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BINDING STUDIES OF THE ANTITUMORAL RADIOPHARMACEUTICAL ^{125}I-CROTOXIN TO EHRLICH ASCITES TUMOR CELLS

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ABSTRACT

The development of tools for functional diagnostic imaging is mainly based on radiopharmaceuticals that specifically target membrane receptors. Crotoxin (Crtx), a polypeptide isolated from *Crotalus durissus terrificus* venom, has been shown to have an antitumoral activity and is a promising bioactive tracer for tumor detection. More specific radiopharmaceuticals are being studied to complement the techniques applied in the conventional medicine against breast cancer, the most frequent cause of death from malignant disease in women. Crtx's effect has been shown to be related with the overexpression of epidermal growth factor receptor (EGFR), present in high levels in 30 to 60% of breast tumor cells. Our objective was to evaluate Crtx as a tracer for cancer diagnosis, investigating its properties as an EGFR-targeting agent. Ehrlich ascites tumor cells (EAT cells) were used due to its origin and similar characteristics to breast tumor cells, specially the presence of EGFR. Crtx was labeled with ^{125}I and binding experiments were performed. To evaluate the specific binding *in vitro* of Crtx, competition binding assay was carried out in the presence of increasing concentrations of non-labelled crotoxin and epidermal growth factor (EGF). Specific binding of ^{125}I-Crtx to EAT cells was determined and the binding was considered saturable, with approximately 70% of specificity, high affinity (Kd = 19.7 nM) and IC_{50} = 1.6 x 10^{-11} M. Our results indicate that Crtx's interaction with EAT cells is partially related with EGFR and increases the biotechnological potential of Crtx as a template for radiopharmaceutical design for cancer diagnosis.

1. INTRODUCTION

Breast cancer remains the major cause of cancer morbidity and mortality in women throughout the world [1]. There is an urgent need for early diagnosis and therapy for the patients, as this type of cancer may exist for a long period as noninvasive or invasive disease. Through nuclear medicine is possible to attach radionuclides to polypeptides, getting radiopharmaceuticals and, then, perform imaging diagnosis as the single photon emission tomography (SPECT) or the positron emission tomography (PET). More specific radiopharmaceuticals are being developed to complement the techniques applied in the

conventional medicine against breast cancer, the most frequent cause of death from malignant disease in women.

New molecules are being studied as targeted delivery of radionuclides or chemotherapeutic drugs to their site of action within an organism. The development of tools for functional diagnostic imaging is mainly based on radiopharmaceuticals that specifically target membrane receptors or other biomarkers, such as antibodies (^{125}I- rituximab) [2], glucose (^{18}FDG) [3], neurotransmitter analogs (^{18}F-DOPA) [4] and peptides as octreodite and DOTA labeled with iodine ($^{123/125}$I) [5].

Crotoxin (Crtx), a polypeptide isolated from *Crotalus durissus terrificus* venom, has been shown to have potent antitumoral activity against a variety of murine and human tumor cell lines [6]. Crtx is a complex formed by two subunits, one acidic with non-enzymatic function and one basic that is a PLA_2 formed by a single chain of 122 amino acid residues [7][8]. It displays cytotoxic activity against various tumor cell lines and appears to be highly active toward the ones that express a high density of epidermal growth factor receptors (EGFR); however, the precise mechanism of the cytotoxicity remains to be determined [9]. Our group has observed that Crtx is a promising bioactive substance against breast tumor cells and interact with them for until 72 hours allowing good quality images of tumor [10].

The search for new tracers can be based on molecular interaction with specific receptors. Overexpression of EGFR has been implicated in the formation of various malignancies including breast cancer. This specific cell-surface receptor is part of the ErbB family of receptor tyrosine kinase and one of its specific ligand is the epidermal growth factor, EGF. 30%-60% of human breast tumor cells present 100 times higher levels of EGFR than normal epithelial tissues [11]. Similarly to those receptors observed on breast tumor cells, EGFR have been identified on Ehrlich ascites tumor cells (EAT cells) [12], a tumor that is primarily derived from a murine mammary adenocarcinoma [13].

