

Knottins As A Structural Basis For The Stabilization Of Radio Pharmaceuticals

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Abstract

The aim of the current research was to study synthesize a peptide tropic to the prostate-specific antigen and containing a cysteine knot.

Materials and Methods: A comparative study of the efficiency of binding in vitro 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 ¹⁷⁷Lu-PSMA-617 is carried out. Three prostate cancer cell lines were used in the research.

Results: Synthesized DOTA-Knot/C0-C1, DOTA-Knot/C1-C2 and DOTA-Knot/C2-C3 peptides containing the GTIQYPFSWGY sequence inserted into U5-Sth1a knottin are more stable in blood plasma and saline and also show a similar degree of binding to LNCaP, PC3 cells compared with the ¹⁷⁷Lu-PSMA-617 radiopharmaceutical.

Conclusion: Modified peptides with a peptide tropic to the PSMA antigen inserted into the structure of U5-Sth1a toxin demonstrate the greatest stability.

Keywords: prostate cancer, knottin, spider toxin, radiopharmaceuticals, stability.

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 (1-3). 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). Hormonal therapy and chemotherapy are the less radical treatment methods (6-8). 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 (14-16). 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) (17-20). 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 (24-26).

Some studies show that knottins or peptides with cysteine knots can be a solution to these problems (27-29). 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 (28-31).

There are studies about the possibility of obtaining target peptides with cysteine knots and their greater efficiency compared with peptides lacking them (23, 32-34). So, the aim of this work became the study 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.

Material and methods

Three cell cultures of prostate cancer were used in the research:

- LNCaP (obtained from lymph nodemetastases),
- PC3 (obtained from bone metastases),
- CHO-K1 (fibroblast-like ovarian cells).

All conducted actions in relaters of the presented research have been 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.

PSMA antigens with the GTIQYPFSWGY sequence³⁵ were synthesized which were inserted into U5-scytotoxin-Sth1a toxin (isolated from the venom of a Scytodes thoracica spider) (36-37) 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 (Figure 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. The peptides were separated from the resin with a cocktail containing Trifluoroacetic acid (92.5%), Triisopropylsilane (5%) and deionized water (2.5%) after synthesis. After that, the cleaved peptides were precipitated by means of cold Methyltert-butylether and dried (38). Analysis of the synthesis results was carried out using the methods of high-performance liquid chromatography and mass spectrometry (Figure 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% B (39).

Mass spectrometric analysis was carried out on a FLEX series MALDI-TOF MS hardware-software complex (Bruker Daltonics, Germany) (40).

For further research, the peptides were labeled with active lutetium in the form of $^{177}\text{LuCl}_3$ 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 min. for knottins and 95 ° C for 20 minutes for PSMA-617 (41).

Analysis and purification of the peptide before and after synthesis was carried out using HPLC with a UV detector (Shimadzu, Japan) on an Eclipse XDB-C18, 5 μm , 4.6x150 mm column (Agilent technologies, USA) with a mobile phase of 0.1% trifluoroacetic acid in water or acetonitrile, at a wavelength of 214 nm. Activity was measured using a Gabi radio detector (Raytest, Germany). Peptides with radiochemical purity > 95% were used (42). The stability of the peptides was studied for 168 hours in saline (pH-7.4) and human plasma. For this purpose, NaCl (0.9%) - 900 mL 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 mL, 4.25 MBq) and PSMA-617 (100 mL, 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) (Figure 1 and Figure 2) after 24, 48, 96 and 168 hours (43). To study the stability in plasma, peptides in a volume of 100 mL (10 MBq) were added to human plasma and incubated at +37°C for 168 hours (43-44). 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 min. 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 (45-46).

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 177Lu-PSMA-617 (47-48) was used as a compare sample. 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 (NaI(Tl)) (49). 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) (50-51).

Results

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 was conducted (Figure 1). 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%.

