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RADIOIODINATION AND BIODISTRIBUTION OF LEUCUROLYSIN-B ISOLATED FROM *BOTHROPS LEUCURUS* IN MICE BEARING EHRLICH

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ABSTRACT

Integrins are family of heterodimeric cell surface adhesion receptors able to recognize and bind to proteins in the extracellular matrix (ECM). This recognition is mainly through the RGD domain present in both the cell surface as the protein in the ECM. Various integrins have been identified as regulators of tumor progression. The RGD domain is also found in some snake venoms named disintegrins. Disintegrins inhibit cell-matrix and a cell-cell interaction mediated by integrin and has been shown that these proteins are able to inhibit metastasis in processes dependent on integrin. The disintegrin-like (ECD), as well as RGD-disintegrin are also able to bind to cell surface integrins and inhibit their adherence to the natural ligands. Leucurolysin-B (Leuc-B) is a metalloproteinase class P-III isolated from *Bothrops leucurus* (BLV) and possesses a disintegrin-like domain (ECD). The goals of this work were to synthesize a radioactive probe analog to Leuc-B using radioiodine ^{125}I and evaluate the interaction of ^{125}I-Leuc-B in tumor cells through the study of biodistribution in animals bearing Ehrlich tumor. ^{125}I-Leuc-B was synthesized using lactoperoxidase with high yield (90%) and specific activity of $1.2x10^{-7}$Bq/mmol. It was observed that ^{125}I-Leuc-B had very fast clearance from the blood stream (T1/2= 0.01 h). Tumor uptake of ^{125}I-Leuc-B gradually increased up to (2 min) and remained for a quite long period. The tumor/normal tissue uptake ratios of ^{125}I-Leuc-B were 1.77 (tumor/normal paw) and 8.44 tumor/skeletal muscle. The results suggest that ^{125}I-Leuc- B may constitute a good template for development of a tool for detection of solid tumors.

1. INTRODUCTION

Tumors are a group of uncontrolled growing cells. Tumors may be either benign or malignant [1].

Benign tumors are well-differentiated, because they differ slightly in appearance and behavior from their tissue of origin. These tumors are slow growing and noninvasive, do not spread throughout the body, and will often have a fibrous tissue capsule around them. All these characteristics make benign tumors easy to remove completely in most cases.

Benign tumors are rarely a threat for life, unless they range a huge size. However, they may cause significant problems if they are located in a vital organ, such as brain or intestine. In fact, the most significant alteration that a benign tumor can make in its host organism is that of spatial encroachment on surrounding normal tissue, leading to obstruction or replacement of the normal tissue by tumor [2].

Malignant tumors often grow rapidly. Cells show marked de-differentiation, or anaplasia; in other words, they do not look like the cells from which they originated. Cells emit cellular expansions through the normal tissue nearby, making complete excision a task virtually impossible. Even after radical surgery these tumors frequently re-grow [2].

The cancer treatment can be done through surgery, radiotherapy, chemotherapy or bone marrow transplantation. In many cases it is necessary to combine radiotherapy and chemotherapy to achieve a better result of treatment [3].

Although the knowledge on development of cancer is growing considerably, just a few advances in the diagnosis and therapy has been achieved. Faced with this scenario, it is clear the need for new substances more specifics with low toxicity to the patient, which can be used for diagnosis and treatment of cancer. Membrane receptors overexpressed in tumor cells are promising target candidates for development of diagnostic tools.

The integrins are membrane heterodimeric proteins composed of two subunits (α 120 to 180 kDa and β 90 to 110 kDa) [4] that recognize the sequence Arg-Gly-ASP (RGD) present in proteins of the extracellular matrix (ECM), such as fibronectin, fibrinogen, and vitronectin [5]. RGD and RGD-like sequences can also be found in some snake venom components where they are called disintegrins.

