826*

Correspondence: Annette R. Khaled
E-mail: annette.khaled@ucf.edu
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Use of cytotoxic peptides for cancer treatment is an emerging therapeutic direction with promising potential; however, in many instances the intracellular actions of these peptides remain unknown. Finding ways to systematically decipher the intercellular targets and functions of rationally designed or endogenously sourced therapeutic peptides to better define clinical use is a significant challenge that needs to be addressed. Here we describe our recent results outlining the activity of CT20p, a peptide derived from the pro-apoptotic protein Bax, which specifically kills metastatic cancer cells but not normal epithelial cells. Using breast cancer as a model system to test the peptide, we found that CT20p can be delivered to cells by polymeric hyper branched nanoparticles and that, once intracellular, have profound effects on cytosolic proteins and organelles in a cancer-specific fashion that are different from the parent protein. In cancer cells, CT20p localizes to the mitochondria, forms pores in mitochondrial-like membranes, alters mitochondrial movement and membrane polarization, and impairs cytoskeletal reorganization, leading to cell detachment and death. Further, we discuss our strategy for determining the molecular actions of CT20p that could help inform the activities of other currently available antimicrobial and anticancer therapeutic peptides.

Keywords: therapeutic peptides; Bax, transmembrane domain; breast cancer; mitochondria; cytoskeleton; cell death

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Developing peptides as therapeutic agents for treating cancer offers many benefits over small molecule drugs. In clinical applications, peptides modeled after proteins with known functions may have reduced off-target effects and a decreased chance of developing drug resistance, which results in low toxicity. Peptides are also cost-effective to produce and are amenable to modifications. A major advantage is the potential to design peptides that target specific protein-protein interactions or organelles, like the mitochondria [1, 2]. While organelle targeting sequences, such as mitochondrial targeting sequences, are known and characterized, their use is largely restricted to targeting non-peptide cargo rather than as a component of a "peptide drug" [3]. One strategy is to derive peptides from previously characterized, endogenous cellular proteins. This approach takes advantage of the information available on the localization and function of the protein from which the peptide is derived and could inform of the potential molecular target and action of the peptide. Recently, we reported that a 20 amino acid α-helical peptide developed from the α9 transmembrane domain of the pro-apoptotic protein, Bax, has cytotoxic actions for cancer cells [4]. This peptide, henceforth referred to as CT20p, is amphipathic and shares similarities with anti-microbial peptides (AMPs), such as the presence of a tryptophan and two C-terminal lysines. While CT20p is capable of forming pores in mitochondrial-like lipid vesicles, in mammalian cells the peptide targets mitochondria and disrupts the cytoskeleton in a manner

that causes cell detachment and death[4-6]. Hence, while derived from an apoptotic protein and retaining cytotoxic activity, CT20p, due to its inherent features, may have unique actions unrelated to the parent protein that could have significant clinical impact.

Pore-forming activity of CT20p

Initial biophysical studies of CT20p revealed that the peptide has the capacity to form membrane pores [5, 6]. Using dye-loaded lipid vesicles, we determined that CT20p formed large pores in zwitterionic or anionic membranes that contained up to eight peptide molecules [5]. Kinetics of pore formation revealed that it was a two-staged process, involving nucleation and assembly steps [5]. The pore structure formed by CT20p in simple lipid membranes was analyzed and found to be a novel α/β-ring structure, with insertion in the membrane being dependent on the presence of two C-terminal lysine sin the peptide [6]. These results suggested that one of the ways peptides like CT20p could act is through pore formation that causes membrane permeabilization. CT20p is not unique in its ability to form pores. AMPs with cancer cell killing activity, such as Magainins or Cecropins, can dock in the membranes of target cells and cause membrane depolarization by forming organized pore structures (reviewed in [7]). Peptides derived from the α5 and α6 helices of Bax also caused permeabilization of lipid membranes, forming toroidal-like pores [8, 9]. However, the membrane selectivity and biological relevance of peptide pore formation remains unknown, since most of these studies were performed using artificial lipid vesicles. The question remains – is forming a pore in cell membranes the main mechanism of action of peptides like AMPs or CT20p or is there potential, based on the structure and sequence of the peptide, for more complex biological activity? To answer this, we examined the effect of CT20p in mammalian cells. We initially determined that CT20p in aqueous solutions was not permeable through the plasma membrane. Because CT20p is a hydrophobic peptide, encapsulation using hyperbranched polymeric nanoparticles enabled its intracellular delivery to cells [10]. Once within cells, we found that CT20p had activities that went beyond simple membrane permeabilization and interfered with key cellular components required for survival, such as the mitochondria and cytoskeleton.

