3-NAntC: a novel Crotoxin B-derived peptide for the treatment of triple-negative breast cancer

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Article

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Abstract

Breast cancer is the most prevalent type of tumor and a major leading cause of cancer mortality. Triple-negative breast cancer (TNBC) has the worst prognosis due to its malignant characteristics and the absence of efficacious treatments. Crototoxin, a protein in \textit{Crotalus} genus snake venom, has proven antitumor activity against aggressive solid tumors, but marked toxicity in humans. Crototoxin B-derived peptides were synthesized and evaluated \textit{in vitro} for their antitumor activity, which resulted in the discovery of 3-NAntC. 3-NAntC (1µg/mL) treatment for 72 hours decreased the MDA-MB-231 cells viability to 49.0%±17.5% \((p < 0.0001)\), while the same condition resulted in the viability of HMEC cells at 98.2%±13.8%. 3-NAntC exhibited higher antitumoral activity \textit{in vitro} than cisplatin and similar effect of doxorubicin. 3-NAntC reduced MDA-MB-231 cell proliferation and caused a G2/M arrest. 3-NAntC primarily induced apoptosis, with a lower necrosis occurrence compared with doxorubicin. 3-NAntC caused a low LDH release, and its cytotoxicity was not impaired by the autophagy inhibitor 3-MA. In zebrafish \textit{in vivo} model, 3-NAntC was very well tolerated, showing no lethal effect and a low malformation rate at \(\leq 75\)mg/mL. 3-NAntC is a novel synthetic peptide with promising antitumor effects \textit{in vitro} against TNBC cells and with low toxicity \textit{in vivo}.

Introduction.

Data from the American Cancer Society estimate that 1.9 million new cases of cancer and more than 600,000 deaths from cancer would occur in 2022 in the USA \cite{1}. Breast cancer (BC) is considered the most common tumor worldwide and the second leading cause of cancer mortality in the USA and Europe \cite{2, 3}.

Triple-negative breast cancer (TNBC) is a BC type that accounts for 15–20% of neoplasias in this tissue. TNBC cells are aggressive, invasive, highly proliferative, and there is limited availability of treatment alternatives, all leading to the poorest prognosis among BC types \cite{3}. TNBC cells exhibit deficient expression of receptors commonly present in BC: receptors for estrogen (ER) and progesterone (PR), and the human epidermal growth factor receptor 2 (HER2). Therefore, TNBC cells cannot be treated with drugs that modulate such receptors, thus being the only BC type that lacks tumor-specific targeted therapies \cite{3, 4}.

The treatments available for TNBC mainly comprise of surgery, radiotherapy, and chemotherapy. Chemotherapy with anthracyclines (e.g., doxorubicin), taxanes (e.g., paclitaxel), alkylating agents (e.g., cyclophosphamide), antimitabolites (e.g.: capecitabine) and platin-containing drugs (e.g., cisplatin) may be employed in the neoadjuvant and/or adjuvant settings. Neoadjuvant chemotherapy is considered a standard approach prior to surgery, which includes mastectomy or lumpectomy \cite{3–5}. There are novel drugs recently approved that can be employed for the treatment of TNBC, including Poly ADP Ribose Polymerase (PARP) inhibitors for patients with BRCA1/2 mutations, immune checkpoint inhibitors and antibody drug conjugates \cite{3}. Notwithstanding, due to the severe side effects, toxicity and limited efficacy of current therapies, new treatment options for TNBC are urgently needed.
Drug Research and Development based on natural proteins is a successful strategy. Poisons and venoms from animals are sources of proteins with a wide range of pharmacological activities. There are at least eleven approved drugs that were developed based on natural venoms and/or poisons, the majority from snake venoms, such as captopril, enalapril, batroxobin and eptifibatide [6]. The first snake venom protein purified and crystallized was Crotoxin (CTX), the major venom component from Crotalus durissus terrificus, the South American rattlesnake [7]. Crotoxin antitumoral activity was demonstrated in vitro and/or in vivo in cell lines derived from breast (MCF-7) and lung (A549, SK-MES-1 and SPCA-1) aggressive human tumours [8–11]. Crotoxin reached the clinical development stage, and it was tested in a phase I trial in patients with solid tumors refractory to conventional therapy. Crotoxin administration for 30 days reduced the tumor mass by > 50% in 2 of 23 patients and complete regression was observed in one patient, however, clinical trials were discontinued due to marked toxicity in humans [12].