These proteins are involved in several biological processes, such as adhesion, migration and induction of cellular signaling essential for the survival, proliferation and cellular differentiation [4]. Changes in cell adhesion can cause various diseases such atherosclerosis, cancer and variety inflammatory conditions [6]. Not surprisingly, various integrins have been identified as regulators of tumor progression [7].

Some disintegrins have also been shown to inhibit experimental metastasis as an integrin-dependent process, and therefore, over the last few years, many studies have focused on these proteins. For many years it's known that administration of soluble synthetic peptides containing the RGD sequence inhibits the formation of metastasis [8]. However, the inhibitory effect of metastasis promoted by disintegrin containing the RGD sequence is often more powerful than the effect of synthetic RGD peptides. This is probably due to structural complexity of these proteins, as residues adjacent to the adhesive RGD sequence and the three-dimensional structure of disintegrin have been important to link their specific ligands. Thus, the study of natural disintegrins present in the venom of snakes is very important in the search for tools for antitumoral therapy. Recently, it has been demonstrated that peptides containing the RGD sequence can be radiolabeled with gamma and positron emitters and applied for differential diagnosis of tumors [9].

The disintegrin-like (ECD), as well as RGD-disintegrin are also inhibit of aggregation and are also able to bind to cell surface integrins and inhibit their adherence to the natural ligands [10].

Leucurolysin-B (Leuc-B) is a metalloproteinase class P-III isolated from *Bothrops leucurus* (BLV). Leuc-B possess a disintegrin-like domain (ECD) and contains the characteristic motif **HE**XX**H**XX**G**XX**H** of metalloproteinases [11] which suggests that Leuc-B can express antitumoral activity. In fact, recently works showed that Leuc-B evoked dose-dependent cytotoxic effect on breast cancer cells: MCF7 and Ehrlich. Leuc-B presented IC_{50} of (IC_{50}: 1 μM) and (IC_{50}: 0.35 μM) against MCF7 and Ehrlich cells, respectively. Interestingly, Leuc-B was more potent than cisplatin, an antineoplastic drug widely used in clinic (IC_{50}: 3 μM). Our results reveal that BLV constitutes a source of potent antitumoral agents and part of its effect is due to Leuc-B [12]. Further studies are in development in order to evaluate the therapeutical index of Leuc-B as well as the spectrum of antineoplastic effect. If this antitumoral effect is due to the action of ECD disintegrin remains to be evaluated.

The goals of this work were to synthesize a radioactive probe analog to Leuc-B using radioiodine ^{125}I, and evaluate the interaction of ^{125}I-Leuc-B in tumor cells through the study of biodistribution in animals bearing Ehrlich tumor.

2. MATERIALS E METHODS

2.1. The venom

The venom of *Bothrops leucurus* was obtained from several specimens from State of Bahia and kept at the serpentarium of the Ezequiel Dias Foundation, Belo Horizonte, Minas Gerais. The venom was lyophilized and kept at - 20 ° C until use.

2.2. Purification of Leucurolysin- B

The whole procedure purification of Leucurolysin-B was done according to the protocol described by Sanchez et al., 2007.

2.3. The animals

The animal experiments were performed in accordance with the Manual on care in the use of laboratory animals (Institute of Laboratory Animal Resources - Commission on Life Sciences - National Research Council - Washington DC) as recommended by the Brazilian College of Animal Experimentation and according with protocol approved at the Committee of Ethics in animal experimentation of UFMG - CETEA-UFMG. Swiss female mice were used, weighing 25-30 g, obtained in Center bioterismo (CEBIO) Institute of Biological Sciences, University Federal de Minas Gerais (UFMG).

2.4. Radioiodination of Leucurolysin- B

The experiments using radioactive materials were made according to the radiological protection standards adopted by CNEN 3.01.

^{125}I-Leuc-B was synthesized using the lactoperoxidase methods, according to the protocol described by Soares, 2007.