Organelle-targeting and cytotoxicity of CT20p

Using a fluorescently-tagged version of CT20p, we observed that within cells, the peptide homed to mitochondria independently of any apoptotic stimulus [11]. Furthermore, localization of CT20p to the mitochondria and cell-killing occurred in cells deficient in Bax or in which Bcl-2 was over expressed, suggesting that CT20p does not require apoptosis-associated proteins for localization or function [4]. The ability of CT20p to localize to the mitochondria may in part be due to the –KKMG amino acid motif which has similarity to consensus mitochondrial targeting sequences [12].Within hours of cell entry, CT20p promoted mitochondrial hyper polarization and aggregation and impaired mitochondrial movement [11]. These effects deviate from the documented function of the parent protein, Bax, which when activated in mammalian cells causes mitochondrial fragmentation, mitochondrial depolarization and release of cytochrome C that leads to apoptosis - events not observed with CT20p [13, 14]. Interestingly, in yeast the over expression of full-length Bax can promote mitochondrial aggregation and hyper-polarization [15]. This is significant because the yeast genome does not encode any of the Bcl-2 family members, shedding light on alternative activities of Bax that are reflected by CT20p.

The ability of CT20p to promote mitochondrial membrane hyper polarization may account for the findings of Valero et al., in which a peptide derived from the α9 helix of Bax, albeit slightly larger than CT20p (aa 169-192), was a weak inducer of both apoptosis and mitochondrial depolarization [9]. More recently, Andreu-Fernandez et al reported that a peptide, similar to in sequence to CT20p (see Table 1), could permeate mitochondrial-like lipid vesicles and promote the release of cytochrome C from isolated mitochondria but triggered the non-apoptotic, necrotic-like death of intact cells without loss of mitochondrial membrane potential [16]. Despite such findings, it remains unclear whether the peptides tested in these reports produce similar death responses in normal cells. A comparison of the Bax C-terminal peptides discussed is shown in Table 1.

The major death response triggered by CT20p does not appear to be a purely apoptotic process as inhibition of caspases only provided a slight increase in cell viability and did not rescue cells treated with CT20p [4]. We surmise that Bax C-terminal derived peptides, while still cytotoxic, may act differently than full-length Bax to trigger cell death. For example, full-length Bax can form two types of channels in mitochondria, depending on the lipid content and organization of the mitochondria [17]. Further, mitochondria from cancer cells may be inherently different from mitochondria of normal cells based on differing metabolic activities [18]. In fact, we observed differences in mitochondria morphology between MDA-MB-231 cells and MCF-10A cells, with the cancer cell mitochondria being smaller and more fragmented [11]. These basal differences in mitochondrial structure between normal and metastatic breast cancer

Table 1. Comparison of Bax C-terminal Peptides

PEPTIDE NAME	AMINO ACID SEQUENCE	REFERENCE
Bax α9	^{169}-TWQTVTIFVAGVLTASLTIWKKMG-^{192}	[9]
TMD-Bax	^{Ac}-*KK*TWQTVTIFVAGVLTASLTIWKK-^{NH2}	[16]
CT20p	^{173}-VTIFVAGVLTASLTIWKKMG-^{192}	[4-6,11]

cells may account for the differences in CT20p-mitochondrial binding and cytotoxicity observed in cancer compared to normal cells. However, our data revealed that the activities of CT20p encompassed more than the mitochondria. CT20p also interfered with mitochondrial movement, actin polymerization and integrin levels, implicating that the peptide was also targeting the cytoskeleton [11]. This suggested that CT20p, unlike the parent protein, Bax, has global actions in the cancer cell, which ultimately lead to cell detachment as the precipitating event that triggers cell death. Indeed, the underlying cause of detachment may be related to the ability of CT20p interact with cytosolic proteins to alter the surface expression of integrins (e.g. α5β1) and attenuate actin polymerization [11].