Crotoxin is a heterodimeric complex with a first basic, toxic phospholipase A2 (PLA2, Component B, CB or crotoxin B) and a second acidic, non-toxic, non-enzymatic component (Component A or CA). The CA component increases the toxicity of CB component. CA and CB components have isoforms with slight variations in enzymatic and pharmacological properties [7, 13]. CTX complexes can be classified into two classes depending on their toxicity and enzymatic activity. CBb, CBc and CBd isoforms (class I) complexed to CA are more toxic, have less phospholipase A2 activity and dissociate from CA more slowly compared to class II CBa2 isoform (crotoxin CB1) [13].

Crotoxin activities (e.g.: neurotoxicity, myotoxicity, nephrotoxicity and cardiotoxicity) are associated with specific molecular regions [7]. Notwithstanding, the sequence resulting in toxicity remains unclear, although associations with the N-terminal, IBS (interfacial binding surface) or C-terminal regions were proposed in independent studies [7, 14, 15]. The exploration of crotoxin modifications on the resulting pharmacological and toxic effects was described in the literature. Chemical modifications at four Crotoxin B amino-acids and protein cleavage altered its cellular lethality, myotoxicity, PLA2 enzymatic activity, bactericidal, edema-inducing, anticoagulant and liposome disrupting effects [16].

In this work, we produced different crotoxin B-derived peptides (CBa2 isoform) to characterize the protein regions responsible for the antitumoral and toxic activity. The strategy was aimed at identifying peptides that maintained the pharmacological effects with an improved safety profile.

**Results.**

**Synthesis and characterization of crotoxin B-derived peptides.**

The peptides were obtained based on exploring fragments of crotoxin B isoform CBa2, which is known to exhibit lower toxicity compared to the class I isoforms [13]. Figure 1 displays the amino acid sequence and residues important for crotoxin activity that were employed as basis for the peptides design highlighted. The peptide 3 is called 3-NAntC (Fig. 1).
In vitro effects of the crotoxin B-derived peptides in the viability of breast cancer cell lines.

Crotoxin B-derived peptides 1, 2 and 3 were evaluated in MDA-MB-231 (triple negative breast cancer) and MCF10A (immortalized “normal” breast) cells to determine their antitumor and cytotoxicity profiles (Fig. 2). Peptide 1 (2.5µg/mL) did not decrease MDA-MB-231 cells viability at 24 and 48 hours (94.4% ±4.6% and 90.7%±3.0%, respectively, p > 0.05) when compared to the control treatment without peptides. Although peptide 2 (2.5µg/mL) slightly decreased MDA-MB-231 viability at 24 (81.6%±5.0%, p < 0.05) and 48 hours (85.0%±7.9%, p < 0.001), it also exhibited cytotoxicity against MCF10A benign cells to a similar extent after 48 hours (85.7%±11.2%, p < 0.01). Therefore, peptides originated from the N-terminal regions were unable to selectively reduce cell viability of breast cancer cells. Conversely, peptide 3 (0.8µg/mL) significantly reduced viability of MDA-MB-231 cells after 24 (77.5%±15.0%, p < 0.001) and 48 hours (60.8%±17.9%, p < 0.0001) compared to non-treated cells. The viability was not significantly affected in MCF10A cells at 2.5µg/mL (93.4%±7.4% and 80.5%±5.3% after 24 and 48 hours, respectively, p > 0.05). However, higher concentrations of peptide 3 (20µg/mL) reduced MCF10A cell viability after 48 hours (65.9%±10.3, p < 0.0001). These results indicate the specificity of the peptide 3 activity and the absence of toxic component.

Peptide 3, herein denominated as 3-NAntC, was resynthesized and characterized for activity confirmation. The resulting peptide exhibited high purity (> 95%), molecular mass of 1,645.95 g/mol (Supplementary Figures S1 and S2). 3-NAntC was found as a monomer or as a dimer, with higher abundance of the dimeric form the higher the pH (monomer/dimer ratio of 7.51 at pH 4.0, 6.47 at pH 7.0 and 27.31 at pH 10.0).

3-NAntC activity upon cell viability was then evaluated in a larger panel of breast cancer (MDA-MB-231 and MCF-7), normal immortalized breast cells (MCF10A), human dermal fibroblasts (HDFa) and primary mammary epithelial (HMEC) cells (Fig. 3). In benign cells, 3-NAntC treatment at concentrations ≤ 1.0 µg/mL promotes a slight decrease in cell viability (< 20%). No differences were observed when the cytotoxicity to MCF10A, HDFa and HMEC were compared to the untreated control respective cells (p > 0.05). Conversely, 3-NAntC (1.0µg/mL) significantly decreased the MDA-MB-231 cells viability to 64.6% ±14.5% (p < 0.05) and 49.0%±17.5% (p < 0.0001) after 48 and 72 hours of treatment, respectively. Although 3-NAntC (1.0µg/mL) also induced a decrease in the viability of MCF-7 cells, the effect was moderate and constant even after long exposure periods (54.7%±15.5% and 62.9%±26.0% after 48 and 72 hours of treatment, respectively). The viability of the MDA-MB-231 and MCF-7 cells was significantly lower than what was observed for HMEC cells after 3-NAntC treatment (Figs. 3D-F, p < 0.05).