The radiosynthesis was done by electrophyllic substitution. Lactoperoxidase was added to a phosphate buffer solution containing 16 μg of Leuc-B and 0.5 mCi of $Na^{125}I$. The reaction was initiated by addition of H_2O_2. The reaction was stopped by addition of 0.05 M PBS containing 0.1% BSA. Free iodine not incorporated into the molecule was separated from the labeled molecule by anion exchange chromatography using Dowex 1-X8. The quality control of the radiolabeled molecule was done by paper chromatography on Whatman paper Nº.1 using methanol saturated with potassium iodide as mobile phase. After complete migration the strips of 1 cm of the chromatographic paper were cut and the radioactivity was measured in a gamma counter (Wizard 3"/1480 Wallace Packard).

2.5. Biodistribution assay

For biodistribution evaluation, radiolabeled Leuc-B (20.3 kBq) was intravenously injected into the tail vein of mice (25-30 g) bearing Ehrlich tumor in the hind paw. This tumor is widely used in experimental oncology due to the fact that rapidly developing restricting the time of study, in addition to knowing the quantity and characteristics of the initial inoculated tumor cells [17].

The main organs (blood, thyroid, heart, lungs, liver, spleen, pancreas, stomach, intestine, bladder, kidney, skeletal muscle, bone, brain and tumor) were dissected and washed with saline and weighed. The radioactivity (gamma emission) in each organ was determined by gamma spectrometry with 82% of efficiency for ^{125}I. The results were expressed as percentage of injected dose per gram of each organ (% ID/g) and analyzed in the GraphPad Prism.

2.5. Pharmacokinetic calculations

For pharmacokinetic analysis was used the one-compartment model. This model is particularly useful for pharmacokinetic analysis of drugs that distribute relatively rapidly through-out the body [13].

The characterization of the pharmacokinetic profile of a new molecule can be obtained from the calculation of mathematical parameters calculated from the biodistribution data. The main parameters are the area under the curve (AUC), the volume of distribution (Vd), clearance (Cl), the half-life and bioavailability [13].

The AUC was calculated from the plot of kinetics of blood molecules and was used to determine the half-life, volume of distribution and clearance.

The apparent volume of distribution is the theoretical volume of fluid into which the total drug administered would have to be diluted to produce the concentration in plasma and was calculated by the equation [13]:

$$Vd = Dose/AUC \quad (1)$$

The Clearance (Cl) of a drug is the volume of plasma from which the drug is completely removed per unit time. The amount eliminated is proportional to the concentration of the drug in the blood [13].

The fraction of the drug in the body eliminated per unit time is determined by the elimination constant (k). This is represented by the slope of the line of the log plasma concentration versus time. The plasma clearance (CL) was calculated by the equation [13]:

$$Cl = K \times Vd \quad (2)$$

3. RESULTS

3.1. Radioiodination of Leucurolysin- B

^{125}I-Leuc-B was labeled with high yield (90%) and specific activity ($1.2x10^{-7}$Bq/mmol). Typical chromatographic profiles of ^{125}I-Leuc-B are shown in Fig.1.