Despite of the several properties described for Crtx in the literature, there is little information about its molecular behavior. It is known this polypeptide presents antitumoral action against some cell lines and that it can be related to epidermal growth factor receptor. Because Ehrlich tumor is primarily a murine mammary adenocarcinoma and presents EGFR, there is a special interest in elucidate Crtx binding to them. In the present study, we investigated the use of radio-iodinated-Crotoxin focusing on its specificity, affinity and binding properties to the EGFR on EAT cells. It is a favorable radionuclide linked to a natural polypeptide with antitumoral activity for improvement of tumors detection and early diagnosis.

2. MATERIALS AND METHODS

2.1. Venom and Chemicals

Crotoxin, isolated from lyophilized venom of *Crotalus durissus terrifics*, was supplied by the Ezequiel Dias Foundation (Funed), Belo Horizonte, Brazil. Carrier-free radioactive sodium iodide Na^{125}I was obtained from Amersham Bioscience. All other chemicals unless otherwise specified were purchased from Sigma (St. Louis, MO).

2.2. Preparation of Ehrlich Ascites Cells

Ehrlich ascites tumor cells are primarily derived from a murine mammary adenocarcinoma [13] and present EGFR, which have been already identified and characterized [12]. The cells were maintained in the ascite form by passages in Swiss mice and weekly intraperitoneal transplantation of tumor cells. The ascitic fluid was extracted using a sterile syringe and the viable tumor cells were counted in a Neubauer hemocitometer. The final concentration of tumor cell suspension to be used on the *in vitro* binding assay was adjusted to 5 x 10^6 viable cells/mL of binding buffer.

2.3. Crotoxin Radioiodination

Radiolabeled Crotoxin (^{125}I-Crtx) was prepared using lactoperoxidase method and Na^{125}I according to Soares, 2007 [14]. Briefly, 7.2 µg of polypeptide was reacted with 18.5 MBq of carrier-free Na^{125}I in the presence of lactoperoxidase and H_2O_2 in phosphate buffer at pH 7.4. The reaction was stopped with phosphate buffer containing 1 mg/mL bovine serum albumin, BSA. Radiochemical analysis was performed using ascending chromatography in *Whatman* paper using methanol saturated with KI as eluent. Radiochemical purity and specific activity were determined by gamma spectrometry.

2.4. *In vitro* Receptor Binding of ^{125}I-Crtx and Competition Assays

Specific interaction of ^{125}I-Crtx with EAT cells was evaluated by *in vitro* receptor binding experiment. Firstly, 1 x 10^6 EAT cells in binding buffer medium (10 mM Tris-HCl, 150 mM NaCl, EDTA 0.5 mM, 0.1% w/v human serum albumin) were incubated in small eppendorf tubes with 1 x 10^{-10} M of ^{125}I- Crtx in the absence (total binding) or in the presence of an excess of cold Crtx (non-specific binding). The assay with Crtx/ ^{125}I-Crtx was performed at 25 °C in 1.5 mL tubes in a final volume of 250 µL of binding buffer. Specific binding was calculated by subtracting the non-specific binding from total binding [15]. Nonspecific binding was measured in parallel experiments using an excess (1000-fold) of unlabeled polypeptide and the values were subtracted from the total binding.

In competition studies increasing concentrations of non-radiolabeled Crotoxin (1 x 10^{-12} M to 1 x 10^{-7} M) were pre-incubated at 25 °C for 15 minutes with EAT cells. Aliquots of a single concentration of ^{125}I-Crtx (1 x 10^{-10} M) were added to the eppendorf tubes and further incubated for 1 hour at 25 °C. Following incubation, bound radioactivity (^{125}I-Crtx that really bound to the cells) was separated from free radioactivity (^{125}I-Crtx that did not bind to the cells) by centrifugation (13,000 *g*, 5 min) and the pellet was washed with 1 mL of ice-cold binding buffer 1% w/v BSA. This process was repeated three times to remove excess free radioactivity. The bound radioactivity was counted in an automatic gamma-spectrometer (1480 Wizard 3" – Wallac). IC_{50} value for the polypeptide, determined as the ligand concentration that inhibits 50% of the maximum specific binding, was calculated using *Prisma* software. All assays were run in triplicate.