3-NAntC-derived peptides 4–12 were then synthesized to explore the effects of amino acid modifications into their activity upon cell viability (Supplementary Figure S3). Peptides 5, 9 and 11 are cyclic due to intramolecular disulfide bonds between the N- and C-terminal cysteine residues. Although peptides 4–12 exhibited a significant decrease in cell viability in vitro, for MDA-MB-231 cells at concentrations ≥ 0.4 µg/mL, the effects were lower than what was observed for 3-NAntC (Supplementary Figure S4). Another in vitro experiment was conducted to evaluate if it was possible to reproduce 3-NAntC activity with the
combination of the peptides 6 and 7, which contain the same amino acids from 3-NAntC divided into two fragments. Although their combinations reduced cellular viability, their effect was lower than what was observed for 3-NAntC. These data further support that 3-NAntC is the smallest crotoxin B-derived peptide that maintains a potent inhibitory activity upon the viability of TNBC cells.

The effect of 3-NAntC on MDA-MB-231 cells was compared with two chemotherapeutic drugs, doxorubicin and cisplatin (Fig. 4). 3-NAntC exhibited significant decrease in MDA-MB-231 viability in vitro at concentrations ≥ 0.4 µg/mL at 24 and 48 hours and ≥ 0.2 µg/mL at 72 hours when compared to control (Figs. 4A-C). However, a lower effect of 3-NAntC was observed in HDFa cells at doses ≤ 1.0 µg/mL. The cellular viability of HDFa cells treated with the peptide was higher than 80% at all conditions (Figs. 4D-F). 3-NAntC presented a higher inhibitory effect upon MDA-MB-231 viability than cisplatin as early as 48 hours of treatment, and its biological effect at 72 hours is similar to that observed for doxorubicin at 48 hours (Figs. 4A-C). However, 3-NAntC and cisplatin had a lower effect on the viability of the HDFa cells treated with doxorubicin (Figs. 4D-F).

Effect of 3-NAntC in cell proliferation and cell cycle progression.

A series of assays were conducted to elucidate the mechanism of action associated with 3-NAntC activity on cell viability. The effects on cell proliferation were assessed using the BrdU labeling method. 3-NAntC significantly decreased the proliferation rate of MDA-MB-231 cells at doses ≥ 0.2 µg/mL in all conditions (Fig. 5A) and the maximum proliferation inhibition (71.1%±27.1%, p < 0.0001) was reached at 0.6 µg/mL following a 48-hour treatment. Interestingly, the proliferation reduction was observed even at concentrations of 0.2 µg/mL that did not impair cellular viability. Additionally, 3-NAntC did not alter the proliferation of HDFa cells at concentrations up to 1.0 µg/mL for 72-hour exposures (Fig. 5B).

Cell cycle progression assay was conducted using PI to quantify DNA content (Fig. 5C). Since 3-NAntC did not affect the proliferation of HDFa cells, only MDA-MB-231 cells were studied through this method. For the 48-hour treatment, 3-NAntC at 0.8 µg/mL reduced the number of cells in G0/G1 (-8.72%±4.03%, p < 0.01), while increasing G2/M (+8.08% ±1.84%, p < 0.05). Thus, 3-NAntC impaired cells to proceed to G0/G1 stage, while leading to G2/M phase arrest.

Mechanism of 3-NAntC-induced cellular death.

A Flow cytometry analysis was performed using Annexin V and PI labelling was conducted to determine 3-NAntC effects on the apoptosis/necrosis cellular profile (Figs. 6A-6C). 3-NAntC primarily induced apoptosis in tumor cells and reduced necrosis compared to doxorubicin. At 24 hours, 3-NAntC presented a much lower percentage of necrotic cells than doxorubicin. A significant increase in the apoptosis rate was observed at 24 and 48 hours after treatment with 3-NAntC at 0.8 µg/mL (+20.9%±11.1%, p < 0.05 and +12.4%±3.8%, p < 0.001, respectively), while maintaining the percentage of cells in necrosis. At 72 hours, 3-NantC increased the apoptosis rate at all tested doses reaching a maximum rate of 36.9%±6.6% (p < 0.0001) at 0.8 µg/mL.
The LDH release assay was conducted to determine the effects of 3-NAntC in damaging the cellular plasma membrane. 3-NAntC treatment did not exhibit any consistent increase in LDH release of MDA-MB-231 cells for the 24- and 48-hour treatments (Fig. 6D), indicating that the peptide does not induce cell death by necrosis or pyroptosis in these conditions [17]. Although at 72 hours there was an increase in LDH release, this result could be explained due to the absence of macrophages to finish an apoptosis death by engulfing the post-apoptotic cells [18].

