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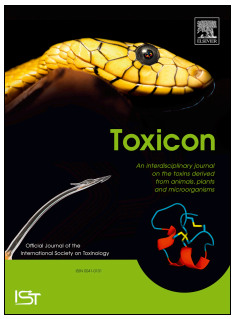

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Crotoxin from *Crotalus durissus terrificus* venom: *in vitro* cytotoxic activity of a heterodimeric phospholipase A_2 on human cancer-derived cell lines

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Abstract

Crotoxin (CTX), a heterodimeric phospholipase present in venom of snakes of the genus Crotalus, has demonstrated a broad spectrum of pharmacological properties, such as antimicrobial, hemostatic, and antitumoral. However, the precise mechanism of its cytotoxicity and antitumoral properties remains to be determined. Therefore, in the present study, we isolated crotoxin (F1 CTX) through two steps DEAE-Sepharose and Heparin-Sepharose FF chromatography. The C-terminal sequence of the A- and B-chain protein fragment was determined by LC-MS/MS mass spectrometry, which showed 100% identity to crotoxin structure. In order to investigate its cytotoxic effects, we demonstrated that the F1 CTX fraction at 0 to 30 µg/mL concentrations for 72 h presented a heterogeneous response profile on nine human cancer-derived cell lines from four tumor types (pancreatic, esophagus, cervical cancer, and glioma). The glioma (GAMG and HCB151) and pancreatic (PSN-1 and PANC-1) cancer cells showed a higher sensitivity with IC_{50} of <0.5, 4.1, 0.7 and <0.5 µg/mL, respectively. Conversely, F1 CTX does not reduce the viability of normal cells. On the other hand, cervical (SiHa) and esophagus (KYSE270) cancer cell lines presented higher resistance, with IC_{50} higher than 30.2 and 8.7 µg/mL, respectively. Moreover, F1 CTX did not affect cell cycle distribution under the conditions evaluated and seems to be more cytotoxic than cytostatic. The pro-apoptotic effect of F1 CTX treatment was demonstrated in glioma (HCB151) cell line. In addition, crotoxin revealed a potential to initiate cell responses such as DNA damage in glioma (HCB151) and pancreatic cancer by H2AX activity induction. Conversely, F1 CTX does not reduce the viability of normal cells. Importantly, the comparison of F1 CTX effect with standard chemotherapeutic agents demonstrated a greater cytotoxic potential in the majority of tumor types (glioma, pancreatic, and cervical cancer). On the other hand, F1 CTX was less cytotoxic in esophageal cell lines compared to the gemcitabine agent used in clinical practice. Therefore, this work showed that F1 CTX has a cytotoxic activity and pro-apoptotic potential, contributing to the knowledge about the F1 crotoxin properties as well as its possible use in cancer research, particularly in glioma and pancreatic cancer cell lines.

Keywords: Crotoxin; snake venom; cytotoxicity; antitumor; glioma; pancreas

1. **Introduction**

Snakes of the genus *Crotalus* produce and secrete a complex of biologically active substances, among them the phospholipase A_2 (PLA_2) (Calderon et al., 2014). Snake venom PLA_2 (svPLA2s) comprises a large class of molecules that catalyze the hydrolysis of sn-2 position phospholipids, releasing fatty acids and lysophospholipids. svPLA2s are related to a broad spectrum of biotechnological activities, including antitumoral, antimicrobial, and hemostatic. The cytotoxic activity has been suggested to be a result of different cell death mechanisms, which has been demonstrated *in vitro* in melanoma, neuroblastoma, and leukemic cell lines (Baldi et al., 1998; Iglesias et al., 2005; Yan et al., 2007; Rodrigues et al., 2009).

Among the PLA_2 isolated from snake venom, crotoxin (CTX), a β-neurotoxin, is a heterodimeric complex composed of a toxic phospholipase (PLA_2) fraction, associated with the non-enzymatic fraction crotapotin (Sobrinho et al., 2016). Parallel to its toxic effects, numerous scientific findings have demonstrated the application of CTX in the pharmacological field, with a broad spectrum of functional properties (Donato et al., 1996; Costa et al., 1997; Sampaio et al., 2010). CTX cytotoxicity is mediated by specific interactions with cell surface receptors and associated with transmembrane ligands, which are involved in cell damage (Krizaj et al., 2000; Montecucco et al., 2008).

The antitumor and antiproliferative activity of CTX in different cancer cell lines including leukemia, cervix, ovarian, lung, colon, renal, melanoma, and brain has been assessed in *in vitro* and *in vivo* studies. According to these studies, the antiproliferative activity occurs through apoptotic mechanisms, triggered by changes in mitochondrial membrane potential, cytochrome C release, and caspase-3 activation (Corin et al., 1993; Newman et al., 1993; Costa et al., 1998; Brigatte et al., 2016). CTX may also induce cell death by activation of autophagy mechanisms, which was demonstrated in breast tumor cells (Yan et al., 2007). Moreover, the ability of CTX to cause cytocidal and memory antitumor immunity in mice have been determined in lymphoma, adenocarcinoma, human lung squamous cell carcinoma and esophageal cancer cell lines (Ferguson, Duncan, 2009; Wang et al., 2012; Qin et val., 2016). Studies of CTX structure and function have brought relevant information for applications of this molecule in the treatment of different types of cancer, including phase 1 clinical studies for the treatment of refractory solid tumors associated with conventional therapies. CTX has

been investigated for its effective application in malignant tumor treatments, *in vivo* or *in vitro*, alone or in combination with antitumor drugs (Rübsamen et al., 1971; Aird et al., 1989; Rudd et al., 1994; Donato et al., 1996; Ye et al., 2011; Han et al., 2014; He et al., 2016). However, new molecular mechanisms must be elucidated to apply the molecule in neoadjuvant antitumor therapies.

To better understand the antitumor properties of CTX, in the present study, we isolated crotoxin from *Crotalus durissus terrificus* snake venom, evaluated its response profile in nine human cancer-derived cell lines, and analyzed its cytotoxicity, antiproliferative and pro-apoptotic potential.

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2. **Materials and Methods**

2.1. *Reagents and venom*

The yellowish *Crotalus durissus terrificus* venom was purchased from Koemitã Me (Mococa, SP, Brazil). Sepharose G-75 and Heparin-Sepharose FF were purchased from Amersham Life Science, Inc. All of the other reagents used in this study were of analytical grade and purchased from Sigma Chem. Co, Merck and/or Synapse Biotechnology.