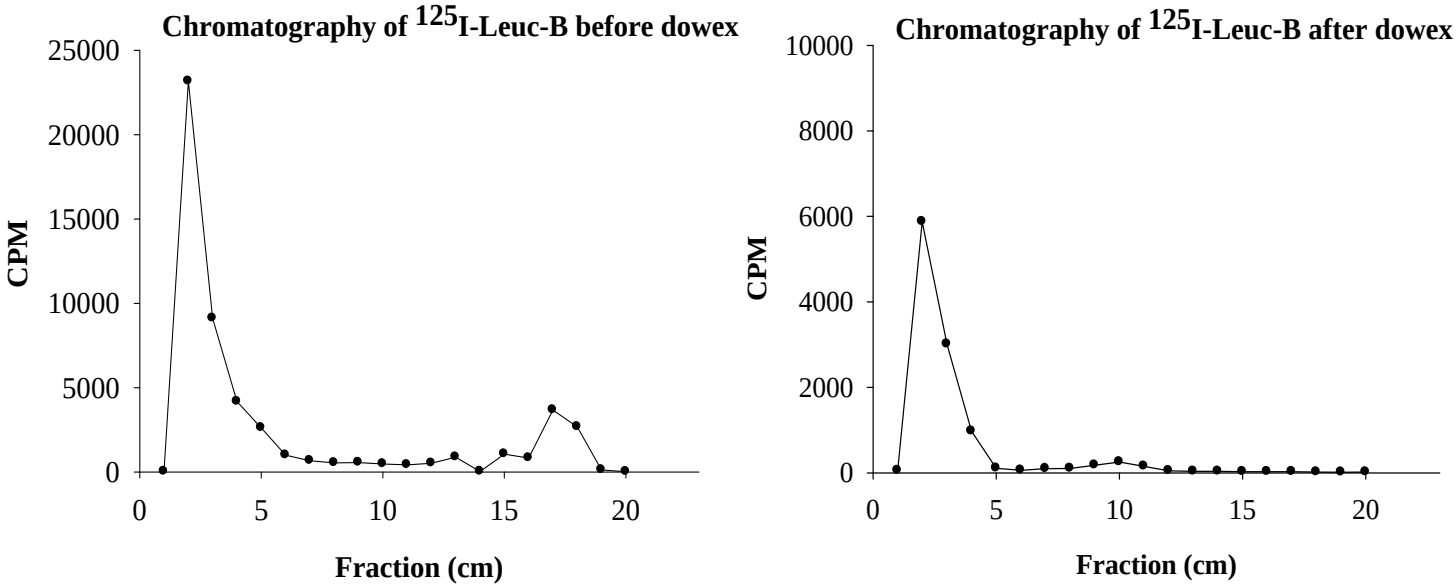

Figure 1. Quality control of radiolabeled by chromatography on Whatman paper using methanol saturated with potassium iodide as mobile phase. After the race the tapes were cut and radioactivity was measured in a gamma counter with the efficiency of 82% for ^{125}I.

3.2. Biodistribution assay

The biodistribution profile of ^{125}I-Leuc-B in Swiss female mice bearing Ehrlich tumor after different times from administration is shown in Fig. 2.