**Cell viability in the presence of the autophagy inhibitor 3-MA.**

Cell viability assay was conducted in the presence and in the absence of 3-MA to determine the effects of 3-NAntC upon autophagy induction. The results showed that 3-MA was unable to revert the decrease of MDA-MB-231 viability promoted by the treatment with 3-NAntC (Supplementary Figure S5). Therefore, 3-NAntC did not induce cell death through autophagy.

**In vivo tolerability in zebrafish.**

In vivo experiments in zebrafish embryos were performed to evaluate 3-NAntC toxicity. Low doses of 3-NAntC (1.5 to 150 µg/mL) and a higher dose (75 mg/mL) showed no lethal effect in vivo and no significant non-lethal (malformations) events (Supplementary Figures S6 and S7). However, at the dose of 150 mg/mL 3-NAntC caused 45% lethality after 48- and 72- hours of treatment and 50–60% of the live embryos exhibited malformations (Supplementary Figure S6). It was not possible to determine the LD$_{50}$ (dose that kills 50% of the embryos) even using high doses of 3-NAntC.

**Discussion.**

Crotoxin is a potent heterodimeric neurotoxin with promising activity against tumoral cells, however with limited applicability in the clinic due to marked toxicity in humans [19]. In a phase I clinical trial, crotoxin was administered intramuscularly to patients with solid tumors resistant to existing therapies for thirty days. Neuromuscular toxicity, including diplopia, strabismus, nystagmus and eyelid ptosis, were the main adverse events observed in 18 of 23 patients [12, 20]. These results were in line with the local myotoxicity and muscle necrosis following its in vivo administration to rats [21] and systemic skeletal muscle damage observed in mice [22]. Considering its toxicity in recent clinical trials using crotoxin, researchers are evaluating the use of intravenous administration to reduce local toxicity, as well as gradual dose escalation to induce neurotoxic tolerance [20].

The molecular mechanism and the aminoacid residues involved in crotoxin pharmacological activities, including the antitumoral and neurotoxic effects, have not been fully elucidated [13, 23]. In this context, the identification of crotoxin B amino acids responsible for the activity against tumoral cells is a promising strategy to develop new crotoxin-based peptides without myotoxicity, nephrotoxicity and cardiotoxicity [13]. The development of venom-based substances has already resulted in approved drugs for several diseases, such as exenatide for Type 2 diabetes, ziconotide for chronic pain, lepirudin and desirudin for thromboembolic disease and eptifibatide for acute coronary syndrome [24]. In oncology,
venom-based synthetic peptides have reached the clinical development stage, including the chlorotoxin-derived peptide $^{131}$I-TM601 for the treatment of gliomas and the soricidin-derived peptide SOR-C13 for solid tumors overexpressing the TRPV6 ion channel [25, 26]. Synthetic peptides based on PLA$_2$ present in Bothrops brazili, Bothrops jararacussu, Bothrops asper and Agkistrodon piscivorus piscivorus snake venoms were shown to exhibit cytotoxic activity against human cancer cell lines [27–29]. For crotoxin B, although no previous assessment of the structure-activity relationship for the antitumoral effect was described in the literature, previous studies showed that the protein cleavage resulted in significantly reduced myotoxicity and PLA$_2$ activity, while only partially affecting antibacterial activity and in vivo lethality in mice [16].

In the present work, crotoxin B-derived peptides were shown to impair the viability of the breast cancer cell lines MCF-7 and MDA-MB-231, with lower negative effect against benign cell lines. Crotoxin activity in breast cancer cells had been previously demonstrated against MCF-7 and ER$^+$ aromatase-overexpressing breast cancer (MCF-7aro) cells [10, 19]. To the best of our knowledge however, this is the first time that crotoxin activity is being explored for the development of novel therapies for TNBC. 3-NantC, a peptide originated from crotoxin C-terminal region was able to selectively reduce viability of breast cancer cells. However not the peptides from the N-terminal region. This result is aligned with the antitumoral activity observed for peptides derived from PLA$_2$ C-terminal region from venoms from other snake species, such as Bothrops brazili and Bothrops jararacussu [27–29].

3-NantC decreased the proliferation rate of tumor cells, which impairs tumor growth, invasion and metastasis [30]. This mode of action has a complementary effect with the cell viability impairment, as any remaining viable cells will be prevented from multiplying. 3-NantC was shown to result in cell cycle G2/M phase arrest in MDA-MB-231 cells, which causes cells to accumulate before the final step of division, thus being more susceptible to apoptosis [31]. Indeed, a higher apoptosis rate was observed in MDA-MB-231 cells treated with 3-NantC in the flow cytometry assay with Annexin V and PI labelling. Comparatively, crotoxin was shown to induce cell cycle arrest at the G2/M phase in MCF-7aro cells [19], however G0/G1 and S phase arrest was observed in lung (SPCA-1) and esophageal (Eca-109) tumors [9, 32].