2.2. *Purification of Crotoxin (CTX)*

C. durissus terrificus crude venom (0.5 g) was applied to a DEAE-cellulose column, which had previously been equilibrated with 0.05 M Tris-HCl, pH 7.5 (buffer A), and the protein was eluted with 0.05 M Tris-HCl, pH 7.5 in a linear gradient of NaCl (0–1 M); 2 mL fractions were collected. The eluted fraction with crotoxin activity was concentrated in an ALPHA 2-4 LD plus Freeze-Dryer. Of the F4 CTX fraction, 50 mg were applied to the Heparin-Sepharose FF column (HiTrap, heparin (HP), 5 mL), which had previously been equilibrated with 0.01 M sodium phosphate, pH 7.0. The protein was eluted in linear gradient of NaCl (0–1.5 M) at a flow rate of 2.5 mL/min, and 3 mL fractions were collected. The F1 CTX fraction was pooled and functional and biochemical characteristics were assessed. Chromatography was performed using the ÄKTAprime and liquid chromatography system (GE Healthcare). Next, F1 CTX was subjected to identification and cytotoxic effects evaluation. All purification and isolation procedures were performed at room temperature.

2.3. *Identification of Crotoxin: internal peptide fragments*

The purity of F1 CTX was determined by 15% (w/v) SDS-PAGE in a Tris-glycine buffer of pH 8.3, for 120 min at 20 mA/100 V. The molecular mass was measured using the Carestream Molecular Imaging Software (Carestream Health, Inc., 1994-2011) and at 10–260 kDa molecular weight calibration standards (Espectra^{TM} Multicolor Broad Range).

The identification of crotoxin was determined by mass spectrometry. A coomassie brilliant blue-stained crotamine band (approximately 2 µg/spot) was cut out of the polyacrylamide gel (13%) and digested “in-gel” for peptide mass fingerprinting and for internal

sequence determination. Five micrograms of isolated F1 CTX were reduced in 10 mM DTT, alkylated in 50 mM of iodoacetamide, and then trypsinized in 20 ng/μL trypsin after gel electrophoresis, according to the protocol reported by Shevchenko et al. (1996), with some modifications. Tryptic fragments of the peptide were scored by the bond cleavage, and charges on the C-terminus (a ions) and N-terminus (b ions) were generated. Peptide identification was performed in a liquid chromatography-tandem mass spectrometer using a C18 nanocolumn (LC-MS / MS Q-TOF PREMIER^{TM}). The resulting spectra were analyzed using Mascot (Matrix Science) in the NCBI protein databases, with carbamidomethylation as the fixed modification. The similarity between peptide sequences was assessed with BLAST.

2.4. *Cytotoxic activity evaluation*

2.4.1. *Cell lines and cell culture*

Nine immortalized human cancer-derived cell lines were used in the cytotoxicity assays, comprising brain (glioma), pancreatic, cervical, and esophageal tumor models. In addition, immortalized keratinocytes (hacat cells - ThermoFisher) and murine fibroblasts (NIH/3T3 (ATCC® CRL-1658™) were used as non-cancer cells. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM 1X, high glucose; Gibco, Invitrogen) or Le Roswell Park Memorial Institute medium (RPMI-1640 1X, Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen) and 1% penicillin/streptomycin solution (Gibco, Invitrogen), at 37℃ and 5% CO_2, as previously reported (Silva-Oliveira et al., 2016; Silva et al., 2018). Authentication of cancer cell lines was conducted by the Diagnostic Laboratory of Barretos Cancer Hospital (São Paulo, Brazil) as reported (Dirks et al., 2005; Silva-Oliveira et al., 2016; Teixeira et al., 20016). The cell lines identities were confirmed by genotyping, with the exception of U373, which was shown to be a subclone of the U251 lineage. In addition, a primary tumor culture (HCB151) was obtained from a glioblastoma biopsy and provided by the Department of Neurosurgery of the Barretos Cancer Hospital (Cruvinel-Carloni et al., 2017).

2.4.2. *Cell viability assay*

The cytotoxic effect of F1 CTX or standard chemotherapeutic agents (paclitaxel (Sigma

- *T7402*), gemcitabine hydrochloride (Sigma - G6423), temozolomide (Sigma - *T2577**)*** and cisplatin (Sigma - *479306*) was analyzed by the MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], (Cell Titer 96 Aqueous cell proliferation assay-MTS, PROMEGA). Cell viability was determined by the survival rate, after incubation with the MTS reagent (2h; 37℃, 5% CO_2) in the cell culture (until a maximum 5 x 10^3 cells/well), which was treated with increasing concentrations of F1 CTX (0.5–30 µg/mL or standard chemotherapeutic agents (0.5–150 µg/mL); 72h), and compared to the control group (1% DMSO, final concentration). The absorbance was measured spectrophotometrically (λ_{490nm}) in an automatic microplate reader system (Varioskan, Thermo). The results obtained in the cell viability assay with the different concentrations of F1 CTX or standard chemotherapeutic agents were converted to percent viability in relation to the control (1% DMSO) ± SD. All the assays were done in triplicate and repeated at least three times The half-maximal inhibitory concentration (IC_{50}) was calculated by non-linear regression analysis using GraphPad PRISM 5.1 (GraphPad Software, La Jolla California USA), as previously reported (Teixeira et al., 2016).

2.4.4. *Effects of CTX on cell cycle and apoptosis*

The effects of F1 CTX on cell cycle and apoptosis was evaluated in HCB 151 (drug-sensitive) and SiHa (drug-resistant) cell lines (1×10^6 cells/well) using a concentration equivalent to IC_{50} value of each cell line for 24 h. For cell cycle analysis, cells were examined using PI stain to determine DNA content and analyzed using Cycle Test kit (BD Biosciences) following the manufacturer's recommended protocol. Apoptosis assays were performed using the Annexin V-FITC kit (BD Biosciences) according to the manufacturer's recommendations. The distribution profile (G1, S, and G2/M) and the percentage of apoptotic cells were characterized by flow cytometry using the BD FACSCanto II reagent kit (BD Biosciences) and analyzed with BD FACSDiva software (BD Biosciences).

2.4.5. *Molecular Analysis by Western Blot*

In a direct immunoblot experiment, HCB151, PANC-1, and SiHa cell lines (1×10^6 cells/well) were exposed to IC_{50} concentration values of F1 CTX for 24h. Protein samples,

previously obtained by cell lysis, were separated and analyzed by SDS-PAGE and transferred to a Hybond-C nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) using a mini turbo transfer system (Trans-Blot® BioRad) as reported (Plescia et al., 2005). Membranes were blocked for 1h (5% milk powder in TBS/0.1% Tween (TBS-T; pH = 7.6) and incubated overnight (4℃) with the primary antibodi es (p-H2AX, p-AKT, AKT, p-P44/42 (MAPK- ERK1/2), p44/42, p21, and β-tubulin) diluted 1:1000 (Cell Signaling Technology). The washed membranes were incubated with the secondary antibody and coupled to horseradish peroxidase, 1:5000 (Cell Signaling Technology). β-tubulin was used as loading control. Protein bands were determined by chemiluminescence (ECL Western Blot Detection Reagents, RPN2109, GE Healthcare) and signal intensity was assessed with the ImageQuant™ LAS 4000 mini photographic documentation system (GE Healthcare). The densitometry analysis of immunoblots was performed with the Image J software (version 1.41; National Institutes of Health).

5. *Statistical analysis*

Student's t-test was used to compare each experimental group with the control group. The p value <0.05 indicated a significant difference between the samples. Graphs and statistical analysis were performed in GraphPad Prism 5.1 software.