Another binding competition experiment was carried out using different conditions. To examine the possible direct interaction of Crtx with the epidermal growth factor receptors, the assay was prepared using the specific ligand EGF as competitor. 1 x 10^6 EAT cells were pre-incubated with increasing concentrations of cold EGF (5 x 10^{-12} a 5 x 10^{-7} M) at 37 °C for

120 min in a final volume of 250 μL of binding buffer supplemented with 0.1% BSA. Following the pre-incubation, aliquots of ^{125}I-Crtx (1 x 10^{-10} M) were added to each eppendorf tube and incubated at 25 °C for 60 min. At the end of the incubation period, the cells were centrifuged and rinsed three times with cold binding buffer 1% BSA to aid removal of unbound ^{125}I-Crtx. The radioactivity was counted in a gamma-counter.

2.5. Saturation Binding Assay of ^{125}I-Crtx and Scatchard Analysis

To obtain a saturation curve of Crtx binding, EAT cells (1 x 10^6) in binding buffer were incubated in epperdorf tubes with 1 x 10^{-10} M of non-radiolabeled Crtx for 15 minutes. Increasing concentrations of ^{125}I-Crtx were added to the tubes to give a range of concentrations of 3 x 10^{-12} to 2 x 10^{-8} M. After incubation of 60 minutes, all tubes were centrifugated and the unbound radioligand was removed as described for competition experiment. The assay was performed in 1.5 mL tubes in a final volume of 250 μL of binding buffer medium and the specific binding was calculated as detailed previously. The amount of ^{125}I-Crtx required to saturate receptors was measured and analyzed to determine the radioligand equilibrium dissociation constant (Kd), a useful gauge of the receptor binding affinity of a radioligand [15]. The number of receptors/cell (Bmax) was determined from a nonlinear fitting of the binding data.

The plots and binding constants were obtained using the *Prisma* software, with non-linear regression for Scatchard transformation of data. To simplify the study, we consider the analysis with one site binding interaction.

3. RESULTS AND DISCUSSION

3.1. Crotoxin Radioiodination

Radiolabeling with the radionuclide ^{125}I was performed by the lactoperoxidase method. The yield of the labeling reaction was approximately 90 % and the specific radioactivity of ^{125}I-Crtx was 31.9 ± 2.3 TBq/mmol. Fig. 1 shows the chromatografic pattern of ^{125}I-Crtx on ascendant chromatography in Whatman paper n°1 before and after the purification with Dowex 1-X8. It can be observed that the radiolabeling reaction produced just a few amount of free iodine contaminant that could be efficiently eliminated by ion exchange chromatography providing good radiochemical purity and yield.

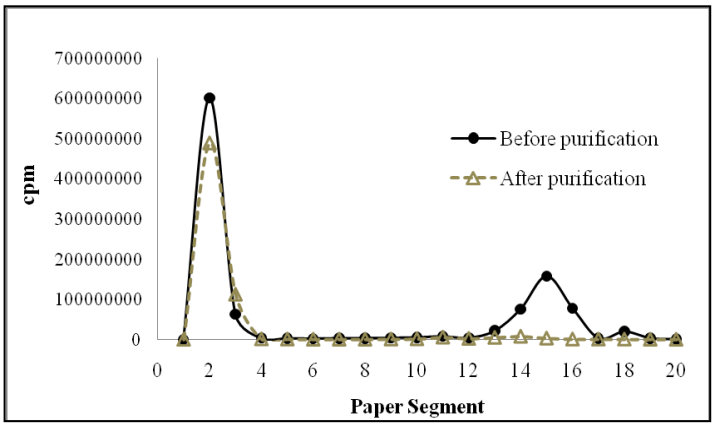

Figure 1. Radiochromatogram of labeled Crtx before (full circle) and after (open triangle) the purification by anionic exchange chromatography.

3.2. *In vitro* Receptor Binding of ^{125}I-Crtx

The specific binding, detected in the presence of 1000-fold molar excess of unlabelled Crtx was kept around 70 % of the total binding (Fig.2). Specific binding was stable for at least three hours of polypeptide interaction, showing its stability on the binding buffer medium described on item 2.4.

