# PSMA-Specific Peptide with Inhibitor Cystine Knot for Prostate Cancer Treatment

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**ABSTRACT**

**Purpose:** A comparative study of the efficiency of binding *in vitro* and *in vivo* with prostate cancer cells of artificial peptides based on U5-scytotoxin-Sth1a toxin with an inserted peptide tropic to the prostate-specific membrane antigen and the radiopharmaceutical $^{177}$Lu-PSMA-617 is carried out.
**Objective and Methods:** The stability of peptides, toxicity, efficiency of binding to LNCaP, PC3 and CHO-K cells, as well as the biodistribution in a BALB/c mice model with inoculated prostate adenocarcinoma cells are studied using radiometric methods (radio-HPLC, TLC, scintillator radiometer).
**Results:** Our results show that the synthesized DOTA-Knot/C0-C1, DOTA-Knot/C1-C2 and DOTA-Knot/C2-C3 peptides containing the GTIQPYPFSWGY sequence inserted into U5-Sth1a knottin are more stable in blood plasma and saline and also show a similar degree of binding compared with the $^{177}$Lu-PSMA-617 radiopharmaceutical. Biodistribution studies of $^{177}$Lu-PSMA-617, $^{177}$Lu-DOTA-Knot/C0-C1, $^{177}$Lu-DOTA-Knot/C1-C2 and $^{177}$Lu-DOTA-Knot/C2-C3 in prostate adenocarcinoma-bearing BALB/c mice demonstrate that, for peptide $^{177}$Lu-DOTA-Knot/C1-C2, the tumor binding index is 20.4% ID/g, while for the radiopharmaceutical $^{177}$Lu-PSMA-617, it is 11.9% ID/g.
**Conclusion:** Thus, the modified peptides with a peptide tropic to the PSMA antigen inserted into the structure of U5-Sth1a toxin demonstrate the greatest stability, both in saline and in blood plasma, and good binding both to cell cultures and to tumors in the body.

## INTRODUCTION

Prostate cancer is the most common cancer in men worldwide, second only to lung cancer. In 2018, 1,276,106 new cases and 358,989 deaths from this type of cancer in men were detected. [1, 2, 3]. The ten-year prognosis of death due to prostate cancer ranges from 3-18% in men without concomitant chronic diseases and can reach 33% if present [4].

Classical therapy for localized prostate cancer includes expectant tactics, which consists in conducting a series of biochemical blood tests and prostate biopsies to determine the possibility of metastasis and choose a further treatment strategy [4]. Prostate cancer treatment may include surgery such as radical prostatectomy [5]. Less radical treatments are hormonal therapy [6, 7] and chemotherapy [7, 8]. However, the most common treatment of prostate cancer is radiation therapy and brachiotherapy [7, 9, 10].

In recent years, more and more attention has been paid to targeted radionuclide therapy [11]. The essence of targeted radionuclide therapy is the delivery of a radionuclide to the tumor using a drug with a high affinity for tumor cells [12]. Targeted radionuclide therapy uses the same mechanism for destroying tumor cells as in external radiotherapy; radiation damage to cells, however, has a large therapeutic index due to the selective accumulation of the drug in tumor tissues [13]. Peptides are of most interest for targeted therapy now [14, 15, 16]. Recent studies have shown the possibility of using targeted radionuclide therapy in the treatment of prostate cancer using peptides, tropic glutamate carboxypeptidase II (prostate-specific antigen, PSMA) [17, 18, 19, 20]. It is

assumed that PSMA is a multifunctional protein involved in nutrient uptake, cell migration, and proliferation [21]. However, the choice of PSMA as a target for targeted radionuclide therapy is due to its hyperexpression on the membrane of prostate cells [22] and the ability of PSMA to internalize [23], which allows the radiotherapy drug to concentrate in tumor cells.

However, peptides as a means of delivery of radionuclides have a number of disadvantages, such as low preoral bioavailability, insufficient in vivo stability, short shelf life, weak binding to target membrane proteins, and poor transmembrane transport [24, 25, 26].

