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Use of Natural Peptides with a Cysteine Knot of Arthropods as a Carrier of a Peptide Tropic to Glutamate Carboxypeptidase II

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ABSTRACT
The aim of the investigation. Synthesis of a peptide with a cysteine knot, affinity to glutamate carboxypeptidase II, evaluation of its binding to glutamate carboxypeptidase II on the surface of LNCaP prostate cancer cells. Comparison of peptide binding to a cysteine knot, affinity to glutamate carboxypeptidase II with the parent peptide, affinity to glutamate carboxypeptidase II.
Materials and Methods. Peptide with sequense GTIQPYPFSWGY, affinity to glutamate carboxypeptidase II, was inserted into the peptide with the cysteine knot U5-Sth1a (isolated from the spider venom of the family Scytodes thoracica). The peptides were synthesized using a peptide synthesizer (ResPepSL, Intavis), the synthesis result was monitored by high performance liquid chromatography (NGC Quest, Bio-Rad) and mass spectrometry (MALDI-TOF MS, Bruker Daltonics). As an object of study, we used prostate cancer cell cultures LNCaP (synthesizes glutamate carboxypeptidase II on the surface), PC3 and DU145 (they do not synthesize glutamate carboxypeptidase II). In the course of the study, the initial tropic glutamate carboxypeptidase II peptide and hybrid peptide were labeled with 6-FAM NHS fluorescent dye, then added to the cell culture medium, then fluorescence was evaluated and compared.
Results. Greater binding of the peptide to the cysteine knot with glutamate carboxypeptidase II on the surface of LNCaP culture cells was noted, compared with the original peptide, affinity to glutamate carboxypeptidase II. An increase in the binding of the hybrid peptide to glutamate carboxypeptidase II with time, in the range from one hour to one day, was also noted.

Keywords: peptides, prostate cancer, targeted medicine, glutamate carboxypeptidase II, PSMA

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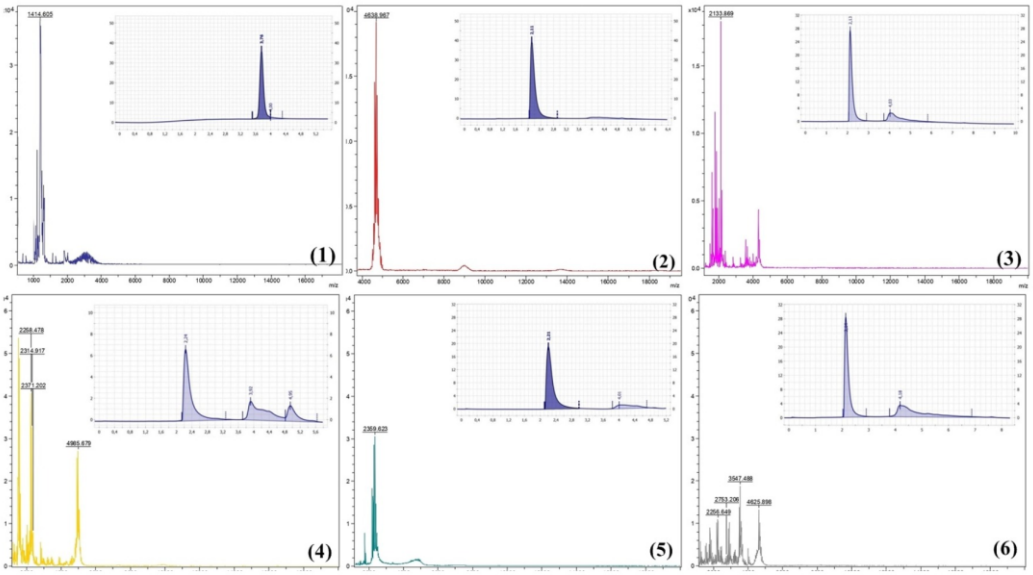
INTRODUCTION
Prostate cancer is the most common cancer in men worldwide, second only to lung cancer. In 2018, 1,276,106 new cases and 358,989 deaths from this type of cancer in men were detected [1]. The ten-year prognosis of death due to prostate cancer ranges from 3-18% in men without concomitant chronic diseases and can reach 33% if present [2]. 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 to choose a further treatment strategy [2]. Treatment for prostate cancer may include surgery such as radical prostatectomy [3]. Less radical treatments are hormone therapy [4,5] and chemotherapy [5]. However, the most common treatment for prostate cancer is radiation therapy and brachiotherapy [5]. In recent years, more and more attention has been paid to targeted radionuclide therapy [7]. The essence of targeted radionuclide therapy is the delivery of a radionuclide to a tumor using a drug with a high affinity for tumor cells [8]. Targeted radionuclide therapy uses the same mechanism of destruction of 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 [9]. Peptides are of the greatest interest for targeted therapy [10]. 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) [11,12]. It is assumed that PSMA is a multifunctional protein involved in nutrient uptake, cell migration, and proliferation [13]. However, the choice of PSMA as a target for targeted radionuclide therapy is due to its overexpression on the membrane of prostate cells [14] and the ability of PSMA to internalize [15], which allows the radiotherapeutic agent 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 [16,17,18].
Some studies show that knottins, or peptides with cysteine knots, can be a solution to these problems [19,20]. A distinctive feature of such peptides is the presence of a structure of three disulfide bonds [20]. 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 [21]. This knot imparts chemical, thermal, and proteolytic stability to the peptide [21,22]. There are already works showing the possibility of obtaining target peptides with a cysteineknot, and their greater efficiency than without it [23,24]. The aim of this work is to synthesize a peptide that is tropic to the prostate-specific antigen and contains a cysteine knot and to compare its binding efficiency using the example of prostate cancer cell cultures.