The research has demonstrated 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 (Figure 2). It can be seen that 177Lu-DOTA-Knot / C0-C1, 177Lu-DOTA-Knot / C1-C2 and 177Lu-DOTA-Knot / C2-C3 are more stable than 177Lu-PSMA-617 both in saline and in plasma. The radiochemical purity of 177Lu-DOTA-Knot / C0-C1, 177Lu-DOTA-Knot / C1-C2 and 177Lu-DOTA-Knot / C2-C3 peptides has retained over 70% for 168 hours in saline and for 96 hours in blood plasma. 177Lu-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.

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 is depicted in figure 3.

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 shows the degree of binding of 177Lu-PSMA-617, 177Lu-DOTA-Knot / C0-C1, 177Lu-DOTA-Knot / C1-C2 and 177Lu-DOTA-Knot / C2-C3 peptides with LNCaP, PC3 and CHO-K1 cell lines after incubation in 3 hours. It can be seen that all the peptides bind sufficiently to the LNCaP culture, especially 177Lu-PSMA-617 and 177Lu-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 amount of antigens on the surface of cancer cells is higher, the degree of binding to these cells is greater.

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 (52). 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 (53). 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 (54), a polyethylene glycol molecule can be attached to peptides (55), or peptides can be incorporated into polymer micro- and nanoparticles (56). 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 (57). 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 (58). These peptides are highly stable in the internal environment of the body. Stability in plasma can be up to 72 hours (59-60). 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.

We have replaced the natural sequences of U5-scytotoxin-Sth1a knottin (a venom component of the spider family *Scytodes thoracica*) with the sequence GTIQYPFSWGY 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 (Figure 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 (Figure 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 (Figure 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 (Figure 4). It can be noted that the incorporation of the peptide into the knottin construct does not greatly affect its ability to bind to target sites on the cell surface.

Conclusion

Our study has shown that the synthesized DOTA-Knot / C0-C1, DOTA-Knot / C1-C2 and DOTA-Knot / C2-C3 peptides containing the GTIQYPFSWGY 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.

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.

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The authors declare there is no conflict of interests related to this article.