Some studies show that knottins, or peptides with cysteine knots, can be a solution to these problems [27, 28, 29]. A distinctive feature of such peptides is the presence of a structure of three disulfide bonds [28]. In a typical cysteine knot peptide, the first and fourth, second and fifth cysteine residues form disulfide bonds; a disulfide bond formed between the third and sixth cysteine residues passes through these first two disulfides, creating a macrocyclic knot [30]. This knot imparts chemical, thermal, and proteolytic stability to the peptide [28, 29, 30, 31]. There are studies showing the possibility of obtaining target peptides with cysteine knots, and their greater efficiency compared with peptides lacking them [32, 33, 34, 35].

The aim of this work is to synthesize a peptide tropic to the prostate-specific antigen and containing a cysteine knot and to compare its binding efficiency using prostate cancer cell cultures as an example.

## MATERIALS AND METHODS

Three cell cultures of prostate cancer were used in the work: LNCaP (obtained from lymph node metastases), PC3 (obtained from bone metastases), CHO-K1 (fibroblast-like ovarian cells).

PSMA antigens with the GTIQPYPFSWGY sequence [36] were synthesized which were inserted into U5-scytotoxin-Sth1a toxin (isolated from the venom of a Scytodes thoracica spider) [37, 38] at the beginning of the sequence (DOTA-Knot / C0-C1), after the first cysteine (DOTA -Knot / C1-C2) and after the second cysteine (DOTA-Knot / C2-C3). A DOTA chelator was attached to each sequence (Fig. 1).

Peptides were synthesized using a ResPepSL peptide synthesizer (Intavis) in accordance with a standard protocol based on solid-phase synthesis using a protective F-moc (fluorenylmethyloxycarbonylchloride)-AA group on TentaGel resin where acetic anhydride was used as "cappingmixing" (0.5M 2- (1H-benzotriazol-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate) activator, N-Methylmorpholine catalyst, Dimethylformamide, N-Methyl-2-pyrrolidone solvents, 20% piperidine deprotector. After synthesis, the peptides were separated from the resin with a cocktail containing Trifluoroacetic acid (92.5%), Triisopropylsilane (5%) and deionized water (2.5%). After that, the cleaved peptides were precipitated by means of cold Methyltert-butylether and dried. Analysis of the synthesis results was carried out using the methods of high-performance liquid chromatography and mass spectrometry (Fig. 1).

High performance liquid chromatography was carried out by means of anion exchange chromatography on a Bio-Rad NGC Quest chromatograph equipped with a photometric detector. Detection was performed at a wavelength of 280 nm. The column was anion-exchange Agilent PL-SAX 4.6x150 mm, 1000A, 10 mM (Part No: PL1551-3102). Gradient elution was used; eluent A was deionized water with the addition of 20 mM Tris-HCl; eluent B was deionized water with the addition of 20 mM Tris-HCl and 1 M NaCl. Gradient elution protocol was: 2.95 ml - 0% B; 10 ml - 0-50% B; 3.5 ml - 100%.

Mass spectrometric analysis was carried out on a FLEX series MALDI-TOF MS hardware-software complex (Bruker Daltonics, Germany). For further research, the peptides were labeled with active lutetium in the form of $^{177}$LuCl3 in 0.05 N hydrochloric acid. The reaction mixture consisted of lutetium chloride with an activity of 3.5 MBq, dissolved peptide (final concentration was 0.05 mg / ml) and 0.2 M sodium acetate buffer with pH 4. Labeling was carried out using a Gaia synthesis module (Raytest, Germany) at 80 ° for 10 minutes for knottins and 95 ° C for 20 minutes for PSMA-617.

Analysis and purification of the peptide before and after synthesis was carried out using HPLC with a UV detector (Shimadzu, Japan) on an Eclipcse XDB-C18, 5 μm, 4.6x150mm column (Agilent technologies, USA) with a mobile phase of 0.1% trifluoroacetic acid in water /

acetonitrile, at a wavelength of 214 nm. Activity was measured using a Gabi radio detector (Raytest, Germany). Peptides with radiochemical purity > 95% were used.