Seeking to understand 3-NantC's mechanism of action, peptide-induced autophagy was evaluated since this process can be associated with treatment-induced cellular death or with drug resistance [33]. Cellular viability following 3-NantC treatment was not affected by the presence of the autophagy inhibitor 3-MA, thus indicating that the peptide does not induce this cellular process in MDA-MB-231 cell lines. This result also indicates a lower potential for the 3-NantC treatment to result in drug resistance via this cellular process. Crotoxin was shown to induce autophagy in MCF-7 and SK-MES-1 cells, but not in MCF-7aro, thus providing evidence that different mechanisms are involved in the pharmacological response depending on the cell line [10, 19]. Moreover, crotoxin-induced autophagy in MCF-7 cells may be associated with cell membrane disruption, since pretreatment with the autophagy inhibitor 3-MA was previously shown to reduce LDH release [10].
Toxicity comparison with the chemotherapeutic drug doxorubicin evidenced higher necrosis occurrence in MDA-MB-231 cells treated with doxorubicin than with 3-NAntC. This result is consistent with the low LDH release following 3-NAntC treatment in comparison with the literature data for doxorubicin [34].

**Conclusions.**

In summary, we described herein the discovery and characterization of 3-NAntC as a novel peptide active against the TNBC cell line MDA-MB-231. 3-NAntC is simultaneously active on impairing the growth and survival of tumoral cells, while having none or few effects over non-tumor cells. 3-NAntC blocks cell cycle progression by inducing G2/M phase arrest. The 3-NAntC mechanism of action seems to be primarily promoted by cell death via apoptosis with lower necrosis and with no evident autophagy involvement.

The present work is the first to describe a synthetic peptide derived from crotoxin component B (CBa2 isoform) that has reduced toxicity and an improved antitumoral activity against TNBC cells. 3-NAntC was shown to be a promising drug candidate for the treatment of TNBC, which will be further explored in additional *in vitro* and *in vivo* preclinical studies.

**Methods.**

**Synthesis and characterization of Crotoxin-derived peptides.**

Peptides were synthesized by WatsonBio (Houston, Texas, USA) through amino acids coupling and high efficiency synthesis in solid phase assisted by microwaves. The peptide was purified using high efficiency liquid chromatography (HPLC). Gradients of two eluents are used: deionized water acidified with 0.1% trifluoroacetic acid (TFA) as phase A and UV/HPLC grade acetonitrile with 0.08% TFA as phase B. UV detector having an $\lambda_{\text{max}}$ of 220 nm (215–220 nm absorption range of amide group) was used. Purification was conducted in a semi-preparatory scale (5 mg of brut sample and flux of 1 mL/min) at room temperature. For HPLC purity characterization, a Varian model Pro Star 210 chromatograph was used with a detector in the ultraviolet region, model Pro Star 330. The columns used were 4.6×250 mm, Boston Green 0DS-AQ and loops of 5 μL, 250 μL, respectively. A gradient of phases A (0.1% trifluoroacetic in 100% water) and B (0.1% trifluoroacetic in 100% acetonitrile) was employed as following: 0.01 minutes (88:22, respectively), 25 minutes (63:37, respectively) and 25.1 minutes (0:100), stopping at 30 minutes. The flow rate was 1.0 mL/min, injection volume of 10 μL and detection at 220 nm. For sequence confirmation, MS/MS followed the HPLC was conducted as follows: Probe: ESI, Probe bias: 4.5 kV, Nebulizer Gas Floe: 1.5 L/min, Detector: 1.5 kV, CDL:-20.0V, CDL Temp: 250°C, Block temperature:200°C, T. Flow: 0.2 mL/min and B. conc: 50%H2O/50%ACN. Peptide sequences were determined based on MS/MS fragmentation spectra using the Andromeda search engine and MaxQuant environment [35]. Peptides 1 and 2 were obtained based on the N-terminal region (NH2 to COOH, HLLQFNKMIKFETRKNAIPF and AIPFYAFY, respectively). Peptide 3 was obtained based on the C-terminal region (MFYPDSCRGPS). Other 9 peptides derived from fragments and/or cyclization of peptide 3 were also produced.
**Cell culture.**