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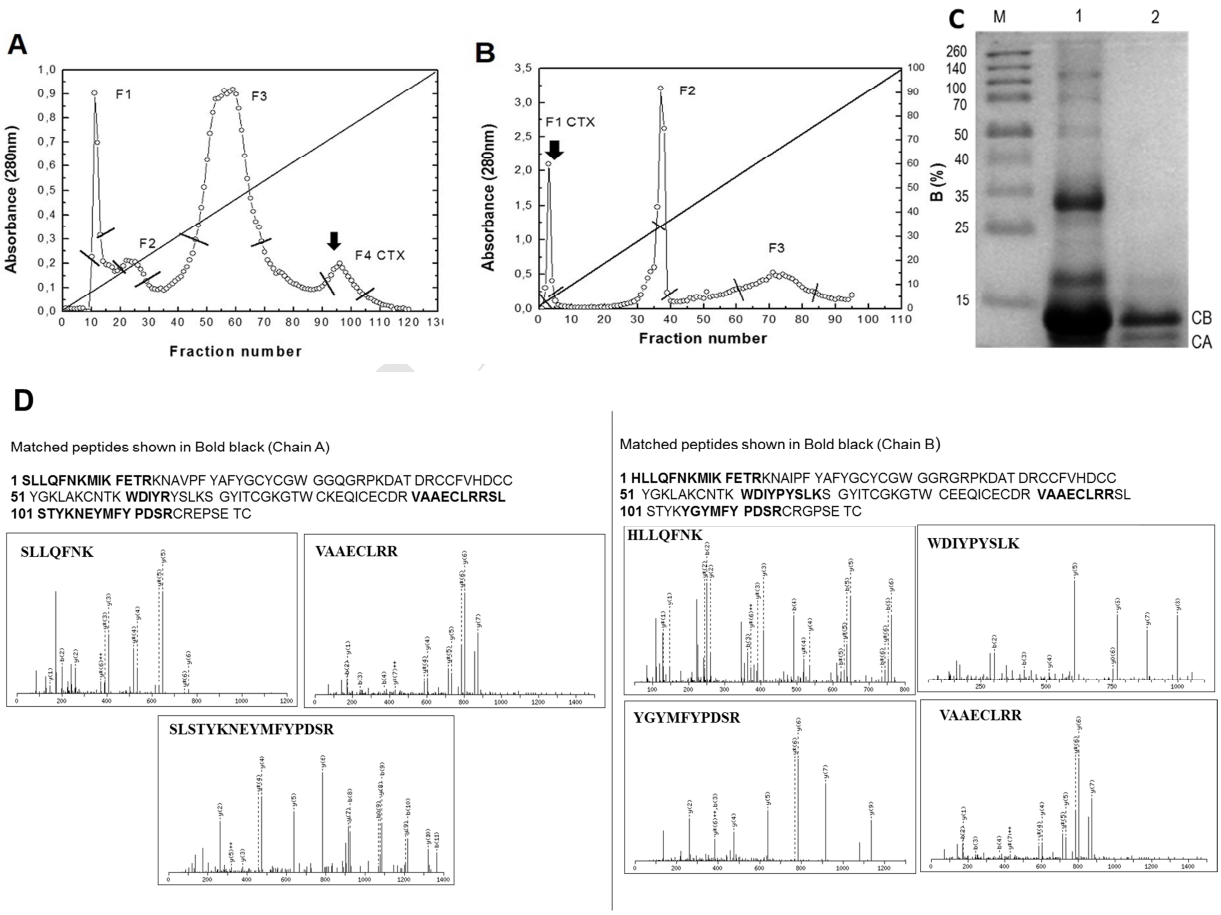
3. **Results**

3.1. *Purification and identification of CTX*

The F1 CTX molecule was isolated in two chromatographic steps: first, by exclusion chromatography using a DEAE-cellulose column (Figure 1A), and second, by bioaffinity chromatography in a Heparin-Sepharose FF column (Figure 1B). The degree of purity was assessed by SDS-PAGE revealing a dimeric, low molecular weight protein consisting of two subunits with apparent molecular weight between 14–15 kDa (Figure 1C). Further peptide fragments identified in the mass fingerprinting corresponding to MS/MS fragmentation of three internal peptide fragments (Chain A) and four fragments (Chain B), confirmed the molecular identity of crotoxin (Figure 1D).

Fig. 1



194 3.2. *CTX cytotoxic profile*

195 CTX cytotoxic potential was determined by MTS assays in brain (glioma), pancreatic,
196 cervical, and esophageal cancer cells at different concentrations (0 to 30 µg/mL). The authors
197 suggested F1 CTX might be a selective anti-cancer agent, discriminating between normal and
198 tumor cells. The dose-response curves showed that cell lines exhibited a heterogeneous
199 cytotoxic profile in response to F1 CTX (Figure 2 and table 1). The mean IC_{50} was 8.6 µg/mL
200 and varied significantly between cell lines, with differences of more than sixty-fold (IC_{50} range:
201 <0.5 – >30 µg/mL) (Table 1). The KYSE 30 (esophageal), GAMG, HCB151 (glioma), PSN-1,
202 PANC-1 (pancreatic), and HeLa (cervical) tumor cells showed higher sensitivity, with IC_{50} of
203 1.0, <0.5, 4.1, 0.7, <0.5, and 2.4 µg/mL, respectively (Figure 2 A, C, E, F, G and H). On the
204 other hand, the KYSE 270 (esophageal), U373 (glioma), and SiHa (cervical) cells exhibited
205 higher resistance, with IC_{50} of 8.7, 30.2 and >30.0 µg/mL, respectively (Figure 2 B, D, and I).
206 In contrast, identical concentrations of F1 CTX did not reduce the viability of normal human
207 keratinocytes (HaCaT) or mouse fibroblasts (3T3) cell lines on the same conditions of assay
208 (Figure 2 J and K).

209 Additionally, we compared the F1 CTX cytotoxic potential with standard
210 chemotherapeutic agents for glioma, pancreatic, esophagus, and cervical cancer. CTX
211 treatment was more cytotoxic in the majority of tumor types (glioma, pancreatic, and cervical
212 cancer). In contrast, F1 CTX was less cytotoxic in esophageal cell lines compared to the
213 gemcitabine agent used in clinical practice (Table 1).