Cancer-selective action of CT20p

A key property of CT20p is selective cytotoxicity towards cancer cells such as the metastatic breast cancer cell line, MDA-MB-231, compared to normal epithelial cells, like the breast epithelial cell line, MCF-10A. We observed that while all cells uptake the peptide encapsulated in nanoparticles, only cancer cells were susceptible to the lethal action of CT20p [11]. The cytotoxicity of CT20pwas manifested by cancer-specific mitochondrial targeting and aggregation, the impairment of act in polymerization, and reduced integrin expression leading to detachment of cells from substrates and eventual cell death [11]. Therefore, understanding the underlying differences between MCF-10A and MDA-MB-231 cells that account for the specific effects of CT20p offers an opportunity to discern how the peptide kills and could reveal new molecular targets that discriminate between normal and cancerous cells.

To investigate the differences between MCF-10A and MDA-MB-231 cells, we resolved lysates from these cell lines by two-dimensional gel electrophoresis following CT20p treatment and compared changes in total protein distribution (Figure 1). Untreated lysates were labeled with the green fluorescent dye Cy3, while CT20p-treated lysates were labeled with Cy5, which fluoresces red. The pooled lysates were then subject to two-dimensional gel electrophoresis, followed by fluorescence detection and analysis. Proteins that were unchanged in both conditions appeared as yellow spots, while proteins that were only present (increased) after CT20p treatment were detectable as red spots, while proteins not present after CT20p treatment (decreased) were detectable as green spots. In MDA-MB-231 cells, the presence of increased red spots indicated that CT20p treatment altered the protein expression profile of the cells, causing new or altered proteins to increase or proteins to decrease. Importantly, similar changes were not seen in MCF-10A cells, with ~95% of the proteins remaining unaffected after CT20p treatment. To further examine the consequences of peptide treatment in MDA-MB-231 cells, several red protein spots were selected for analysis of protein content by mass spectrometry. Peptide identification revealed that CT20p induced changes in proteins involved in metabolic, cellular, and developmental processes, such as enzymes involved in glucose metabolism and components of microfilaments (actin) and microtubules (tubulin), suggesting that the peptide could target key processes essential for the survival and migration of cancer cells. Further exploration of this proteomics approach may potentially reveal specific targets of interest for in-depth study.

Conclusions and future directions

Using Bax, a known mitochondria-targeting apoptotic protein, we derived a cytotoxic peptide that has specific action on cancer cells. Current studies revealed the CT20p targets organelles like mitochondria and the cytoskeleton to disrupt essential cellular functions needed by cancer cells to support their growth and movement. Future studies are underway to temporally coordinate the phenotypic changes observed (i.e.: mitochondrial changes, cytoskeletal changes) with protein expression changes. By proceeding in this manner, we will identify specific proteins that mediate the actions of CT20p, shedding light on how and why CT20p is lethal to cancer cells but not normal cells. Importantly, this approach may also uncover novel proteins specific to the metastatic cancer cell that could serve as diagnostic biomarkers. The potential for discovery of new therapeutic peptides, for example using combinatorial libraries or phage-display libraries, is the first step towards identifying a clinical relevant compound. The next step is to discern the function of the peptide of interest and determine, like CT20p, whether novel activities can be ascribed. Using biophysical and bioinformatics approaches in addition to biological testing, we demonstrated that a peptide from a known protein can