MCF-7 (Luminal breast cancer), MDA-MB-231 (TNBC) and MCF10A (non-tumor mammary gland epithelial cells) were purchased from the Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, RJ, Brazil). HMEC (Primary Mammary Epithelial Cells) and HDFa (Human Dermal Fibroblasts) cell lines were purchased from Gibco. MCF-7 and MDA-MB-231 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% glutamine and 1% antibiotic/antimycotic solution. MCF10A and HMEC were cultured in MEBM medium supplemented with 100 ng/ml cholera toxin, 2.5 mM L-glutamine, 5% horse serum, 10 µg/mL human insulin, 0.5 µg/mL hydrocortisone and 10 ng/mL EGF. Cells were incubated at 37°C under 5% CO₂ and checked for authenticity through polymerase chain reaction (PCR) followed by fragment analysis of eight highly polymorphic microsatellite loci (short tandem repeat - STR) plus gender determination with score ≥ 80%.

**Cell viability assay.**

Cell viability assay was conducted using the MTT method [36]. The cells were plated with 2x10⁴ cells/well in 100 µL culture medium in 96-wells sterile plates for treatments lasting 24 hours and 1x10⁴ cells/well for 48- or 72-hour treatments with or more, completing the volume to. The plate was incubated for 8–16 hours at 37°C and 5% CO₂. The following day, treatments were prepared in culture medium, by applying 200 µL/well at the desired concentration, and the plate was incubated at 37°C and 5% CO₂ for the treatment duration. Afterwards, 20 µL/well of a MTT solution was added at 5 mg/mL to PBS 1x, incubating the dye at 37°C and 5% CO₂ for 2.5 hours. Then, formazan salts were solubilized with 200 µL of DMSO:isopropanol (3:1) and agitated for at least 15 min at room temperature. After salt dissolution, absorbance was measured at 570 nm and relative cell viability was calculated. To evaluate the mechanism of cell death, cell cultures were treated with peptides in the presence or absence of 3-Methyladenine (3-MA) (1nM), is an autophagy inhibitor [37]. After treatment MTT assay was carried out as previously described.

**Cell proliferation assay using the BrdU method.**

Cell proliferation was assessed using the BrdU method [38]. The cells were plated with 2x10⁴ cells/well in 100 µL culture medium in 96-wells sterile plates for treatments lasting 24 hours and 1x10⁴ cells/well for 48- and 72-hour treatments. The plate was incubated for 8–16 hours in an incubator at 37°C and 5% CO₂. The following day, the treatments were prepared in culture medium, by applying 200 µL/well and incubated at 37°C and 5% CO₂ for different times. BrdU-labeling (20 µL/well) was added and incubated at 37°C for 2.5 hours. The reaction was revealed through ELISA with anti-BrdU-POD antibody, followed by washing and substrate solutions. The substrate was developed for 15 min and the reaction was stopped with HCl (6M). Absorbance was measured at 495 nm and relative cell proliferation was calculated.

**Cell cycle progression assay.**
Cell cycle progression assay using flow cytometry and propidium iodide (PI) was conducted to determine the distribution of cells into the different cell cycle stages [39]. Cells were plated in 6-well sterile plates at 2.5x10^5 cells/well, completing the volume with 1 mL culture medium. The plate was incubated for 8–16 hours at 37°C and 5% CO_2. The following day, the treatments were prepared in culture medium, by applying 2mL/well at the desired concentration, and the plate was incubated at 37°C and 5% CO_2 for the duration of treatment. Afterwards, cells were collected in a conic 15 mL tube and washed with 1mL of PBS. Cells were resuspended in 500 µL/tube of PBS and 4.5 mL of ethanol 70 . Then, tubes were closed and kept at 2–4 °C for up to 2 months. Cells were washed with PBS and resuspended with 135 µL of PBS RnaseA solution at 100 µg/mL was added and incubated at room temperature for 30 min. Cells were labeled with 2 µL PI at 100 µg/mL and analyzed at the flow cytometer for size and DNA content.

**Cellular death assessment.**

Labeling with the fluorescent dyes Annexin V and PI enables the discrimination of cellular death by apoptosis and necrosis [40]. Cells were plated in 6-wells sterile plates at 2.5x10^5 cells/well, completing the volume with 1000 µL culture medium. Plates were incubated for 8–16 hours at 37°C and 5% CO_2. The following day, the treatments were prepared in culture medium, by applying 2,000 µL/well at the desired concentration, and the plate was incubated at 37°C and 5% CO_2 for the duration of treatment. Afterwards, cells were collected in a conic 15 mL tube and washed with 200 µL of binding buffer (1x) from the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences). Cells were resuspended in 100 µL/tube of binding buffer (1x) and 1 µL of Annexin V-FITC was added to each tube except the blank. Cells were then incubated for 20 min in ice and in the dark. Then, 50 µL/tube of binding buffer was added and cells were labeled with 2 µL of PI (100 µg/mL) and read at the flow cytometer for size and fluorescent content.