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223 **Fig. 2**

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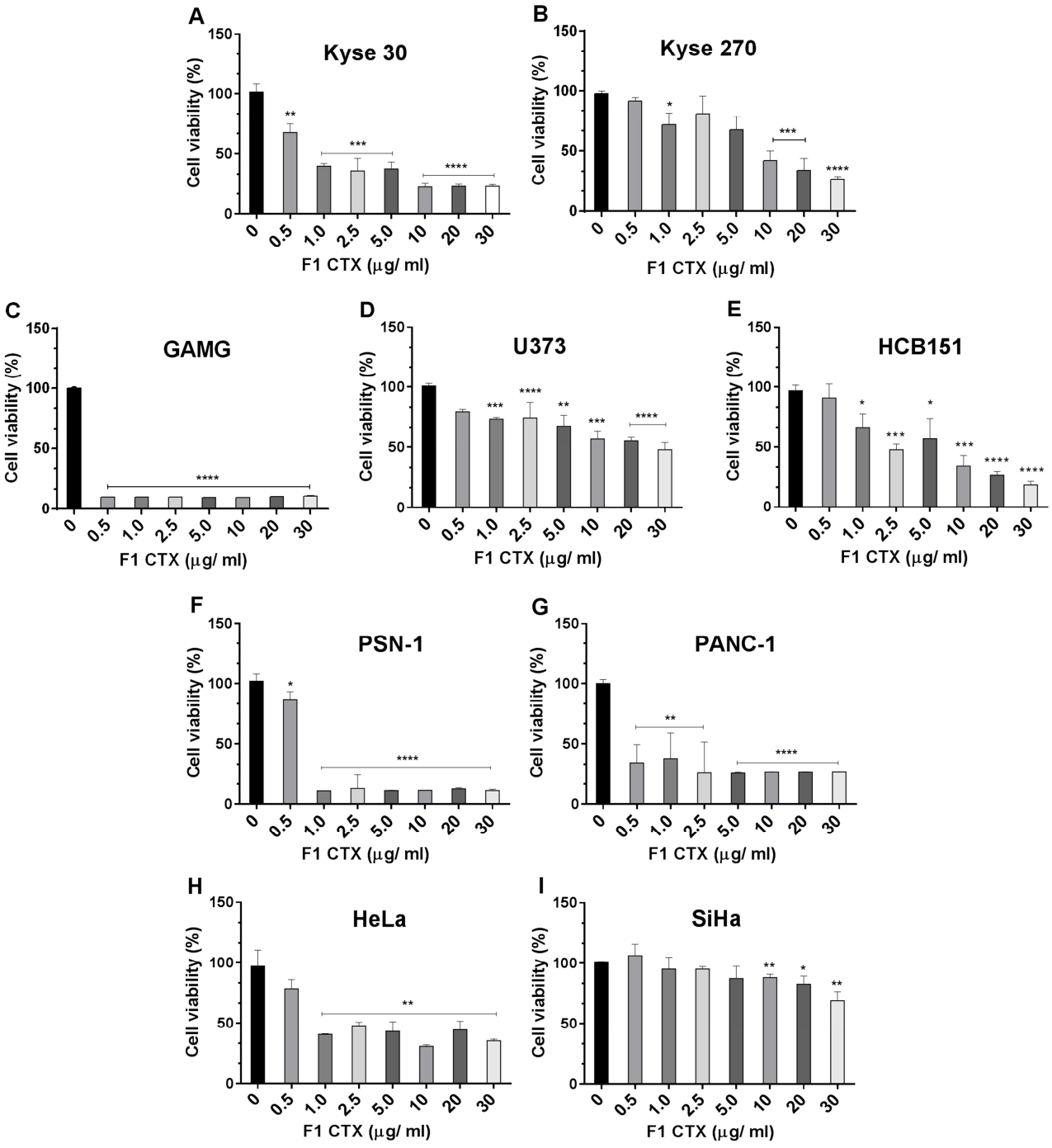

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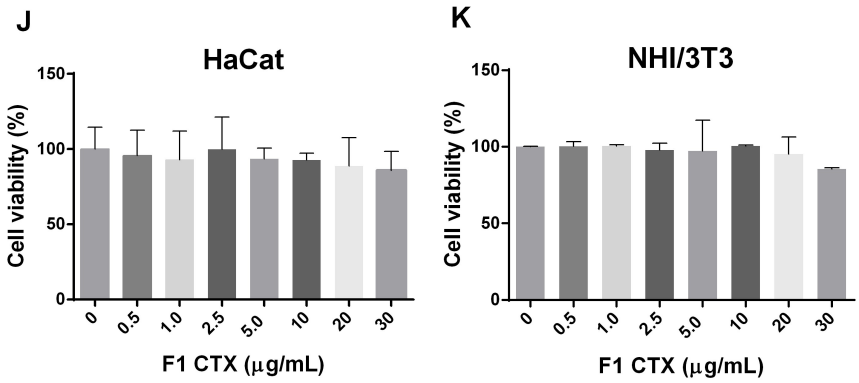

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259 3.3. *CTX antiproliferative and pro-apoptotic effects*

260 Since we observed a cytotoxic effect of F1 CTX in cancer cells, we further determined
261 its effect on cell cycle distribution and cell death using flow cytometric analysis. As shown in
262 Figure 3 (A,B,C,D), no significant effect was observed in drug-sensitive (HCB151 – 4.1μg/mL)
263 or drug-resistant cells (SiHa -30 μg/mL) after toxin treatment, suggesting that CTX at
264 concentrations used has no interference on cell cycle distribution under the conditions
265 evaluated (Figure 3 B.D). Next, we examined the apoptosis-inducing effect of F1 CTX. The
266 basal population of early and late apoptotic cells in the untreated cultures was 0.6% and 0.1%
267 (HCB 151) and 4.8% and 7.2% (SiHa). However, when cancer cells were treated with F1 CTX
268 at IC_{50} value for 24 h, the apoptotic cells (early + late apoptosis) increased by 13.2% and
269 11.0% (HCB 151) compared to the control cells (Figure 4 A,B). No significant effect was
270 observed in SiHa cell (Figure 4 C,D).