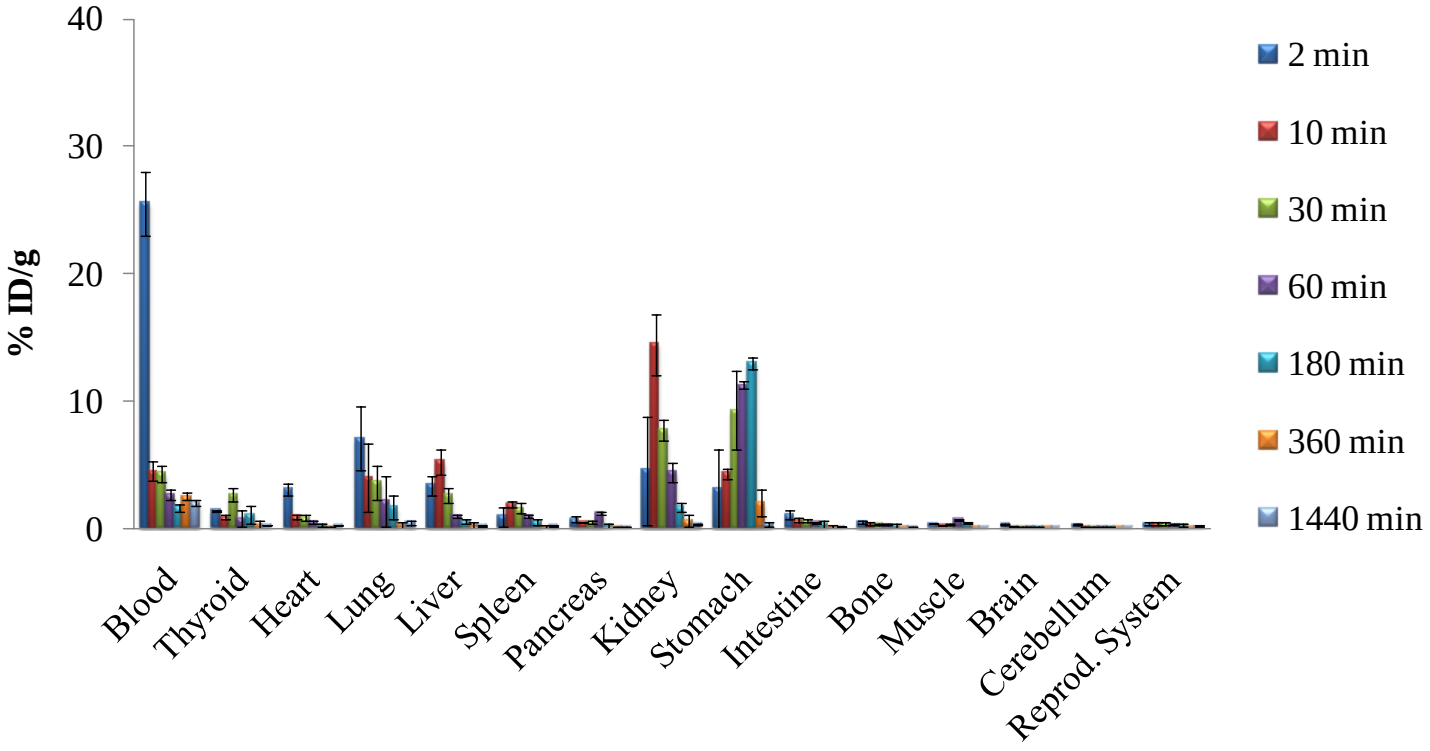

Figure 2. Biodistribution profile of ^{125}I-Leuc-B in Swiss mice bearing Ehrlich tumor.

The results showed that the highest concentration of polypeptide ^{125}I-Leuc-B in blood was reached 2 minutes (25.5 ± 2.5% ID/g) post i.v. injection and then it was gradually reduced, becoming negligible at 1440 minutes (2.01 ± 0.02% ID/g).The half-life ($T_{1/2}$) in the blood was 0.01 hours. The plasma clearance (CL) of ^{125}I-Leuc-B was 414.4 mL/h and the apparent volume of distribution was 3.18 mL (Fig. 3).

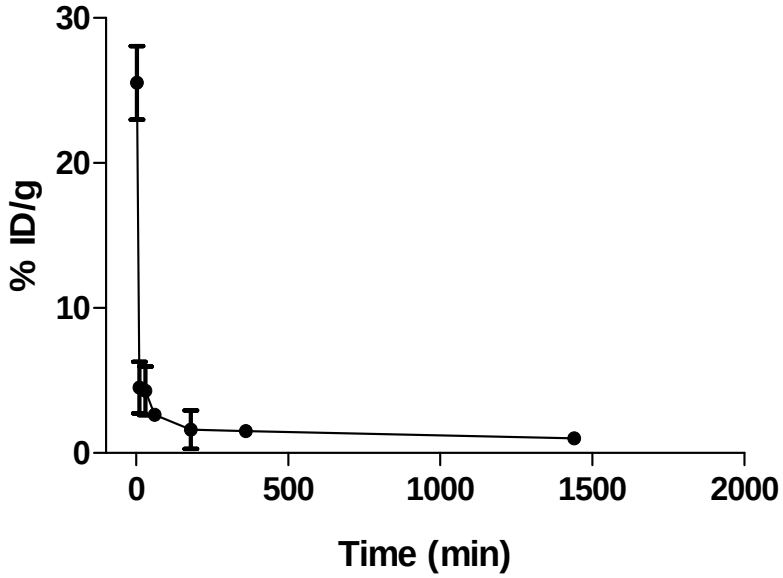

Figure 3. Biokinetics of ^{125}I-Leuc-B in the blood of animals bearing Ehrlich tumor. ^{125}I-Leuc-B was fastly cleared out from the blood after i.v. administration.