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Figures

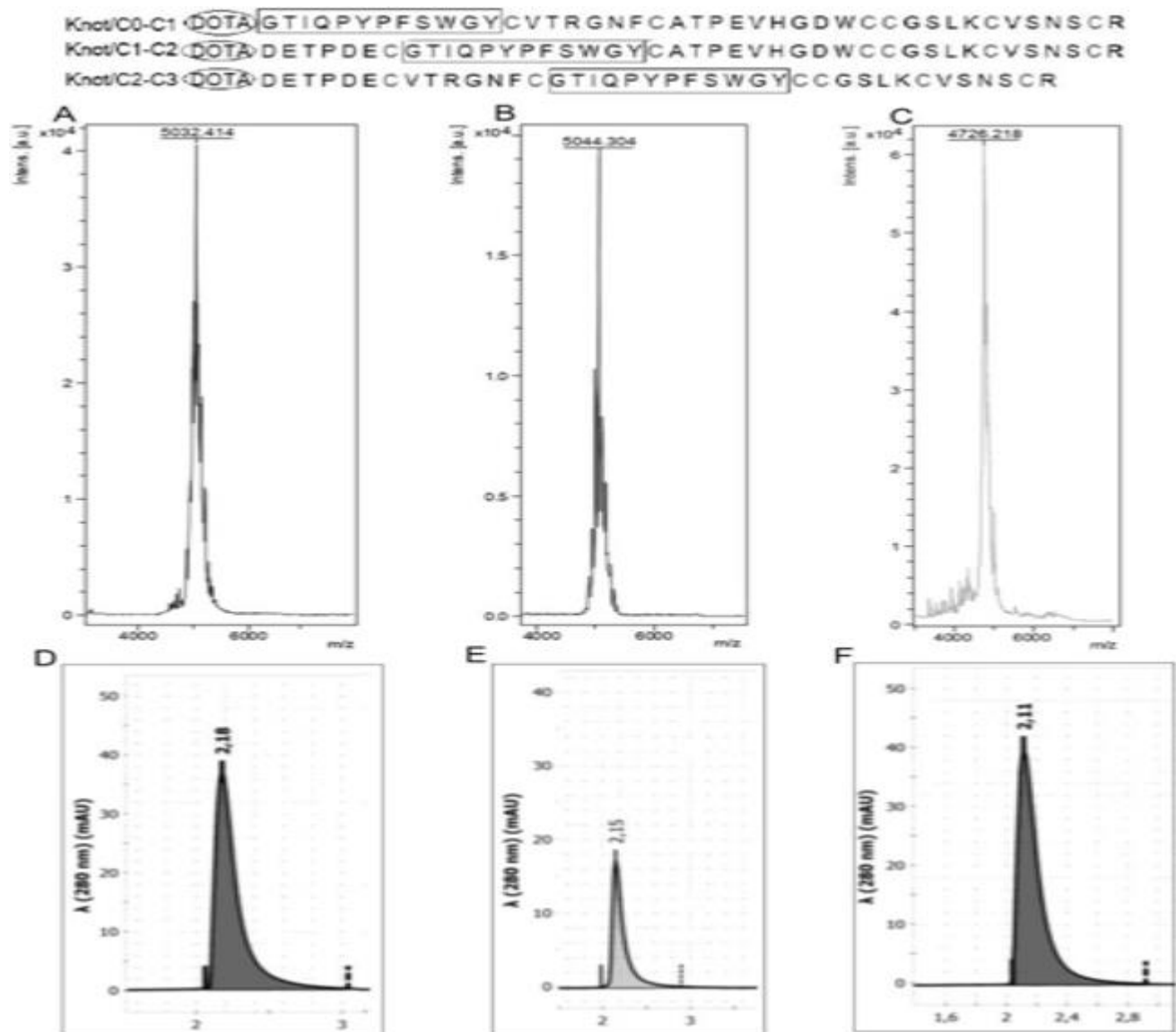


Figure 1: The results of the synthesis of PSMA-tropic peptide with the sequence GTIQPYPSWGY inserted into different regions of U5-Sth1a toxin

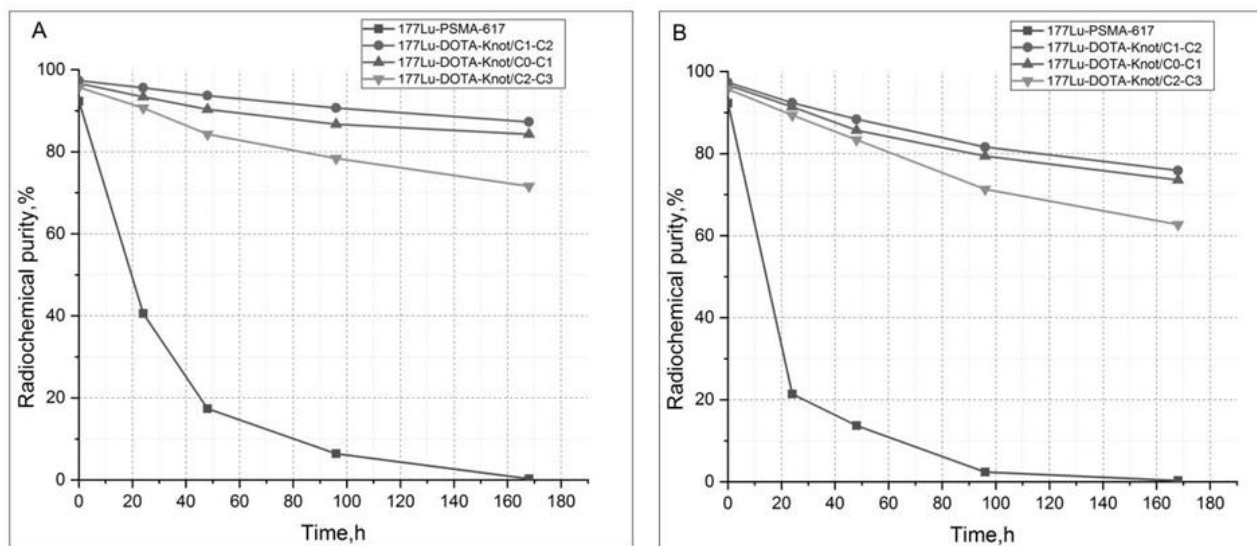


Figure 2: Stability of labeled peptides: 177Lu-PSMA-617, 177Lu-DOTA-Knot / C0-C1, 177Lu-DOTA-Knot / C1-C2 and 177Lu-DOTA-Knot / C2-C3 in saline (A) and in blood plasma (B).