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287 **Fig. 3**

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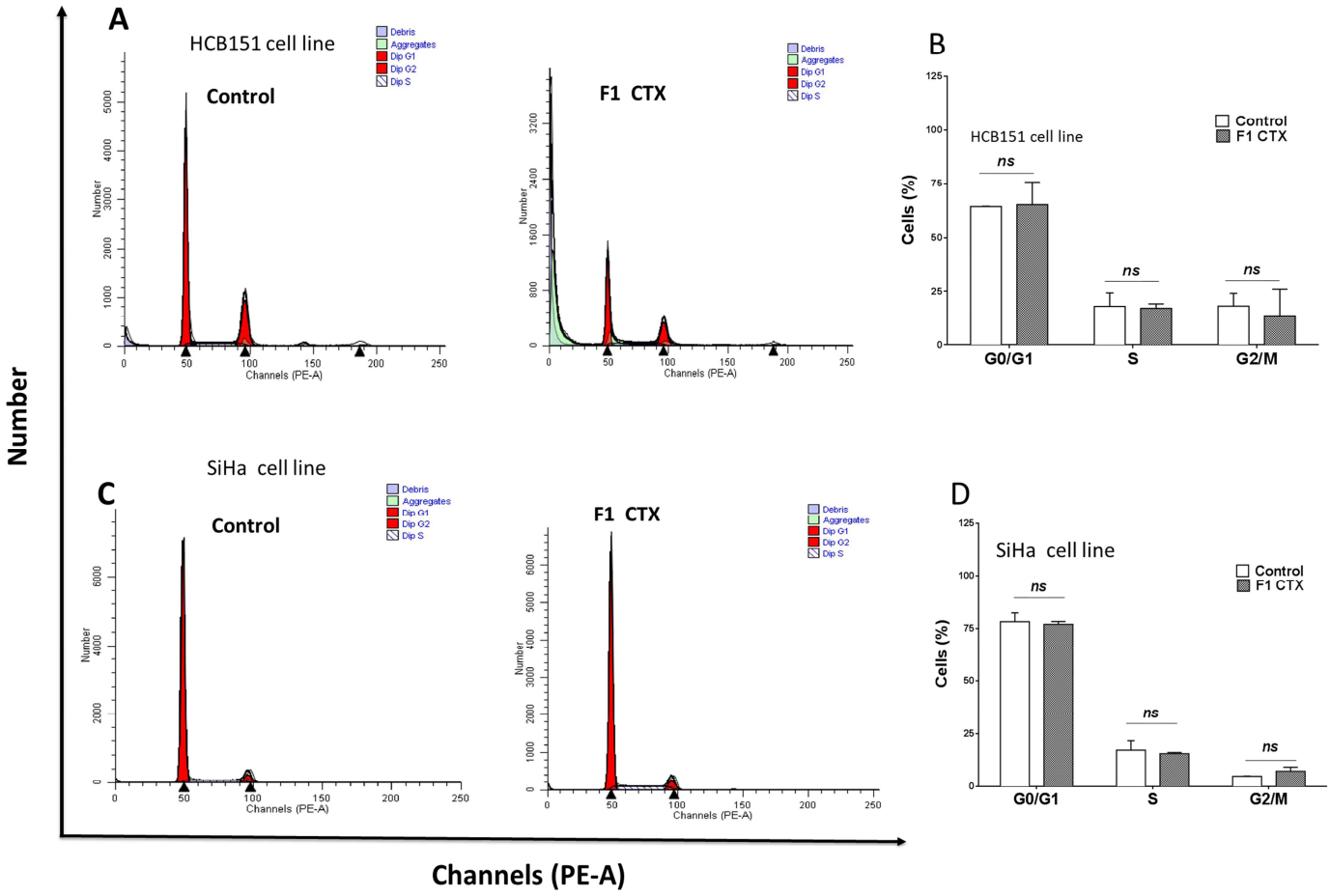

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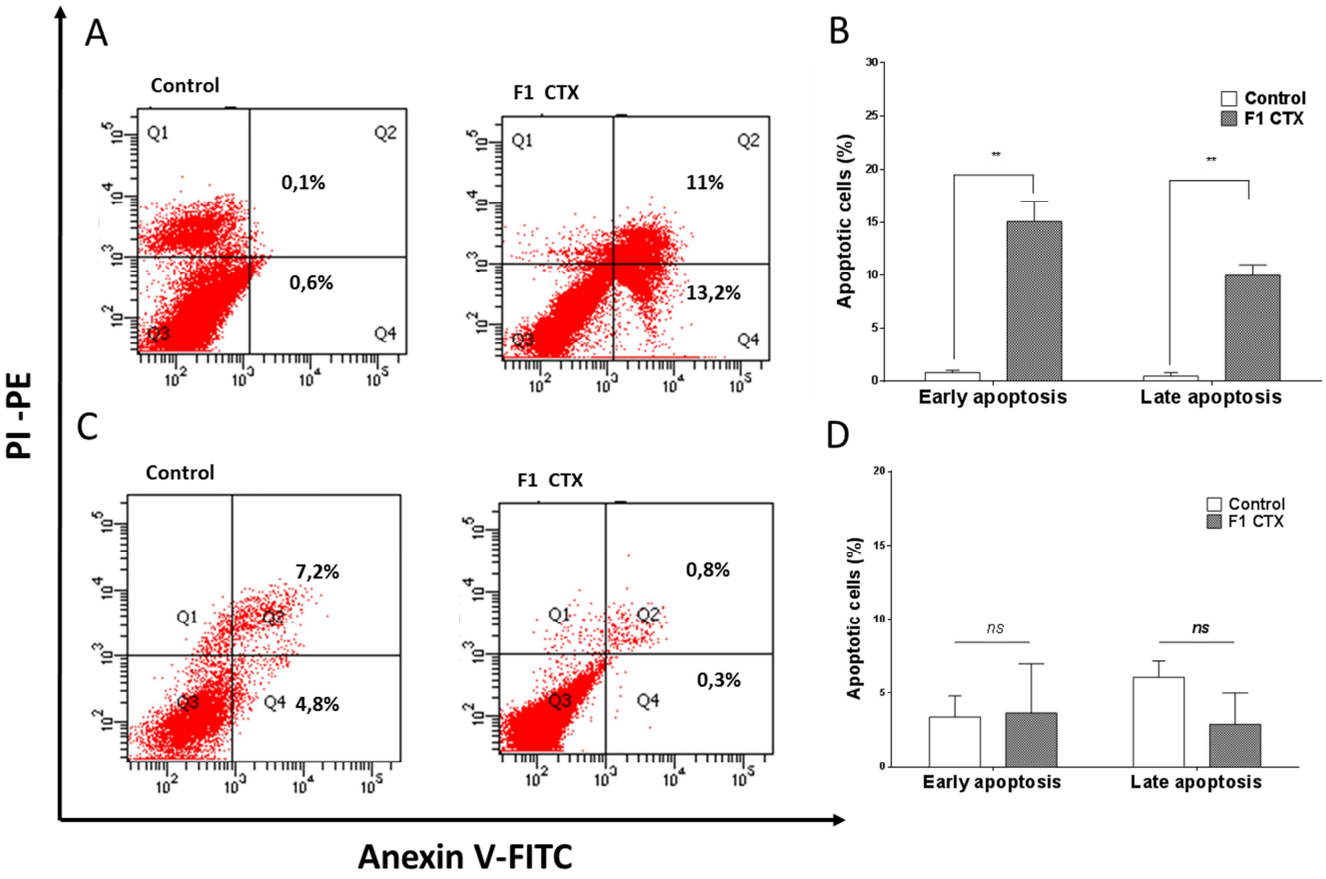
Fig. 4