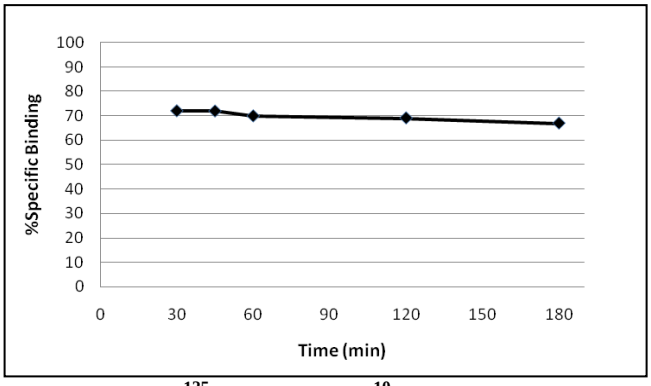

Figure 2. Stability of ^{125}I-Crtx (1 x 10^{-10} M) specific binding to EAT cells at different incubation time in the presence of a 1000-fold cold Crtx excess (1 x 10^{-7} M).

Plotting the specific binding of the radiolabeled ligand (^{125}I-Crtx) *versus* the log of non-radioactive ligand (Crtx) concentration generates an IC_{50} curve (Fig. 3). In the presence of increasing concentrations of non-radiolabeled Crtx it was possible to observe the displacement of the ^{125}I-Crtx of the specific binding site. 1.6 x 10^{-11} M of Crtx caused half-maximal inhibition of the radioligand receptor binding, correlating to the point on the curve found halfway between the high and low plateaus.

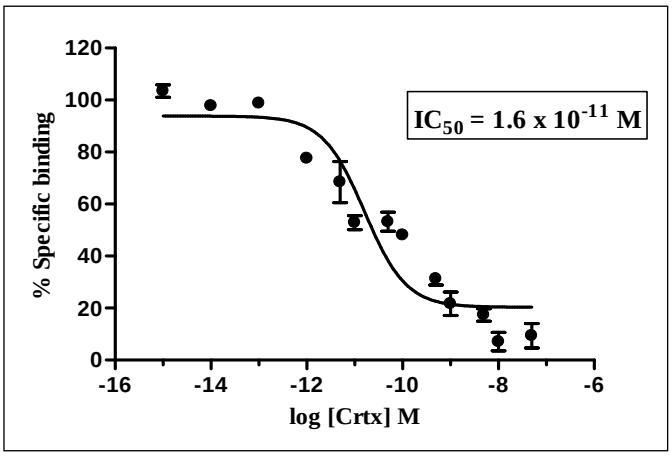

Figure 3. Competitive binding of ^{125}I-Crtx with various concentrations of non-radiolabeled Crtx (competitor) to EAT cells. Cells were incubated with 1 x 10^{-10} M of ^{125}I-Crtx in the presence of 1 x 10^{-12} M to 1 x 10^{-7} M of non-radiolabeled Crtx.

3.3. Competition Binding Assay with EGF

Various concentrations of EGF (5 x 10^{-12} a 5 x 10^{-7} M) were used to examine ^{125}I-Crtx competitive binding to EAT cells and evaluate its interaction with EGFR. The competition curve (Fig. 4) obtained is showed above. Increasing concentrations of cold EGF were used to determine the concentration of Crtx required to reduce the specific binding of a radiolabeled standard by 50%. The competitor, EGF, fails in displaces high amounts of the radioligand. However, the decrease on specific binding values represents a small competition and a relative binding affinity of ^{125}I-Crtx for the EGF receptor, showing that ^{125}I-Crtx binding was partially affected by EGF. This experiment was not performed in triplicate because of the limited amount of EGF.