The stability of the peptides was studied for 168 hours in saline (pH-7.4) and human plasma. For this purpose, NaCl (0.9%) in a volume of 900 μL was added to solutions of peptides $^{177}$Lu-DOTA-Knot / C0-C1, $^{177}$Lu-DOTA-Knot / C1-C2, $^{177}$Lu-DOTA-Knot / C2-C3 (100 μl, 4.25 MBq) and PSMA-617 (100 μl, 10 MBq). Each mixture was incubated at 4 ° C and at 21 ° C in triplicate for each time point and analyzed using radio-HPLC with a Gabi radioactivity detector (Raytest, Germany) (Fig. 1, 2) after 24, 48, 96 and 168 hours [39]. To study the stability in plasma, peptides in a volume of 100 μl (10 MBq) were added to human plasma and incubated at +37 ° C for 168 hours [40, 41]. For control, peptides stored at +4 ° C were used. Their stability was studied using thin layer chromatography (TLC).
For this purpose, the samples were applied to plates covered with silica gel, dried at 40 ° C, transferred to a chamber with pure acetonitrile, and stored for 10 minutes to raise the gradient. After that, the plates were again dried and read using a MiniGita Star TLC with a beta positron detector (Elysia-raytest GmbH, Germany). Each time point was repeated three times.

To study the binding to the culture, cells were seeded into plates in the amount of 100 thousand cells per well of the plate. After the addition of the labeled peptide (50KBq), the cultures were incubated in PBS for 3 hours at 37 ° C in an atmosphere of 5% CO2. The peptide $^{177}$Lu-PSMA-617 [42, 43] was used as a comparison. The activity of the initial peptide solution, PBS solution after incubation and a solution with washed-off cells was measured by means of a Triathler scintillator radiometer (Hidex Oy, Finland) using a sodium iodide crystal (NaITI). The results were processed using Excel.

To analyze the toxicity, CHO-K1 cells were treated with unlabeled peptides (0.5 μM) 24 hours after passage, and the kinetics of the cytostatic response was monitored in real time using the RTCA S 16 xCELLigence 16-well plate system (ACEA Biosciences, USA). [44]

The distribution of peptides was determined at 4 and 24 hours after injection in BALB/c mice inoculated with prostate adenocarcinoma cells, five in each group. Each individual was injected with 100 μl (3.9 MBq) of a labeled peptide solution through the tail vein. Animals were killed using animal care protocols. Tissues were weighed, and their specific activity as a percentage of the administered dose per gram was determined using a Triathler scintillator radiometer (Hidex Oy, Finland).