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), DU145 (obtained from brain metastases).

For work, the following sequences were synthesized: PSMA antigen with the sequence G T I Q P Y P F S W G Y [25] and four hybrid sequences consisting of a peptide with a cysteine knot U5-Sth1a (isolated from the venom of the spider family Scytodes thoracica), into different parts of which the GTI sequence was inserted. Of the five hybrid peptides, only one was successfully synthesized (Figure 1).



(1) GTI
G-T-I-Q-P-Y-P-F-S-W-G-Y

(2) Knottin1

D-E-T-P-D-E-C-G-T-I-Q-P-Y-P-F-S-W-G-Y-V-T-R-G-N-F-C-A-T-P-E-V-H-G-D-W-C-C-G-S-L-K-C-V-S-N-S-C-R

(3) Knottin2

D-E-T-P-D-E-C-V-T-R-G-N-F-C-G-T-I-Q-P-Y-P-F-S-W-G-Y-A-T-P-E-V-H-G-D-W-C-C-G-S-L-K-C-V-S-N-S-C-R

(4) Knottin3

D-E-T-P-D-E-C-V-T-R-G-N-F-C-A-T-P-E-V-H-G-D-W-C-C-G-T-I-Q-P-Y-P-F-S-W-G-Y-G-S-L-K-C-V-S-N-S-C-R

(5) Knottin4

D-E-T-P-D-E-C-V-T-R-G-N-F-C-A-T-P-E-V-H-G-D-W-C-C-G-S-L-K-C-G-T-I-Q-P-Y-P-F-S-W-G-Y-V-S-N-S-C-R

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 and hybrid pptides based on the original sequence G T I Q P Y P F S W G Y.

The synthesis of peptides was carried out on a peptide synthesizer ResPepSL (Intavis) in accordance with a standard protocol based on solid-phase synthesis using a protective F-moc (fluorenylmethyloxycarbonylchloride) group on TentaGel resin, where (CH-CO) $_2$O (HBU (acetic acid anhydride) was used as caTppingmixing - (1H-benzotriazol-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate) in DMF - activator, NMM (N-Methylmorpholine) - catalyst and DMF, NMP (N-Methyl-2-pyrrolidone) and DMF (Dimethylformamide) - solvents, piperidine in DMF - for cleavage of the F-moc group. After synthesis, the peptides were cleaved from the resin with a cocktail containing TFA (Trifluoroacetic acid) (92.5%), TIPS (Triisopropylsilane) (5%) and deionized water (2.5%). The cleaved peptides were then precipitated with cold MTBE (Methyltert-butylether) and dried. The analysis of the synthesis results was carried out by the methods of high-performance liquid chromatography and mass spectrometry.

High performance liquid chromatography was carried out by the method of anion exchange chromatography on a Bio-Rad NGC Quest chromatograph equipped with a photometric detector. Detection was carried out at a wavelength of 280 nm. Column anion-exchange Agilent PL-SAX 4.6x150 mm, 1000A, 10mM (Part No: PL1551-3102). Gradient elution, eluent A - deionized water with

the addition of 20 mM Tris-HCl; eluent B - deionized water with the addition of 20 mM Tris-HCl and 1 M NaCl. Gradient elution protocol: 2.95 ml - 0% B; 10 ml - 0-50% B; 3.5 ml - 100% B. Mass spectrometric analysis was carried out on a MALDI-TOF MS FLEX series hardware-software complex (Bruker Daltonics, Germany).

To study the peptides were labeled with 6-FAM NHS fluorescein (Lumiprobe) according to the standard Lumiprobe protocol [26]. Fluorescein was dissolved in pure DMSO, then mixed with the peptide dissolved in 0.1M NaHCO3 solution and incubated for an hour at room temperature.