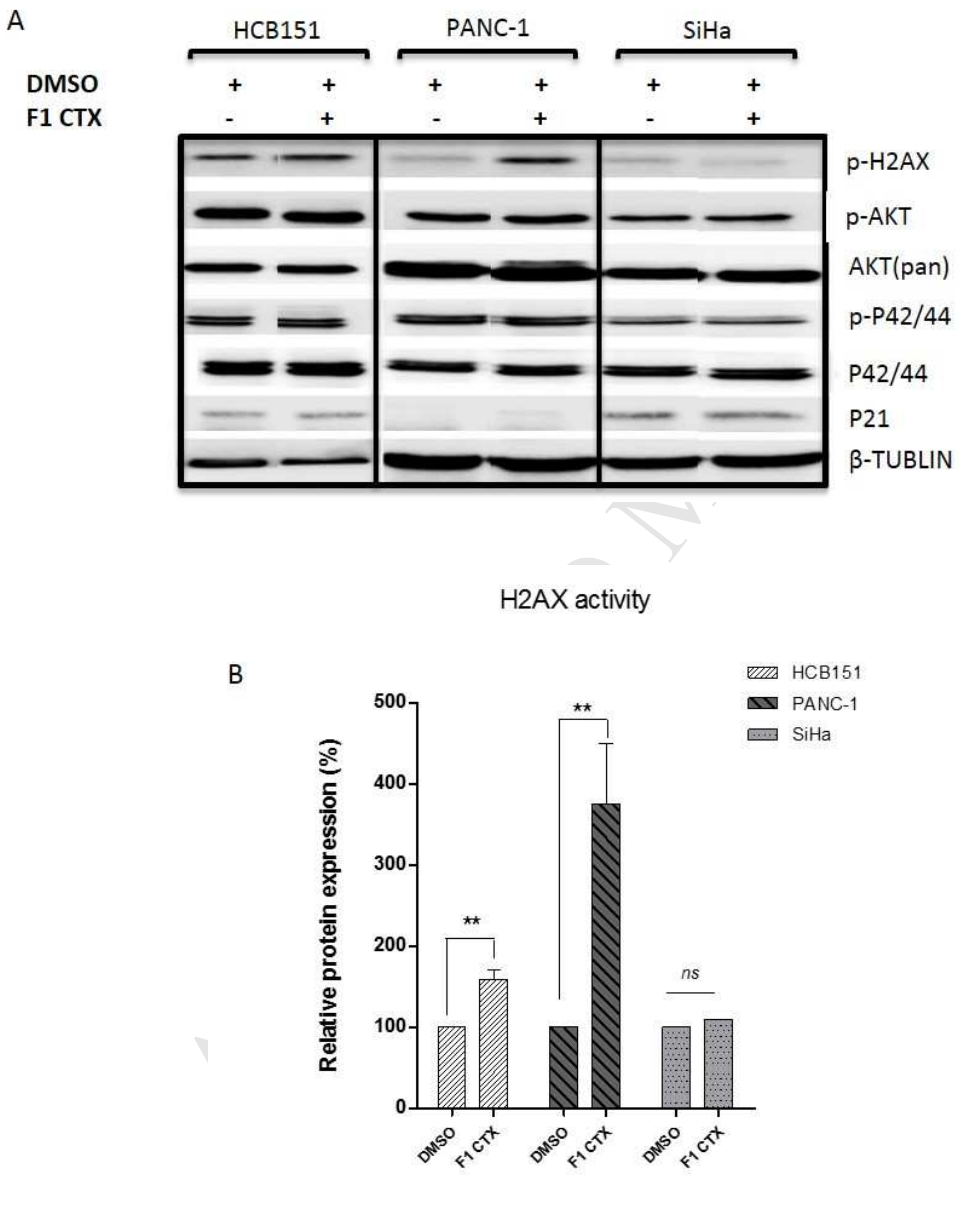


3.4. *Effects of CTX on cell signaling*

To better explore the underlying mechanisms of F1 CTX, we assessed the expression of the main proteins related to proliferation/survival and DNA damage mechanism induced by molecule. The protein expressions after F1 CTX treatment in cancer cells (HCB151, PANC-1 and SiHa) were quantified relative to untreated control cells. As shown in Figure 5, we observed an increase of H2AX phosphorylation, an important marker of DNA damage in HCB151 cells; it was remarkably upregulated in PANC-1 (Figure 5 A, B). p21 expression, an important cell cycle regulator, did not change in any cell lines corroborating the data obtained

through flow cytometry. In addition, no change in response to CTX was observed for p-AKT, AKT (pan), pP42/44 and P42/44 in the three cell lines evaluated (Figure 5 A)

Fig. 5



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329 4. **Discussion**

330 In this study, crotoxin, a phospholipase A2 from *Crotalus durissus terrificus* venom was
331 purified to homogeneity in a two-step procedure using ion exchange chromatography followed
332 Heparin Sepharose affinity chromatographic. Due to its unique structure and surface charge
333 distribution, the heparin matrix was able to interact strongly with some components in the
334 crude venom, whereas crotoxin (F1 CTX) showed a low affinity to the column (Figure 1).
335 Crotoxin is a heterodimeric complex consisting of a weakly basic, toxic, and non-hemolytic
336 PLA_2 (B subunit or crotoxin CB) and an acid, non-toxic, non-enzymatic and hemolytic
337 component known as crotapotin (A subunit or crotoxin CA) that potentiates the toxicity of the
338 molecule, acting as a chaperone protein (Neumann, Habermann, 1955; Fraenkel-Conrat,
339 Singer, 1956; Fraenkel-Conrat, 1971; Marlas, Bon, 1982; Radvanyl, Bon, 1982; Andrião-
340 Escarso et al., 2002; Faure, Saul, 2012). The subunit CB or PLA_2 presents about 14 kDa,
341 isoelectric point 9.7, consisting of a single polypeptide chain of 122 amino acid residues,
342 stabilized by seven disulfide bridges (Aird et al., 1989; Faure, Saul, 2012). The CA subunit
343 (crotapotin) has a molecular weight of 8.9 kDa, isoelectric point of 3.4, and has no enzymatic
344 or toxic activities (Rübsamen et al., 1971; Bon et al., 1989). Faure et al., (2012) have been
345 demonstrated that this subunit comprises three covalently linked polypeptide chains (α, 39
346 residues, β, 35 residues and γ, 14 residues). Our results showed peptide fragments that were
347 generated by mass fingerprinting and confirmed the identity of both A and B subunits of
348 crotoxin (Figure 1). Some authors suggest that combinations of these subunit complexes or
349 post-translational modifications originate the different described isoforms of CTX (Faure et al.,
350 1988; Faure et al., 1991; Faure et al., 1993).

351 We first investigated the cytotoxic profile of F1 CTX action against four different types
352 of solid tumor cell lines. In our studies, the lowest IC_{50} value (>0.5 µg/mL) was found for the
353 pancreatic tumor cell line (PANC-1) and glioma (GAMG), which demonstrated greater
354 sensitivity. Notably, F1 CTX also promoted cytotoxicity in HCB151, PSN-1, PANC-1, HeLa,
355 and KYSE 30 cell lines. In contrast, purified CTX exerted significantly lower cytotoxicity in the
356 esophageal tumor cell line (KYSE 270), presenting an IC_{50}=8.7 µg/mL. In the same way, we
357 observed a greater resistance for glioma (U373, IC_{50}=30.1 µg/mL) and cervical cancer (SiHa,
358 IC_{50}>30 µg/mL) cells. Our study was performed in according to the criterion adopted by

American National Cancer Institute (NCI) to consider an extract promising for preclinical studies when IC_{50} values presented are lower than 30 µg/mL for 72 h (http://www.cancer.gov) (Chou, Talalay, 1984; Suffness, Pezzuto, 1990; Talib, Mahasneh, 2010; Kuete et al., 2013; Trendowski, 2015).