**Cellular death assay using the LDH release method.**

The CyQUANT LDH Cytotoxicity Assay (Thermo Fisher) was employed to determine LDH release as a measure of plasma membrane damage, an important indicator of necrosis and pyroptosis [41]. It is known that LDH extravasation does not occur in apoptosis due to formation of apoptotic bodies and engulfment of material by leukocytes [17, 42]. Cells were plated in 96-wells sterile plates for cell culture at 2x10^4 cells/well for treatments lasting 24 hours and 1x10^4 cells/well for treatments with 48 hours or more, completing the volume to 100 µL with culture medium. The plate was incubated for 8–16 hours at 37°C and 5% CO_2. The following day, treatments were prepared in culture medium, by applying 200 µL/well at the desired concentration, and the plate was incubated at 37°C and 5% CO_2 for the treatment duration. Then, 20 µL of supernatant was transferred to an EIA/RIA plate. The Reaction Mixture (20 µL/well) was added and incubated at room temperature for 30 min in the dark. Afterwards, 60 µL/well of the Stop Solution was added and the absorbance of the reaction mixtures was quantified at 490 nm and 680 nm. The absorbance at 680 nm was subtracted from the value at 490 nm. Relative LDH release compared to the control without treatment was calculated.

**In vivo zebrafish toxicity study.**
Transgenic Tg(fli1:EGFP)y1 zebrafish embryos were raised at 28°C for 48 hours in E3 embryo medium 0.2 mM 1-Phenyl-2-Thiourea (PTU). Unfertilized eggs or larvae that did not appear healthy or exhibited any obvious developmental defects were excluded before treatment onset (~10%). Zebrafish embryos obtained at 48 hours post fertilization (hpf), n = 20 embryos/group, were subcutaneously injected with the 3-NAntC peptide at 0.0015, 0.015, 0.15, 75 and 150 mg/mL diluted in E3 embryo medium containing 0.003% 1-phenyl-2-thiourea (PTU) and the embryos were maintained at 28.5°C. Pictures were taken after 24, 48 and 72 hours of peptide exposure and the percentage of live animals, as well as the percentage of live animals with malformations, were visually analyzed.

**Statement of ethical approval.**

The animal experiments were conducted following institutional ethical guidelines. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of BioReperia AB (Sweden). Data reporting follows the recommendations of the ARRIVE guidelines.

**Statistical analysis.**

The *in vitro* cell viability, cell proliferation, cell cycle progression and cell apoptosis/ necrosis profile were analyzed using the two-way ANOVA followed by Dunnett or Bonferroni post-hoc test to the significance of 5%. LDH release was analyzed using the one-way ANOVA followed by Dunnett post-hoc test to the significance of 5%. GraphPad Prism v6.0 was used for the analysis.

**Abbreviations.**

ANOVA, analysis of variance; BrdU, 5-Bromo-2’-deoxyuridine; CTX, crotoxin; CTX CB, crotoxin component B; CTX CB1, crotoxin component B isoform CB_a2; DMSO, dimethylsulfoxide solution; HPLC, high efficiency liquid chromatography; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PARP, poly adenosine diphosphate – (ADP)-ribose polymerase; TNBC, triple-negative breast cancer.

**Declarations**

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Author contribution.

The authors confirm contribution to the paper as follows: study conception and design: P.H.A.B. and E.M.; data acquisition: P.H.A.B.; data analysis and interpretation: P.H.A.B. and E.M.; draft manuscript preparation: P.H.A.B. and E.M. All authors reviewed the results and approved the final version of the manuscript.

Data availability.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests.

P.H.A.B. and E.M. were employed at PHP Biotech International when this work was conducted. The author(s) declare no other competing interests.

References


Figures

A

N-terminal

<table>
<thead>
<tr>
<th>Peptide 1</th>
<th>Peptide 2</th>
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<tr>
<td>HLLQFNKMIKFEKTEKNAIPYAFYG</td>
<td>CCGWGRGRPRKEDATDRCGFVHDOCKYGLAKNTKEDYTPSYLKSGYITCGKGTWCE</td>
</tr>
<tr>
<td>EQICECQDRVAAECLRSSSTKYGMFTYPSRCRGPEYC</td>
<td></td>
</tr>
</tbody>
</table>

C-terminal

B

Chain C

C-terminal

Chain B

N-terminal

Peptide 1 and 2

1 - 24 aa, Chain B

Peptide 3

108 - 121 aa, Chain C

Figure 1
Amino acid sequence and structure of crotoxin B (PLA$_2$) from *Crotalus durissus terrificus* snake venom.