The peptide was purified from the dye residues by ethanol precipitation. 1 part of the peptide solution was mixed with 9 parts of 95% ethanol cooled at -20 ° and incubated at -20 ° for 2 hours. After incubation, the solution was centrifuged for 30 minutes at 14,000 rpm. After centrifugation, the supernatan was removed, the peptide was resuspended in the culture medium. For the study, cell cultures were seeded in plates, in the amount of 100 thousand cells per well of the plate. After adding the labeled peptide, the cultures were incubated in RPMI-1640 medium with glutamine supplemented with 10% BSA and 100x penicillin-streptomycin solution at 37 ° C in an atmosphere of 5% CO2. The intensity of cellular fluorescence was assessed 1, 3 and 24 hours after the addition of the peptide. The study was carried out using an inverted Nikon Ti-S microscope. The resulting images were processed using the ImageJ program.

RESULTS AND DISCUSSION

In the course of the work, the ability of the hybrid peptide to bind to a prostate-specific membrane antigen on the surface of prostate cancer cell cultures was studied.

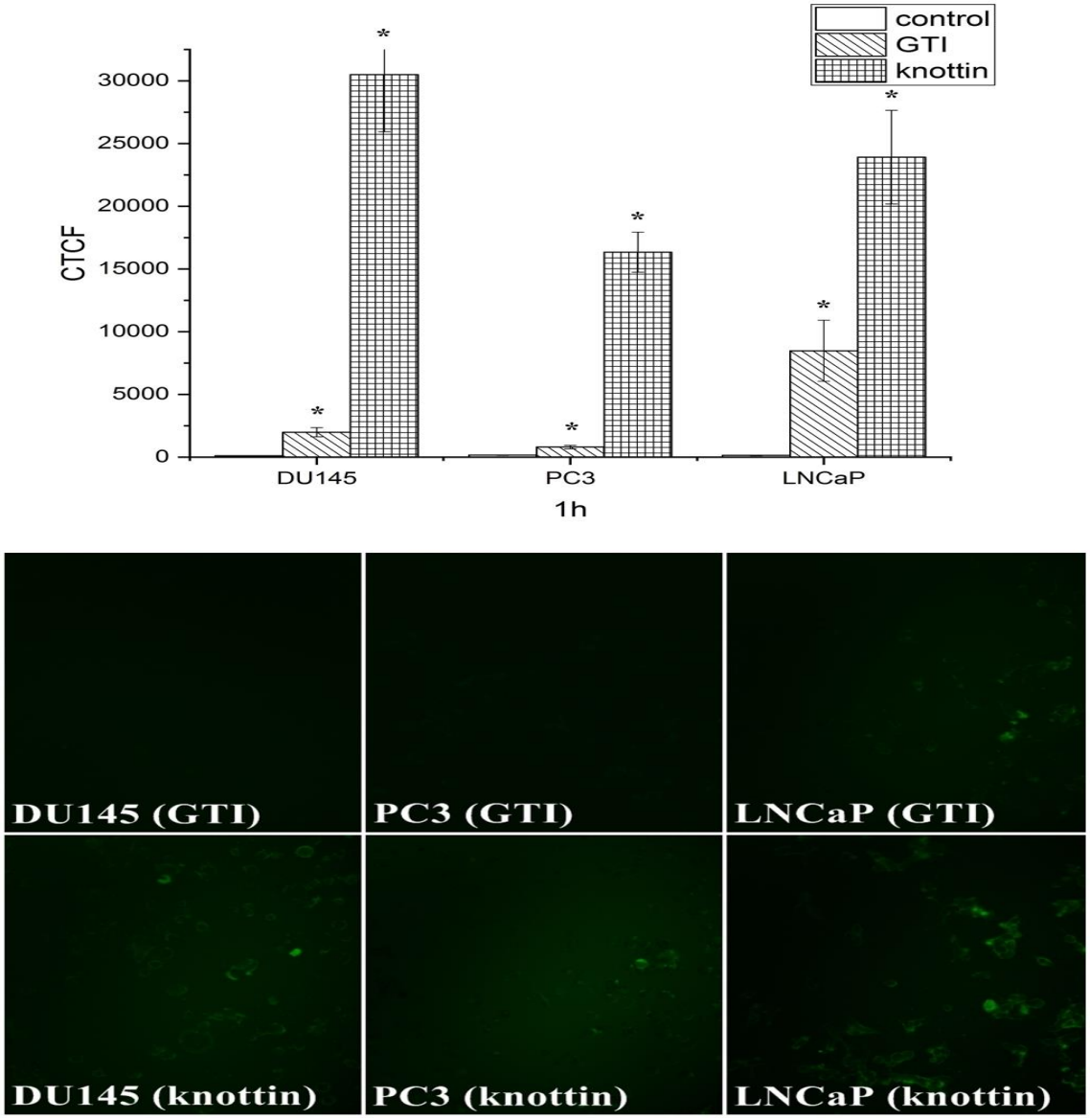


Figure 2. The fluorescence intensity of cell cultures 1 hour after the addition of PSMA-tropic hybrid and original PSMA-tropic peptide.