## RESULTS

Knot/C0-C1 DOTA GTIQPYPFSWGY CVTRGNFCATPEVHGDWCCGSLKCVSNSCR
Knot/C1-C2 DOTA DETPDEC GTIQPYPFSWGY CATPEVHGDWCCGSLKCVSNSCR
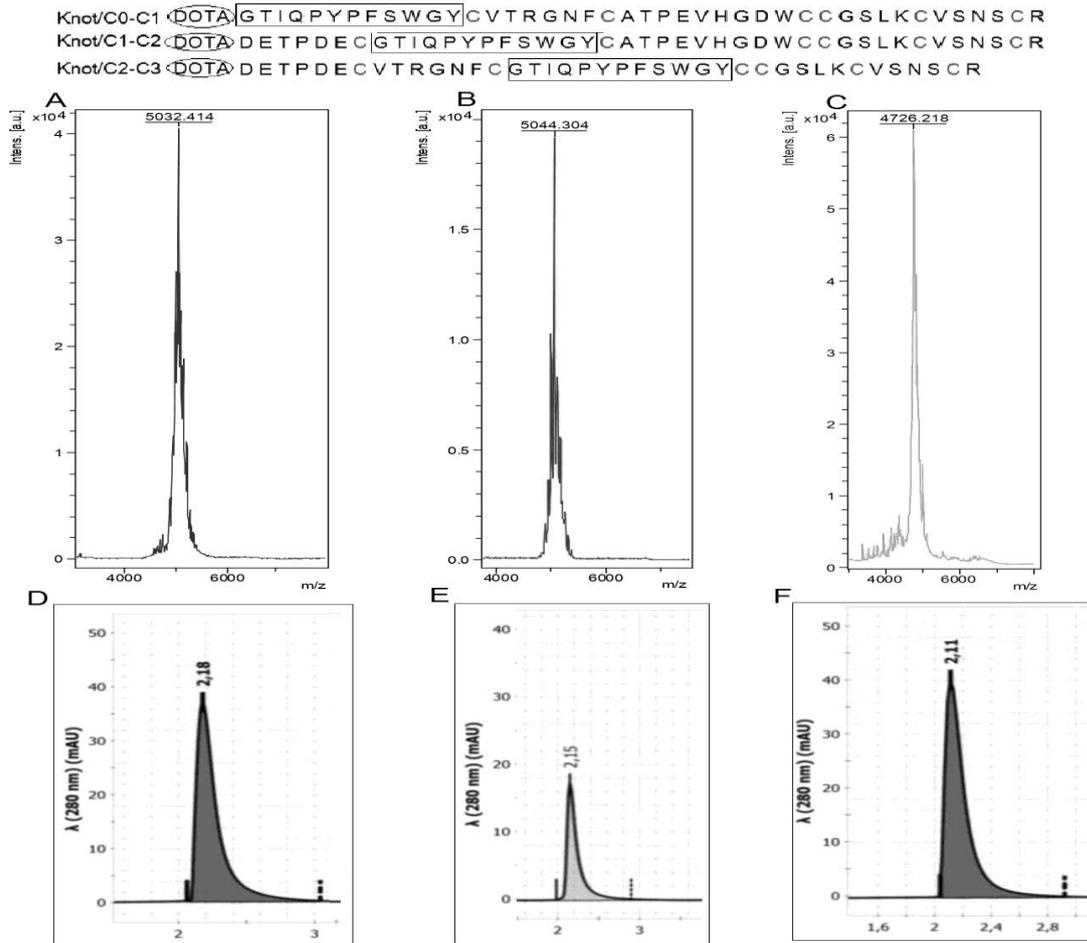Knot/C2-C3 DOTA DETPDECVTRGNFC GTIQPYPFSWGY CCGSLKCVSNSCR



**Figure 1**. The results of the synthesis of PSMA-tropic peptide with the sequence G T I Q P Y P F S W G Y inserted into different regions of U5-Sth1a toxin

Figure 1 shows the results of the synthesis of U5-Sth1a toxin with a PSMA antigen tropic peptide inserted into different domains, having the sequence G T I Q P Y P F S W G Y and an attached DOTA chelator. All three peptides

DOTA-Knot / C0-C1, DOTA-Knot / C1-C2, DOTA-Knot / C2-C3 were synthesized and purified to a degree higher than 95%.
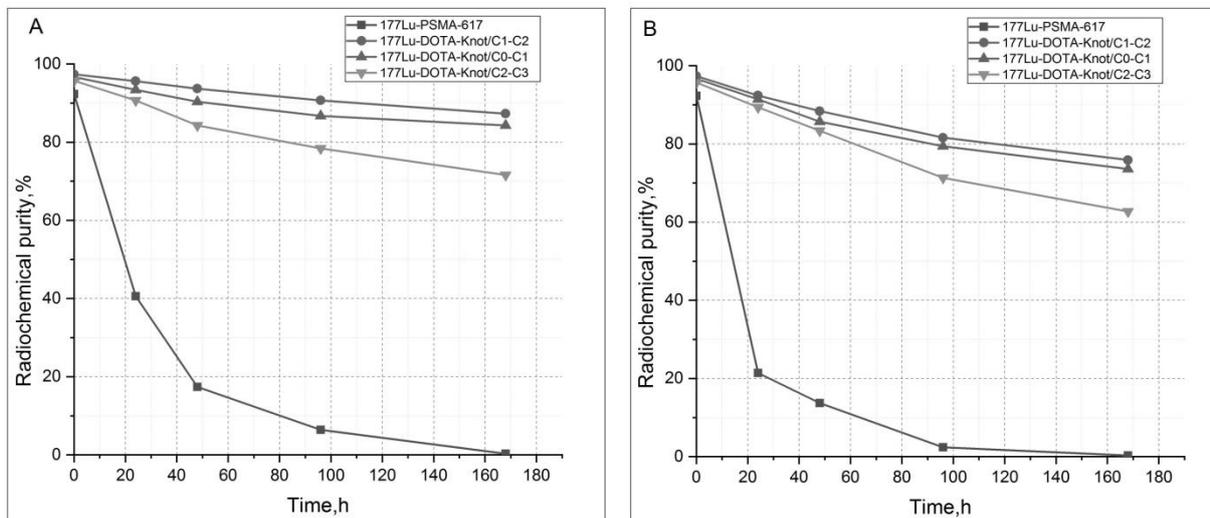
**Figure 2**. Stability of labeled peptides: ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot / C2-C3 in saline (A) and in blood plasma (B).