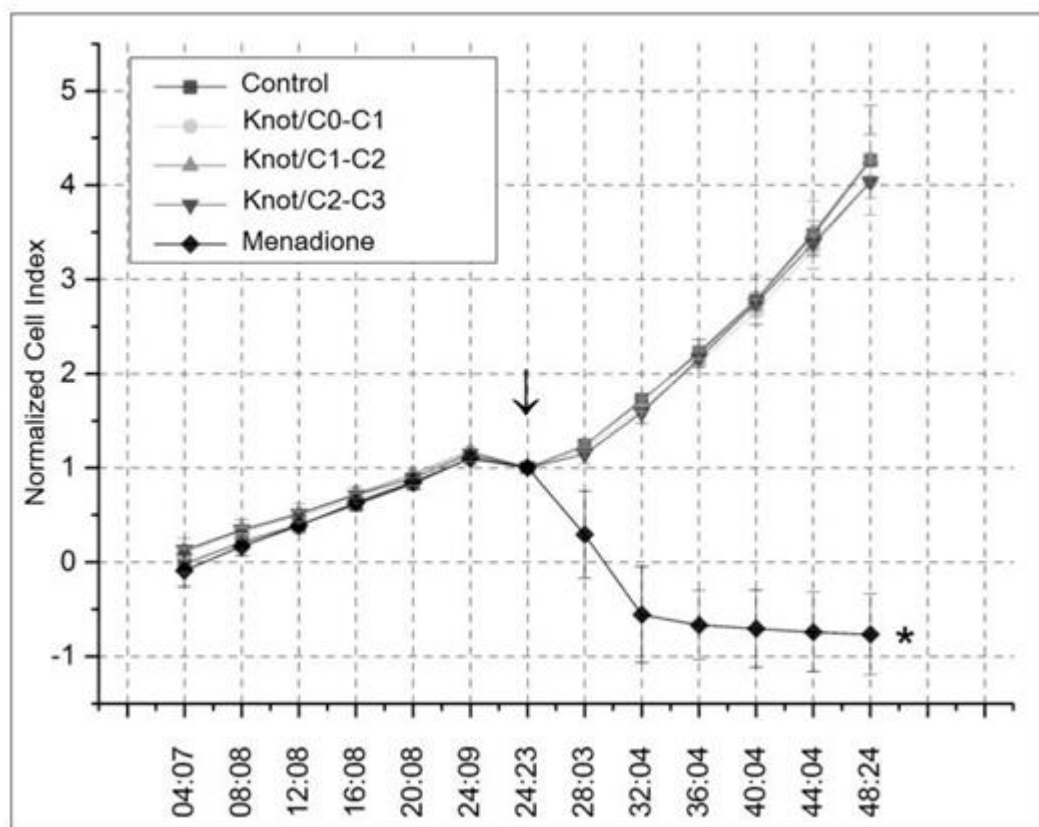


Figure 3: Cytotoxic responses of CHO-K1 cells after the addition (↓) of DOTA-Knot / C0-C1, DOTA-Knot / C1-C2 and DOTA-Knot / C2-C3 peptides and menadione after 24 hours of incubation.

*p> 0.05 when compared with the control.