^{125}I-Leuc-B biokinetic profile in the kidneys and liver showed clear accumulation and retention of the compound in these organs, corroborating with their role in metabolism and elimination of this compound.

In the brain, it was observed negligible radioactivity for all time intervals, similar to background, suggesting that ^{125}I-Leuc-B cannot easily cross the blood brain barrier. The amount of ^{125}I-Leuc-B in the thyroid, heart, lung and spleen followed the kinetics of blood.

The uptake ratio of ^{125}I-Leuc-B in the tumor and non tumoral organs is shown in Tab1. The best setting in the paw with the Ehrlich tumor was achieved in times of 2 min. (3.5 ± 0.5% ID/g). The

Fig.4 shows that ^{125}I-Leu-B has a residence time for the tumor longer than that one for normal paw indicating therefore its potential for differential tumor detection.

Table 1. Concentration ratio of ^{125}I-Leuc-B in tumor and adjacent organs.

Time	2 min	10 min	30 min	60 min	180 min	360 min	1440 min
Tumor/Normal paw	1.77±0,25%	1.04±0.77%	1.24±0,68%	1.37±.0.37%	1.63±0.32%	1.21±0,26%	1.40±0,23%
Tumor/Muscle	8.44±0.25%	6.61±0.41%	7.90±0.24%	2.84±0.25%	4.77±0.18%	18.62±1.25%	9.91±5.5%
Tumor/Bone	5.89±0.5%	4.19±2.5%	7.53±1.8%	6.23±0.7%	9.82±0.9%	12.21±0.26%	9.07±0,3%
Tumor/blood	0.12±0.5%	0.38±0.2%	0.58±2.25%	0.75±0.1%	1.18±3.5%	0.13±0.4%	0.38±0.9%