Figure 2 depicts the stability of lutetium-labeled DOTA-Knot / C0-C1, DOTA-Knot / C1-C2, DOTA-Knot / C2-C3 and PSMA-617 peptides in human saline and plasma for 168 hours. It can be seen that ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot / C2-C3 are more stable than ^{177}Lu-PSMA-617 both in saline and in plasma. The radiochemical purity of ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot /

C2-C3 peptides has retained over 70% for 168 hours in saline and for 96 hours in blood plasma. ^{177}Lu-DOTA-Knot / C1-C2 peptide has shown the highest stability; it retains RCP> 90% in saline and> 80%. in plasma within 96 hours. All these data confirm the hypothesis of an increase in the stability of RPs due to embedding a toxin into the molecule stabilized by disulfide bridges.
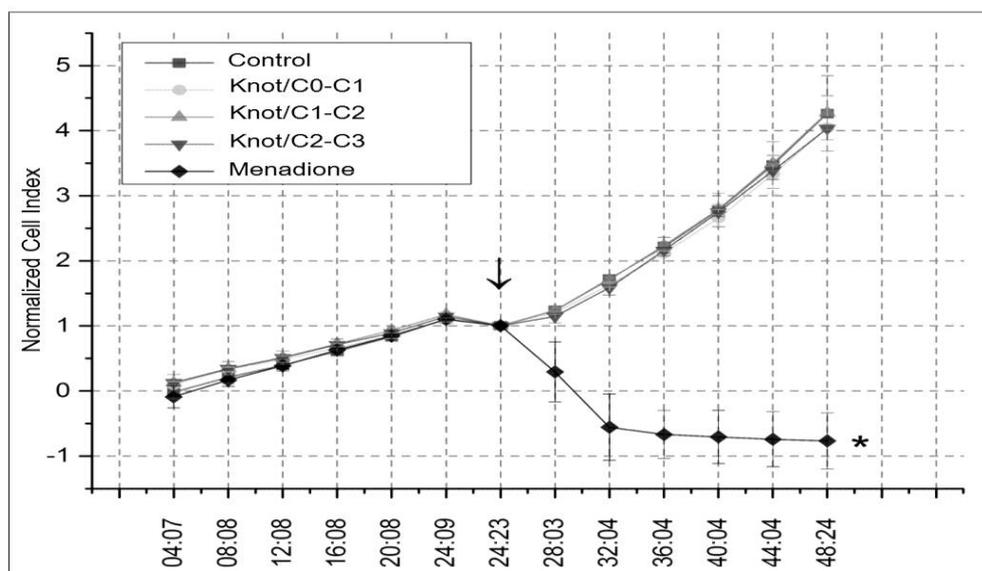


Figure 3. Cytotoxic responses of CHO-K1 cells after the addition (↓) of DOTA-Knot / C0-C1, DOTA-Knot / C1-C2 and DOTA-Knot / C2-C3 peptides and menadione after 24 hours of incubation. (* p> 0.05 when compared with the control).

Figure 3 depicts the cytotoxic responses of CHO-K1 cells after the addition of DOTA-Knot / C0-C1, DOTA-Knot / C1-C2 and DOTA-Knot / C2-C3 peptides and menadione after 24 hours of incubation. As can be seen from the figure, the differences between the treated cells and the control after the addition of peptides are minimal. Positive control