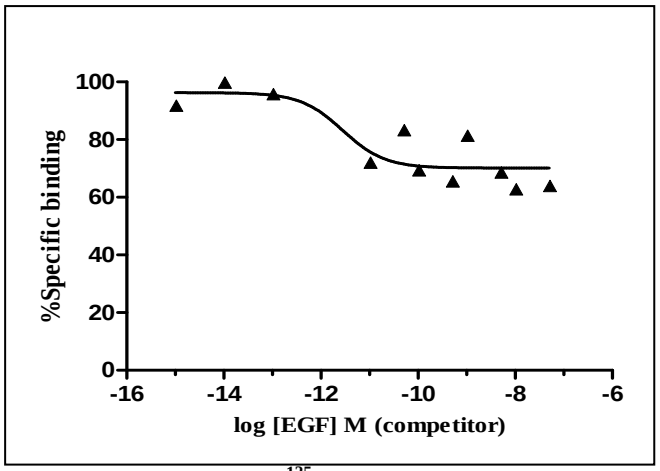

Figure 4. Competitive binding of ^{125}I-Crtx and various concentrations of non-radiolabeled EGF (competitor) to EAT cells. Cells were incubated with 1 x 10^{-10} M of ^{125}I-Crtx in the presence of 5 x 10^{-7} M to 1 x 10^{-11} M of EGF for 2h at 37 °C.

3.4. Saturation Binding Assay of ^{125}I-Crtx and Scatchard Analysis

Incubation of EAT cells with various (3 x 10^{-12} to 2 x 10^{-8} M) of ^{125}I-Crtx in binding buffer resulted in the representative saturation binding curve of the radiolabeled peptide (Fig. 5). ^{125}I-Crtx exhibited a specific and saturable binding to specific sites on Ehrlich ascites tumor cells. Scatchard analysis of the saturation curve (Fig.5, inset) produced a linear plot over the concentration range used and it was possible to calculate the dissociation constant (Kd) equal to 19.68 ± 3.67 nM, showing that these were high affinity binding sites. The Bmax was 24.92 ± 0.06 fmol/1x10^6 EAT cells.

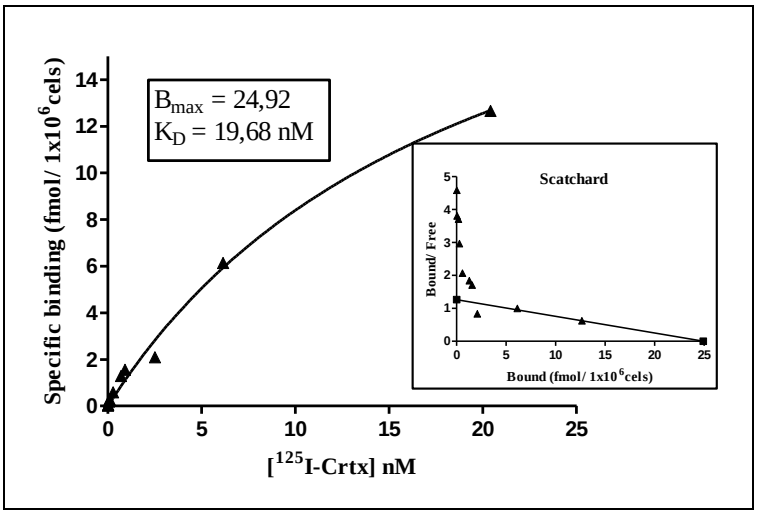

Figure 5. Saturation binding curve showing the effect of radioligand concentration on the specific binding and non-specific binding. The small graph is the scatchard analysis of saturation curve.

4. CONCLUSIONS

Crtx presents an antitumor activity against various tumor cell lines, including Ehrlich ascites tumor cells. This work showed that specific binding of ^{125}I-Crtx to EAT cells was saturable indicating the specificity of this interaction (approximately 70 % of specificity). Using one site binding Scatchard analysis, it was demonstrated the high affinity interaction between Crtx and EAT cells (Kd= 19.7 nM). The partial competition of Crtx with EGF indicates that the specific sites for Crtx include somehow EGFR and suggest that Crtx may interfere with the signaling pathway of this important receptor involved in cell survival and proliferation. Because EGFR is frequently overexpressed in breast cancer tissue and is associated with a poor prognosis, crotoxin is an attractive tool for tumor diagnosis. These results shed more light on the biotechnological potential of Crtx as template for radiopharmaceutical design for cancer.

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