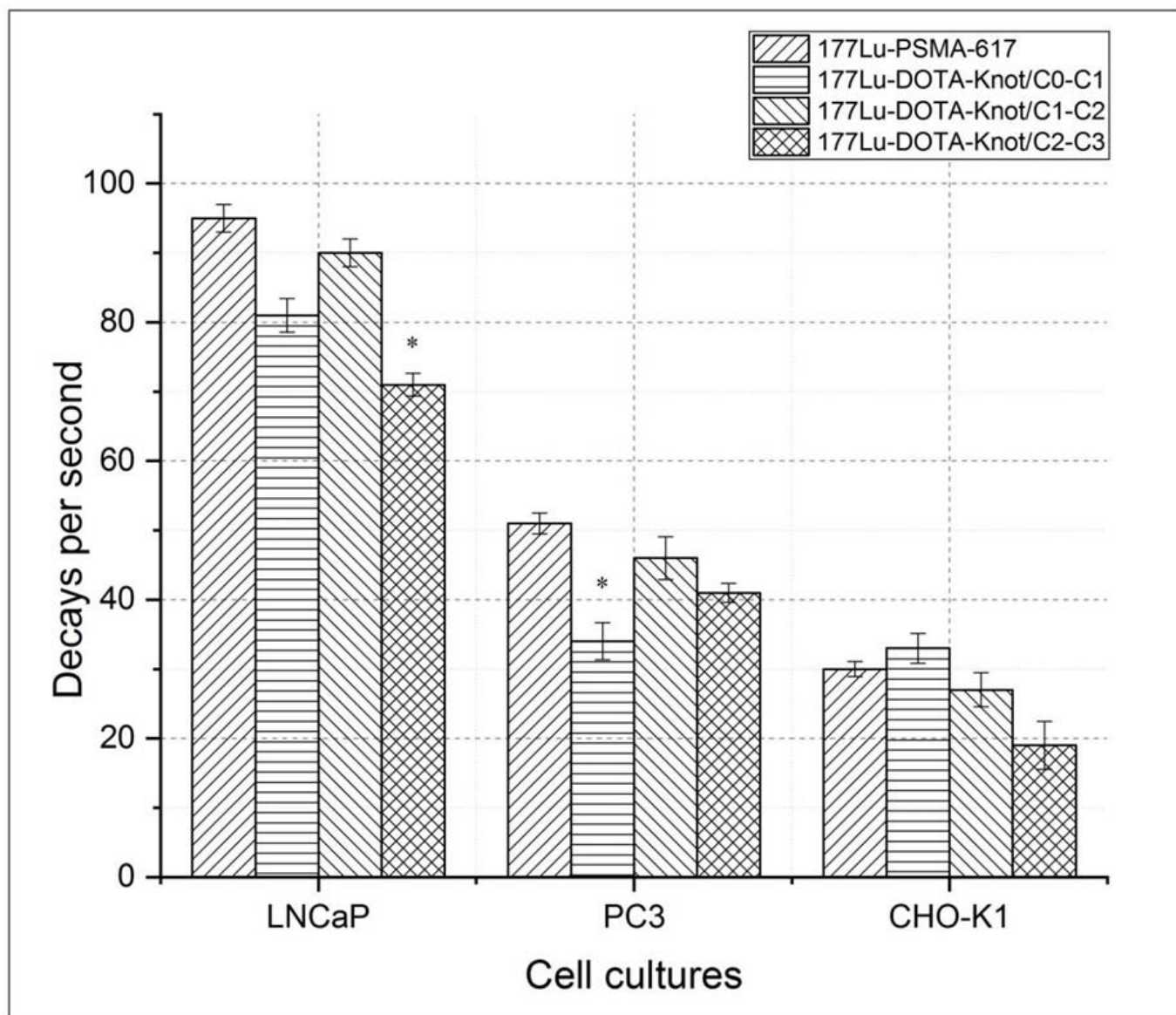


Figure 4: Binding of labeled ^{177}Lu -PSMA-617, ^{177}Lu -DOTA-Knot / C0-C1, ^{177}Lu -DOTA-Knot / C1-C2 and ^{177}Lu -DOTA-Knot / C2-C3 peptides with the membrane of tumor cells of LNCaP, PC3 and CHO-K1 lines after 3 h (* - statistically significant difference ($p < 0.05$) when compared with ^{177}Lu -PSMA-617).