values of the cell index with menadione treatment have negative values (-0.8), indicating cell death. Statistically significant differences were obtained when comparing the data with the control group at p> 0.05. The data obtained indicate the absence of toxicity of the studied peptides for CHO-K1 cells at concentrations of 0.5 μM.
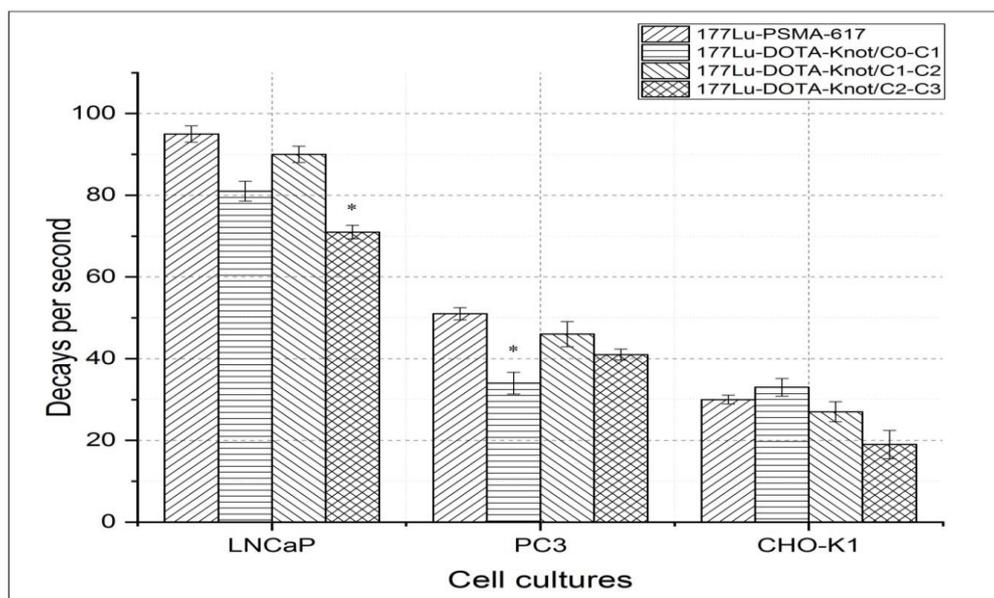
Figure 4. Binding of labeled ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot / C2-C3 peptides with the membrane of tumor cells of LNCaP, PC3 and CHO-K1 lines after 3 hours (* - statistically significant difference (p <0.05) when compared with ^{177}Lu-PSMA-617).

Figure 4 shows the degree of binding of ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot / C2-C3 peptides with LNCaP, PC3 and CHO-K1 cell lines after 3 hours of incubation. It can be seen that all the peptides bind sufficiently to the LNCaP culture, especially ^{177}Lu-PSMA-617 and ^{177}Lu-DOTA-Knot / C1-C2.