Thus, our results demonstrated that the isolated CTX presented cytotoxic effects on different tumor cell lines with a heterogeneous response profile. One of the main objectives of targeted cancer therapy is to selectively eliminate tumor cells while sparing normal tissues. Although, we had no normal counterpart available for many of the tissues evaluated, we used mouse fibroblasts and human normal keratinocytes, which are strongly affected by anti-neoplastic chemotherapies (Plescia et al., 2005) to compare cytotoxic potential of CTX. We demonstrated that CTX had no effect on viability of normal human keratinocytes (HaCaT) or mouse fibroblasts (3T3) cell lines at concentrations tested. Ferguson and Duncan (2009) reported significant cytotoxic activity with *Crotalus durissus terrificus* PLA_2 on colon adenocarcinoma (HT29), melanoma (B16F10), and breast adenocarcinoma (MCF-7) cells with an IC_{50} of 40, 108.3, and 308.6 µg/mL respectively. In order to reduce the toxicity of the molecule, these authors conjugated PLA_2 with dextrin by the polymer masked-unmasked protein therapy method and showed that the conjugate presented a marked reduction in hemolytic activity and greater cellular cytotoxicity, but with an IC_{50} value of 16.3 µg/mL for HT29, and 62.9 µg/mL for MCF-7. In a previous study, Rudd et al. (1994) highlighted that crotoxin displays low toxicity in normal cells, suggesting its selective toxicity to tumors.

The ant proliferative and cytotoxic activities of CTX have been demonstrated through cell cycle arrest and pro-apoptotic mechanisms (Costa et al., 1998; Ferguson, Duncan, 2009; Wang et al., 2012; Han et al., 2014). However, we did not observe interference on cell cycle distribution under the conditions evaluated. This finding seems to be in disagreement with studies with lung adenocarcinoma (A-549), esophagus (ECA-109), and lung carcinoma (SK-MES-1) lines (Rudd et al., 1994; He et al, 2013; Han et al., 2014), which demonstrated growth inhibitory effects by cell cycle arrest. To further explore the role of CTX in cell death mechanism, we investigated its effect in protein expression related to DNA damage, proliferation/survival and cell death. The pancreatic (PANC-1) cell line exhibited a significant upregulation of H2AX activity, suggesting possible DNA damage effect (Ivashkevich et al., 2012; Siddiqui et al., 2015; Ji et al., 2017). Likewise, the glioma cell line (HCB 151) showed a

slight increase of H2AX activity and increased percentage of apoptotic cells (early + late apoptosis) after CTX treatment evaluated by flow cytometer. Therefore, CTX exposure induced alterations in different pathways involved in DNA damage and mortality. Studies have shown that the molecular cytotoxic mechanisms of PLA_2 involve the regulation of different signaling pathways, and that this effect is dependent of enzymatic activity. These studies revealed that the dissociation of the complex and the enzymatic activity are necessary for cytotoxicity, because when the catalytic activity of the complex or the PLA_2 subunit is alkylated, cytotoxic activity is lost (Corin et al., 1993; Soares, Giglio, 2003; Marchi-Salvador et al., 2008; Sobrinho et al., 2016).

The pro-apoptotic effect of CTX has been shown to trigger changes in mitochondrial membrane potential, cytochrome C release, and caspase-3 activation (Donato et al., 1996; Costa et al., 1997; Sampaio et al., 2010; Sobrinho et al., 2016). It has also been demonstrated that CTX induces cell death by autophagy mechanisms in breast and lung cancer cells (Yan et al., 2007; Han et al., 2014) and inhibits the growth of Eca-109 cells *in vitro* via apoptosis induction and G1 phase arrest (Ye et al., 2011). Interestingly, Wang et al (2012) showed that the combination of CTX with tyrosine kinase inhibitor gefitinib (Iressa®) significantly enhanced the antitumor activity of gefitinib and caused increased damage to blood vessels and reduced tumor size *in vivo*. These studies suggest that CTX cell cytotoxicity depends on tumor type, which may indicate distinct mechanisms of action.

In addition, we compared the effect of CTX with standard chemotherapeutic agents for glioma, pancreatic, esophagus, and cervical cancer. Our results demonstrated that crotoxin treatment was more cytotoxic than their corresponding chemotherapeutics in the majority of tumor types (glioma, pancreatic, and cervical cancer). Once the synergy (chemo-sensitization) of known and new compounds are of major interest, this study opens new perspectives for classical chemotherapy. However, the response level seen in practice is still suboptimal and there is an urgent need for improvement (Wiedmann, Mossner, 2013; Voutsadakis, 2011). Our results provide insights for further studies with CTX as an interesting antineoplastic agent in glioma and pancreas cancer lines.

5. **Conclusions**

In conclusion, F1 CTX fraction from *Crotalus durissus terrificus* was purified with

electrophoretic homogeneity. Purified hetero-dimer enzymes showed significant cytotoxicity exhibiting substantial activity against several cancer cell lines, while normal cell lines were not affected. Mechanistically, F1 CTX promotes pro-apoptotic effects and induces a potential DNA damage on pancreatic (PANC-1) and glioma (HCB151) cell lines, revealed through the increase in H2AX activity. Interestingly, F1 CTX demonstrated a greater cytotoxic potential than the specific standard chemotherapeutic agents used in clinical practice in the majority of tumor types (glioma, pancreatic, and cervical cancer). These results add to the functional knowledge database on crotoxin, and provide new insights into the development of antitumor therapy.

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Ethical statement

The experiments followed the methodology recommended by the international ethical standards of the scientific committee of our university (process nº 847/2015).

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680

681 **Figure legends**

682 **Figure 1. Purification and isolation of Crotoxin (CTX). (A)** Ion exchange chromatography
683 of the crude venom of *Crotalus durissus terrificus* on DEAE-c column, which had been
684 previously equilibrated in 0.05 M Tris-HCl, pH 7.5, and eluted in increasing gradient of NaCl
685 (0.1 M) in 0.05 M Tris-HCl, pH 7.5. Fractions of 2 mL were collected and analyzed in a
686 spectrophotometer (λ_{280}nm). **(B)** Heparin-Sepharose FF (HiTrap, heparin (HP), 5 mL) column
687 of the F4 CTX (50 mg) previously equilibrated with 0.01 M sodium phosphate, pH 7.0 and
688 eluted in linear gradient of NaCl (0–1.5 M) at a flow rate of 2.5 mL/ min. Fractions of 3 mL
689 were collected and analyzed in a spectrophotometer (λ_{280}nm). **(C)** SDS-PAGE using a 13%
690 acrylamide-bisacrylamide (w/v) gel in Tris-glycine buffer, pH 8.3. Lane 1: molecular weight
691 markers; lane 2: crude venom (CV) (10 µg); and Lane 3: CTX (10 µg). **(D)** Mass spectrometry
692 of CTX**.** Five micrograms of isolated CTX were reduced in 10 mM DTT, alkylated in 50 mM
693 iodoacetamide, and digested with 20 ng/µL of trypsin. Protein identification was performed in
694 a mass spectrometer coupled to an HPLC using a C18 nanocolumn. The resulting spectra
695 were analyzed using Mascot (Matrix 5 Science) in the NCBI nr protein databases, with
696 carbamidomethylation as the fixed modification. Similar peptide sequences were identified
697 using BLAST. Peptide fragments are observed in b ions (N-terminus), and y ions (C-
698 terminus). a^*, b^*, and y^* represent ion fragments of RKNQ that have lost ammonia (-17 Da). b°
699 represent ion fragments of STED that have lost water (-18 Da).