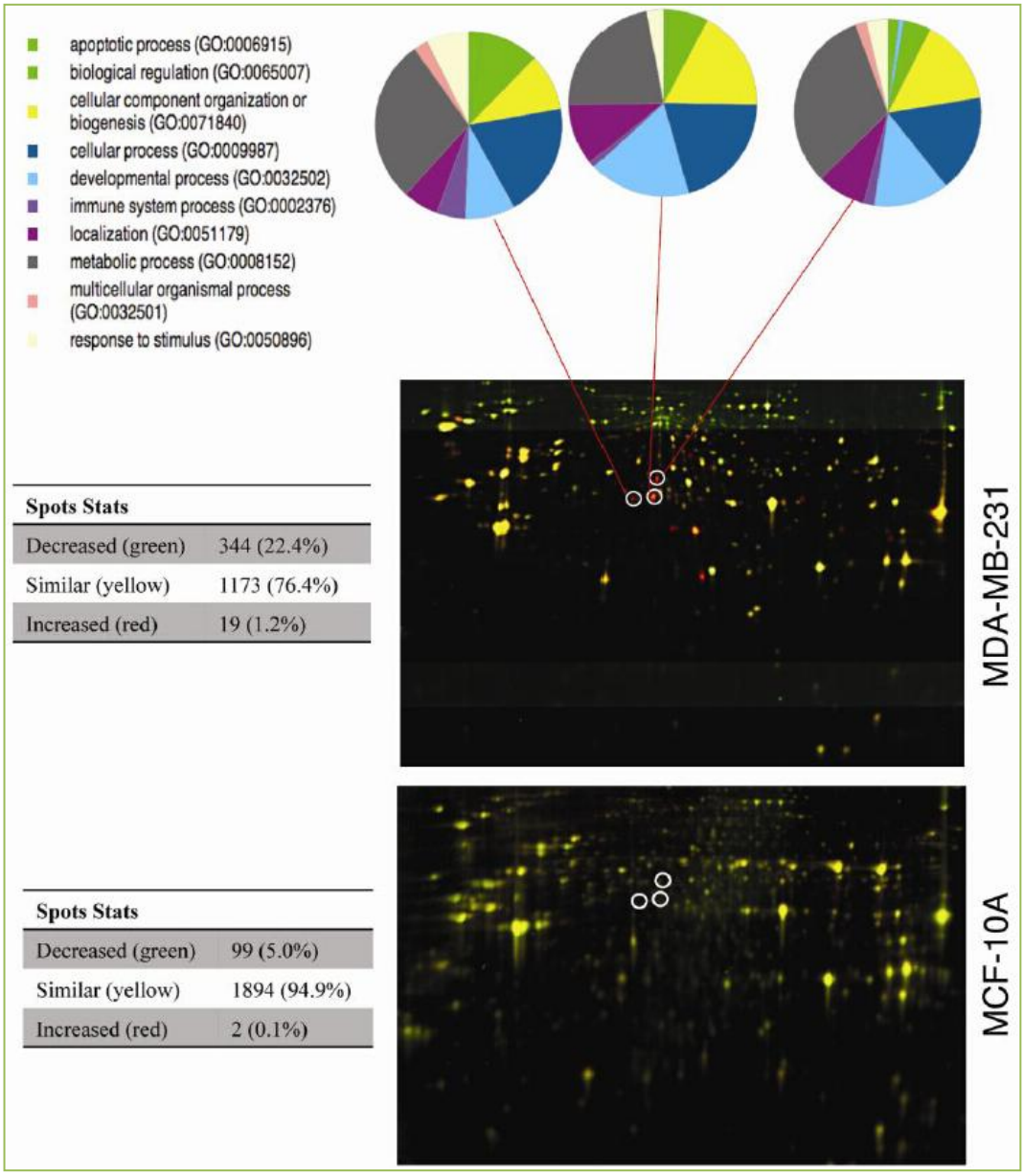

Figure 1. CT20p induces global changes in protein expression in cancer cells but not normal cells. The metastatic breast cancer cell line MDA-MB-231 and the normal breast epithelial cells line MCF-10A were treated with CT20p for 3 hours and lysates (labeled with fluorescent dyes) were resolved by two-dimensional gel electrophoresis. Using DeCyder 2D software (GE Healthcare), the relative expression levels of protein spots were compared between CT20p treated and untreated lysate on each gel. Spots of interest were identified, cut out and sent for mass spectrometry analysis. The pie charts and associated legend show the range of biological functions of the proteins identified in each spot that was analyzed.

have functions that are unique and go beyond that of the parent protein. The implications for future discoveries are significant and suggest that peptide therapeutics is a field where interdisciplinary approaches can best reveal how the human proteome may be mined for compounds with promising clinical application.

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