Less binding has been observed for the PC3 culture, and the least – for CHO-K1. All these data indicate the attachment of peptides to the PSMA antigen to the cell surface. Since the number of antigens on the surface of cancer cells is higher, the degree of binding to these cells is greater.
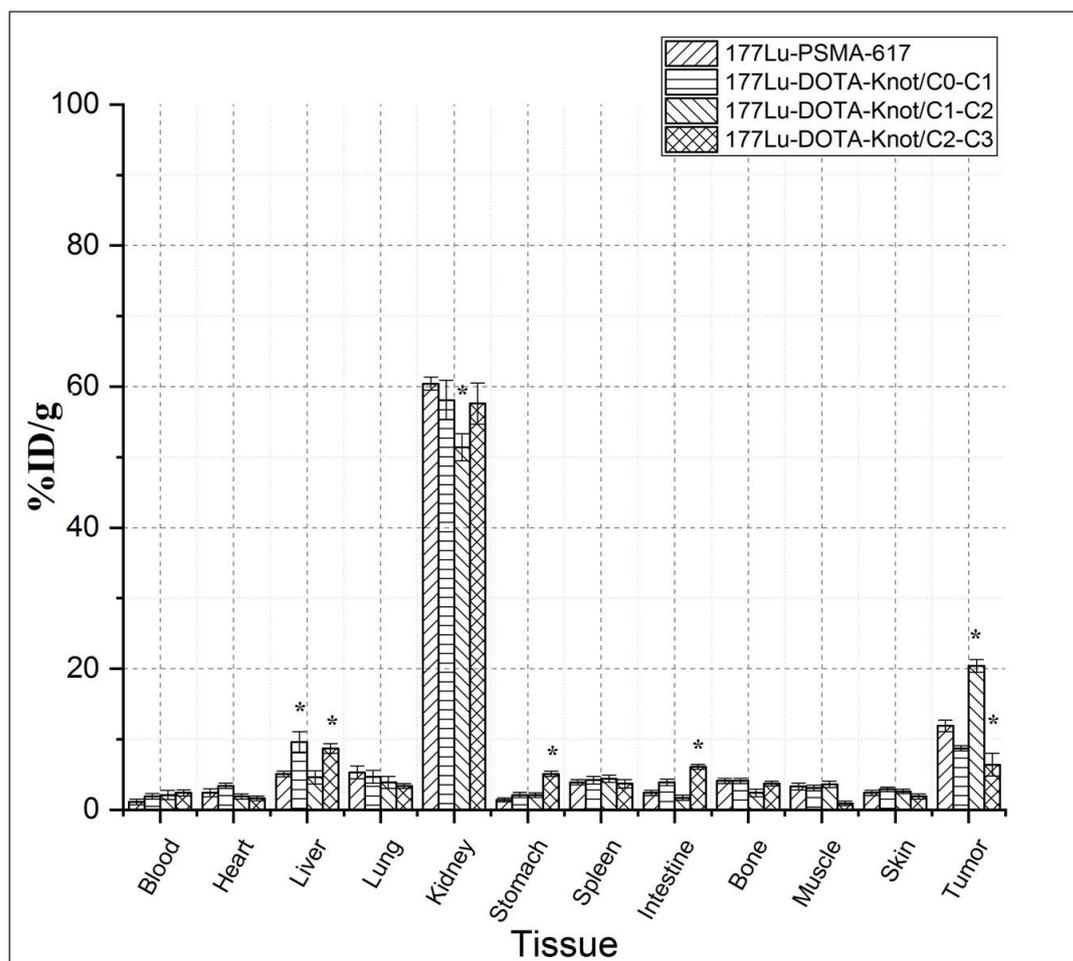


Figure 5. Biodistribution of ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, 177Lu-DOTA-Knot/C1-C2 and ^{177}Lu-DOTA-Knot/C2-C3 in prostate adenocarcinoma-bearing BALB/c mice in 24 hour (* - statistically significant difference (p <0.05) when compared with 177Lu-PSMA-617).

Figure 5 shows the biodistribution of ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot / C2-C3 peptides in BALB/c mice after 24 hours after injection. As can be seen, the greatest binding to tumor cells after 24 hours is observed in ^{177}Lu-PSMA-617 and ^{177}Lu-DOTA-Knot / C1-C2, while the other two peptides accumulate more in the liver (^{177}Lu-DOTA-Knot / C0- C1 and ^{177}Lu-DOTA-Knot / C2-C3), stomach and intestines (^{177}Lu-DOTA-Knot / C2-C3).

## DISCUSSION

One of the ways to increase the effectiveness of RPs is to increase their resistance to the action of blood proteases. The existing radiopharmaceuticals for the treatment of prostate cancer are quite sensitive to the action of plasma enzymes. Under the action of blood proteases, RPs are destroyed and accumulated in the kidneys, liver and

gallbladder. The half-life of many RPs is within 10 hours [45]. An increase in the half-life will increase the radiation load on the tumor and reduce it on other organs, as well as reduce the administered dose of the isotope. Work on increasing the half-life of RPs has been carried out in different directions, for example, peptides can be modified with additional sequences of hydrophobic amino acids, a polyethylene glycol molecule can be attached to peptides [46], or peptides can be incorporated into polymer micro- and nanoparticles [47]. There is another way to increase the stability of RPs in the internal environment of the body, which is associated with the use of natural peptide structures. The most attractive natural peptide construct is peptides containing multiple cysteine bridges. These peptides are usually found in natural toxins. The number of disulfide bridges in such peptides ranges from one to seven, but almost 60% of all toxins have three bridges.