700

701 **Figure 2. Effect of crotoxin (CTX) on cell viability of normal and human cancer-derived**
702 **cell lines.** Viability of tumor cells was determined by the MTS assay after incubation with
703 different concentrations of crotoxin (0.5–30 µg/mL) for 72 h. (A and B) Esophageal cancer

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cells (Kyse 270 and Kyse 30). (C-E) Glioma cells (GAMG, HCB 151 and U373). (F and G) Pancreatic cancer cells (PSN-1 and PANC-1). (H-I) Cervical cancer cells (HeLa and SiHa). (J-K) Normal human keratinocytes (HaCaT) and mouse fibroblasts (3T3) respectively. Results from three replicates are reported as the mean percentage of viable cells ± SD compared to control (DMSO - considered as 100% viability). The results of tree replicates were expressed as the mean percentage ±SD of viable cells relatively to the control (considered as 100% viability). The differences between the means and the significance of the treatments were analyzed with GraphPad Prism (Version 6.01) using Student's t-test. *P<0.05, **P<0.01, ***P<0.001.

Figure 3. Effect of crotoxin (CTX) on cell cycle distribuition. Representative cell cycle analysis by flow cytometry of (A) HCB151 and (C) SiHa cells, in absence (Control - 1% DMSO) or presence of FI CTX (HCB 151, IC_{50} = 4.1 µg/mL, SiHa, IC_{50} = 30 µg/mL), after 24 h. Summary of percentage of cells in each phase of the cell cycle are indicated by bars as the mean ± SD and differences with p <0.005 in the Student's t-test (**).*n.s*. means non-significant. The X-axis represents the channel number (relative DNA content/cell). The Y-axis represents the number of cells/channel.

Figure 4. Effect of crotoxin (CTX) on cell death mechanisms. After 24 h treatment with crotoxin, (A) HCB151 and (C) SiHa cells were fixed, stained with annexin V / PI, and analyzed by flow cytometry using FACScan. The data represent three independent experiments. Non-treated CTR: DMSO (1%) and crotoxin-treated cells (HCB151, IC_{50} = 4.1 µg/mL; SiHa, IC_{50} = 30 µg/mL). (B-D) Bars represent the percentage of apoptotic cells as the mean ± SD and differences with p <0.005 in the Student's t-test (**).*n.s*. non-significant.

728

729

730 **Figure 5. Effect of crotoxin (CTX) on protein profile of human cancer-derived cell lines.**
731 (A) Drug-sensitive (HCB151 and PANC-1) and drug-resistant (SiHa) cells were incubated with
732 crotoxin at the IC_{50} of 4.1 µg/µL, 0.5 µg/µL and 30 µg/µL respectively for 24 h. DMSO (1%)
733 was used as negative control. The cell extracts were subjected to Western blotting to analyze
734 the protein expression levels. B-tubulin was used as internal control. (B) Densitometry with
735 levels of protein activity. Proteins were quantified by the ImageJ program. The asterisks (**)
736 indicate statistical significance ($p < 0.005$) between control and experimental group in the
737 Student's t test. *n.s*. non-significant.

738

739 **Tables**

740

741 **Table 1** The CTX, temozolomide, gemcitabine, paclitaxel, and cisplatin half-maximal inhibitory
742 concentration (IC_{50}) against the human cancer-derived cell lines.

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Table 1 The F1 CTX, temozolomide, gemcitabine, paclitaxel, and cisplatin half-maximal inhibitory concentration (IC_{50}) against human cancer-derived cell lines.

Cell line	F1 CTX Mean IC_{50} ±S.D (μ g/mL)	Temozolomide Mean IC_{50} ± S.D (μ g/mL)	Gemcitabine Mean IC_{50} ± S.D (μ g/mL)	Paclitaxel Mean IC_{50} ± S.D (μ g/mL)	Cisplatin Mean IC_{50} ± S.D (μ g/mL)	Origin	Organism	Tissue	Culture conditions
GAMG	< 0.5	18.8± 2.0	*ND*	*ND*	*ND*	Glioblastoma	Human	Brain	DMEM + 10% FBS + 1% P/S
U373	30.1±1.5	105.7± 1.5	*ND*	*ND*	*ND*	Glioblastoma astrocytoma	Human	Brain	DMEM + 10% FBS + 1% P/S
HCB151	4.1±3.8	17.5±0.5	*ND*	*ND*	*ND*	Primary glioma	Human	Brain	DMEM + 10% FBS + 1% P/S
PANC-1	< 0.5	*ND*	8.8±2.6	*ND*	*ND*	Pancreatic cancer	Human	Pancreas	DMEM + 10% FBS + 1% P/S
PSN-1	0.69±0.08	*ND*	1.4±0.02	*ND*	*ND*	Pancreatic cancer	Human	Pancreas	DMEM + 10% FBS + 1% P/S
KYSE30	0.9±1.7	*ND*	*ND*	0.005± 0.003	*ND*	Squamous cell carcinoma	Human	esophagus	RPMI 1640 + 10% FBS + 1% P/S
KYSE270	8.7±1.4	*ND*	*ND*	0.006±0.002	*ND*	Squamous cell carcinoma	Human	esophagus	RPMI 1640 + 10% FBS + 1% P/S
HeLa	2.4±1.1	*ND*	*ND*	*ND*	6.3±1.8	Cervix carcinoma	Human	Cervix	DMEM + 10% FBS + 1% P/S
SiHa	>30	*ND*	*ND*	*ND*	14.9±3.6	Squamous Cell Carcinoma	Human	Cervix	DMEM + 10% FBS + 1% P/S
NHI/3T3	>30	*ND*	*ND*	*ND*	*ND*	Fibroblast	Mouse	Embryo	DMEM + 10% FBS + 1% P/S
HaCat	>30	*ND*	*ND*	*ND*	*ND*	Normal Keratinocytes	Human	Skin	DMEM + 10% FBS + 1% P/S

FBS. Fetal Bovine Serum; P/S Penicillin/Streptomycin solution; ND. Not done. All the assays were done in triplicate and repeated at least three times.

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Highlights

Crotoxin demonstrated cytotoxic effect on different tumor cell lines.

The molecule had a heterogeneous cytotoxic effect on the different tumor lines.

The glioma and pancreatic cancer cells showed a higher sensitivity.

Cervical and esophagus cancer cell lines presented higher resistance.

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