(A) Amino acid sequence of crotoxin B. The regions that originated the peptides 1, 2 and 3 are shown within the blue box. The residues from the catalytic site are highlighted in yellow, while the residues from the Ca$^{2+}$ binding site are highlighted in green. The residues highlighted in purple are important for crotoxin B toxicity/ lethality, myotoxicity, edema induction, antibacterial, liposome disrupting, enzymatic and anticoagulant activities. (B) Structure of crotoxin B (PDB: 2QOG) with the regions that originated peptides 1 and 2 highlighted with carbon atoms in green and peptide 3 highlighted with carbon atoms in purple.

**Figure 2**

**Evaluation of crotoxin B-derived peptides on cellular viability against TNBC cells and mammary benign cells.** MDA-MB-231 (blue line) and MCF10A cells (black line) were treated with peptides 1, 2 and 3 for 24 and 48 hours at the concentration range of 0.2 – 20 µg/mL. Cellular viability assay was evaluated using the MTT method. Data are shown as mean ± s.e.m. of at least three independent assays. Treatment groups were compared to the control (without peptides treatment) using two-way ANOVA and Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 3

3-NAntC (peptide 3) activity in breast cancer cell lines vs. benign cells. Tumor (MDA-MB-231 and MCF-7) and benign (HDFa, HMEC and MCF10A) cell lines were treated with 3-NAntC for 24, 48 and 72 hours at the concentration range of 0.2 – 1.0 µg/mL. Cellular viability assay was conducted using the MTT method. Data are shown as mean ± s.e.m. of at least three independent assays in triplicate. (A-C) Benign cell lines treated with the same concentrations of 3-NAntC were compared using two-way ANOVA and Bonferroni post-hoc test. (D-F) MDA-MB-231 and MCF-7 tumor cell lines were treated with different concentrations of 3-NAntC and compared with the HMEC benign cell line using two-way ANOVA and Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 4

Comparative decrease in cell viability of 3-NAntC vs. the conventional therapy with doxorubicin and cisplatin. Cellular viability assays were conducted for MDA-MB-231 (A-C) and HDFa (D-F) cell lines using the MTT method. Cells were treated with 3-NAntC, cisplatin or doxorubicin for 24, 48 and 72 hours at the concentration range of 0.2 – 2.0 µg/mL. Data are shown as mean ± s.e.m. of at least three independent assays in triplicate. Treatment groups were compared using two-way ANOVA and Bonferroni post hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 denotes significance vs. the negative control without the drugs.
Figure 5

Antiproliferative effects of 3-NAntC in TNBC and normal cells. (A-B) Cellular proliferation assay using the BrdU incorporation method. MDA-MB-231 and HDFa cells were treated with 3-NAntC for 24, 48 and 72 hours at the concentration range of 0.2 – 1.0 µg/mL. Data are shown as a mean ± s.e.m. of at least three independent assays in triplicate. Treatment groups were compared at the same time of exposure using two-way ANOVA and Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 shows significance vs. the negative control (0 µg/mL), #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 shows significance vs. the same concentration in HDFa cell lines. (C) Cell cycle progression assay. Cells were treated with 3-NAntC for 48 hours at the concentration range of 0.2 – 0.8 µg/mL. A flow cytometry assay was conducted with propidium iodide labeling. Data are shown as a mean ± s.e.m. of at least three independent assays. Treatment groups were compared to the negative control without the drugs using two-way ANOVA and Dunnett post-hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
Figure 6

Effect of 3-NAntC in apoptosis/ necrosis profile of the MDA-MB-231 cell line. (A-C) A flow cytometry with Annexin V and Propidium iodide labelling was performed. Cells were treated with the peptide 3-NAntC (0-0.8 µg/mL) or with doxorubicin (0.2 µg/mL) for 24, 48 and 72 hours. Cells were labeled with Annexin V and propidium iodide and evaluated using flow cytometry. 3-NAntC induced cell death by apoptosis with a very low amount of necrosis. Data are shown as a mean ± s.e.m. of at least three independent assays. Treatment groups were compared vs. the negative control with no addition of peptide treatment at the same time of exposure using two-way ANOVA and Dunnett’s post-hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. (D) LDH release assay. Cells were treated with the 3-NAntC peptide (0-1.0 µg/mL) for 24, 48 and 72 hours and the LDH release was evaluated. There is no significant difference in this biomarker, suggesting no involvement of either necrosis or pyroptosis as a primary cell death mechanism. Data are shown as mean ± s.e.m. of at least three independent assays in triplicate. Treatment groups were compared vs. the negative control (without treatment) at the same time of
exposure using one-way ANOVA and Dunnett's post hoc test, \( *p<0.05, **p<0.01, ***p<0.001 \) and \( ****p<0.0001 \).

**Supplementary Files**

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