These peptides often contain a disulfide pseudo-knot, and therefore they are called cysteine knot peptides or knottins [48]. Three disulfide bridges and one intermediate form a pseudo-knot consisting of a ring (Cys I - Cys IV, Cys II - Cys V) pierced by a third disulfide bridge (Cys III - Cys VI). Cysteine knot peptides have the conserved sequence -Cys-X 3-7 -Cys-X 3-8 -Cys-X 0-7 -Cys-X 1-4 -Cys-X 4-13-Cys-, where X is any amino acid [49]. These peptides are highly stable in the internal environment of the body. Stability in plasma can be up to 72 hours [49, 50]. As can be seen from the description of knottins, they have both conserved and variable aminoacid sequences in their structure. Variable sequences can contain any amino acids.

In our study, we have replaced the natural sequences of U5-scytotoxin-Sth1a knottin (a venom component of the spider family Scytodes thoracica) with the sequence GTIQPYPFSWGY in positions before the first cysteine knot (DOTA-Knot / C0-C1), after the first cysteine (DOTA-Knot / C1-C2 ), after the second cysteine (DOTA-Knot / C2-C3). All peptides were synthesized using an automated peptide synthesizer and were> 95% pure (Fig. 1).

Since the knottin from the spider venom was taken as a basis, toxicity was tested in cell culture. Experiments have shown that our peptide constructs did not have a toxic effect on the cell culture (Fig. 3).

Experiments to study the stability of the synthesized peptides in saline and blood plasma have confirmed our hypothesis that they are more stable than linear peptides. Compared with the widely used peptide PSMA-617, our peptides retained 75-90% radiochemical purity in saline and 60-77% radiochemical purity in blood plasma after 168 hours, while PSMA-617 was completely destroyed (Fig. 2).

Experiments to study the binding of labeled peptides ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2 and ^{177}Lu-DOTA-Knot / C2-C3 with the membrane of tumor cells of LNCaP, PC3 and CHO-K1 cell lines have demonstrated a similar binding degree compared to ^{177}Lu-PSMA-617 (Fig. 4). It can be noted that the incorporation of the peptide into the knotin construct does not greatly affect its ability to bind to target sites on the cell surface.

Experiments to study the biodistribution of ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2, ^{177}Lu-DOTA-Knot / C2-C3 in prostate adenocarcinoma-bearing BALB/c mice in 24 hours have showed that ^{177}Lu-DOTA-Knot/C1-C2 accumulates in the cancer tumor almost twice as much as ^{177}Lu-PSMA-617 (Fig. 5). This is due to the greater stability of the ^{177}Lu-DOTA-Knot / C1-C2 peptide in blood plasma, which leads to prolonged circulation in the bloodstream and more frequent interaction of the peptide with prostate cancer cells.

## CONCLUSION

Our study has shown that the synthesized DOTA-Knot / C0-C1, DOTA-Knot / C1-C2 and DOTA-Knot / C2-C3 peptides containing the GTIQPYPFSWGY sequence inserted into knottin U5-Sth1a are more stable in blood plasma and saline, and also exhibit a similar degree of binding as compared to the ^{177}Lu-PSMA-617 radiopharmaceutical. Biodistribution studies of ^{177}Lu-PSMA-617, ^{177}Lu-DOTA-Knot / C0-C1, ^{177}Lu-DOTA-Knot / C1-C2, ^{177}Lu-DOTA-Knot / C2-C3 in prostate adenocarcinoma-bearing BALB/c mice have demonstrated that, for ^{177}Lu-DOTA-Knot / C1-C2, the tumor binding index is 21% ID/g, while for the radiopharmaceutical ^{177}Lu-PSMA-617, it was 11% ID/g.

Thus, all the data presented show that the modified peptides with a peptide tropic to the PSMA antigen inserted into the structure of U5-Sth1a toxin demonstrate the greatest stability, both in saline and in blood plasma, and good binding both to cell cultures and to tumors in the body.

## LIST OF ABBREVIATIONS
PSMA - Prostate-specific membrane antigen
HPLC - High-performance liquid chromatography
TLC - Thin Layer Chromatography
RPs – Radiopharmaceuticals

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This research study was approved by the Ethics Committee of the Institute of Medicine, Ecology and Physical Culture of Ulyanovsk State University. Legal and ethical approvals were obtained prior to initiation of the research work carried out on animals. The experiments were performed in accordance with the relevant guidelines and regulations.

## CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest related to this article.

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