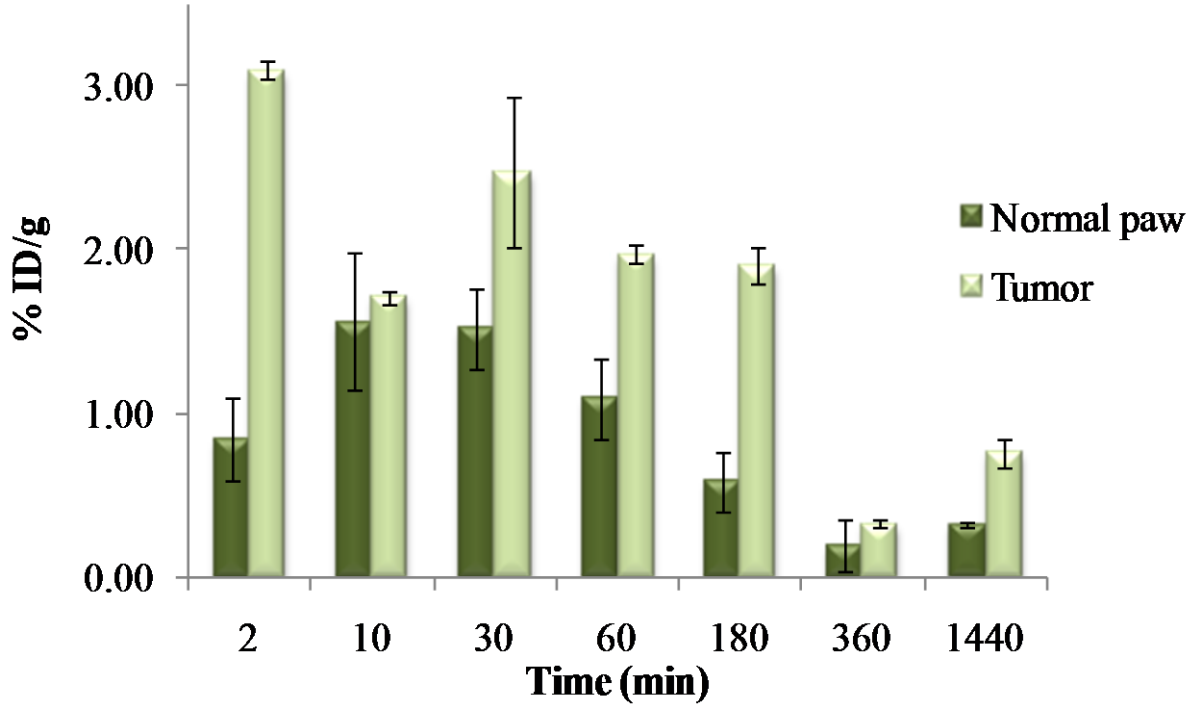

Figure 4. Comparison of the uptake of ^{125}I-Leuc-B in the normal paw and tumor.

4. DISCUSSION

According to World Health Organization (2006) the cancer kills 7 million people, where breast cancer is the second most frequent type in the world and more common among women [14].

The antitumoral biotechnological potential of snake venom compounds has been shown in several studies. It has been shown that snake venom disintegrins, such as; contortrostatin, echistatin [16], salmosin [17] also cause morphological changes in tumor cells, inhibiting adhesion and the metastasis. Jarharagin (ECD-disintegrin)

promotes changes in morphology and biological properties of tumor cells [15]. Gabriel *et a*l (2009) has shown the antitumoral effect of Leuc-B, which contain an ECD-disintegrin motif.

In order to further study to investigate the pharmacological potential of ^{125}I-Leuc-B, its radiolabeling was standardized. Radioactive ^{125}I-Leuc-B probe was synthesized with high efficiency (90%) and satisfactory specific activity (1.2x10$_{-7}$Bq/mmol).

The biodistribution profile of ^{125}I-Leuc-B indicated fast blood clearance (T1/2: 0.01 hours) which may occur mainly through the kidneys after hepatic metabolization. The uptake of ^{125}I-Leuc-B in the other abdominal organs followed the blood kinetics and became negligible after 10 min post injection.

The low uptake of the molecule by the brain, indicates that the ^{125}I-Leuc-B hardly cross the blood brain barrier, which make difficult the use of drugs for the systemic treatment of brain tumors. An alternative to this problem would be the encapsulation of drugs in nanovectors such as liposomes. These molecules have lipophylic characteristics that allowed them to cross the blood brain barrier, with the advantage of reducing the toxic effects of the encapsulated molecule [18].

The accumulation of ^{125}I-Leuc-B in thyroid was similar to background suggesting that radiolabeled molecule probably was not strongly hydrolyzed in the plasma and the molecular structure was kept intact.

Once the molecular structure of ^{125}I-Leuc-B was kept intact, it was able to interact with Ehrlich tumor and discriminate tumor from healthy tissues. If the ^{125}I-Leuc-B specific sites involve integrins receptors or if it is dependent of ECD domain, it remains to be evaluated. To assess the effect of toxicity by Leuc-B studies are under development.

5. CONCLUSIONS

In this work it was demonstrate the synthesis of a radioactive probe with ^{125}I based on ECD-like polypeptide, Leuc-B, with high efficiency and high specific activity preserving its biological activity. It was also demonstrated for the first time the *in vivo* interaction of ^{125}I-Leuc-B with solid tumors. Considering the other radioisotopes of iodine which emit high energy gamma photon and β (^{131}I) or positrons (^{124}I) it may be possible to design labeled Leuc-B analogs for internal radiotherapy and PET imaging, respectively.

All results indicate the potential use of Leuc-B as a template for the development of drugs and/or radiopharmaceuticals for the treatment and diagnosis of malignant tumors.

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