Figure 2 shows the results of a fluorescence study of prostate cancer cell cultures 1 hour after the addition of the hybrid peptide and the original PSMA-tropic peptide. The highest fluorescence is observed in DU145 culture

cells to which the hybrid peptide was added. This means that this peptide binds better to the prostate-specific membrane antigen in a given cell culture. The original peptide binds better to the prostate-specific antigen on the LNCaP cell culture, which is also seen from Figure 2. The overall fluorescence level of the hybrid peptide is much higher than that of the original peptide, which shows its ability in general to better bind to target receptors on the surface of cancer cells prostate gland.

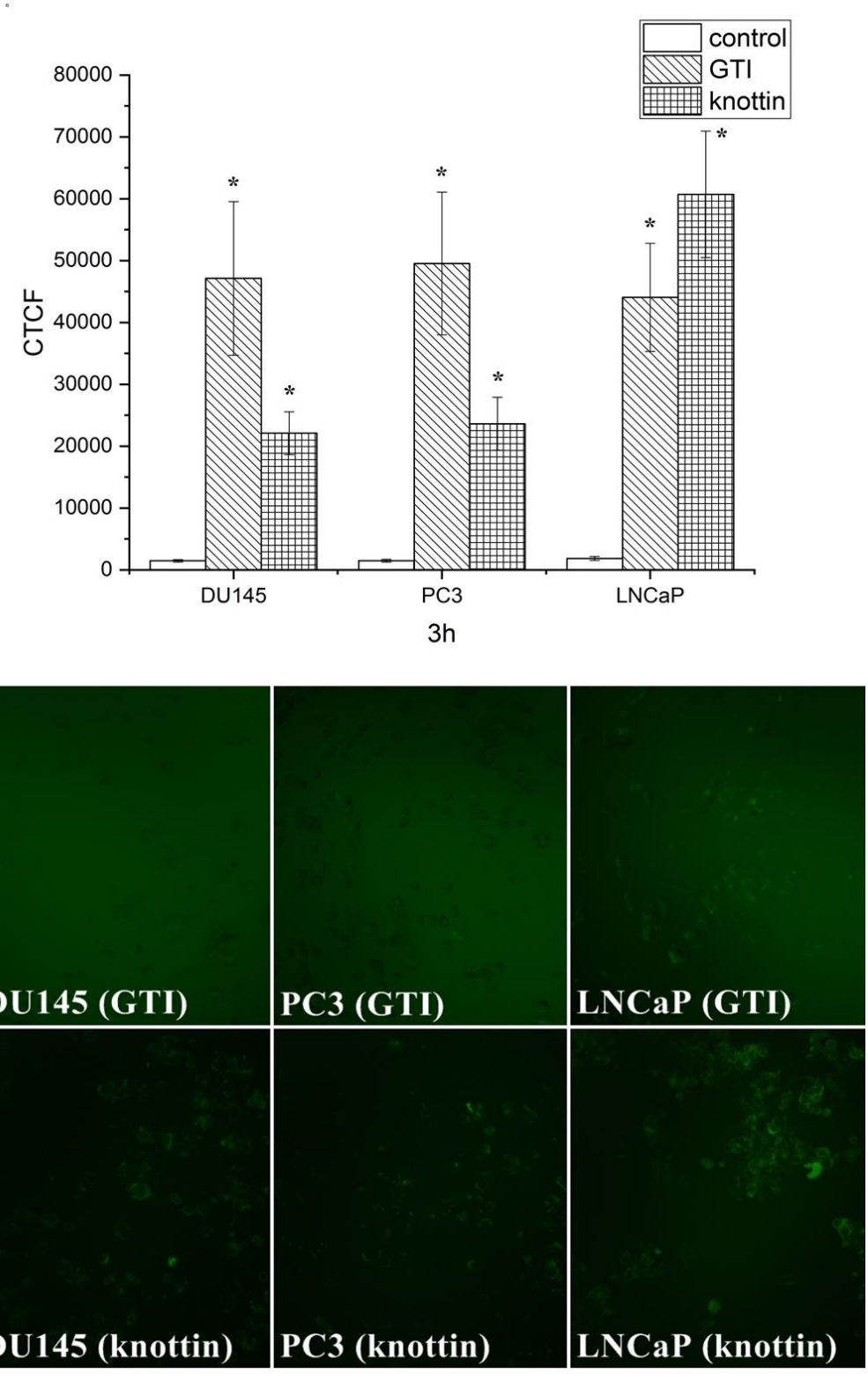
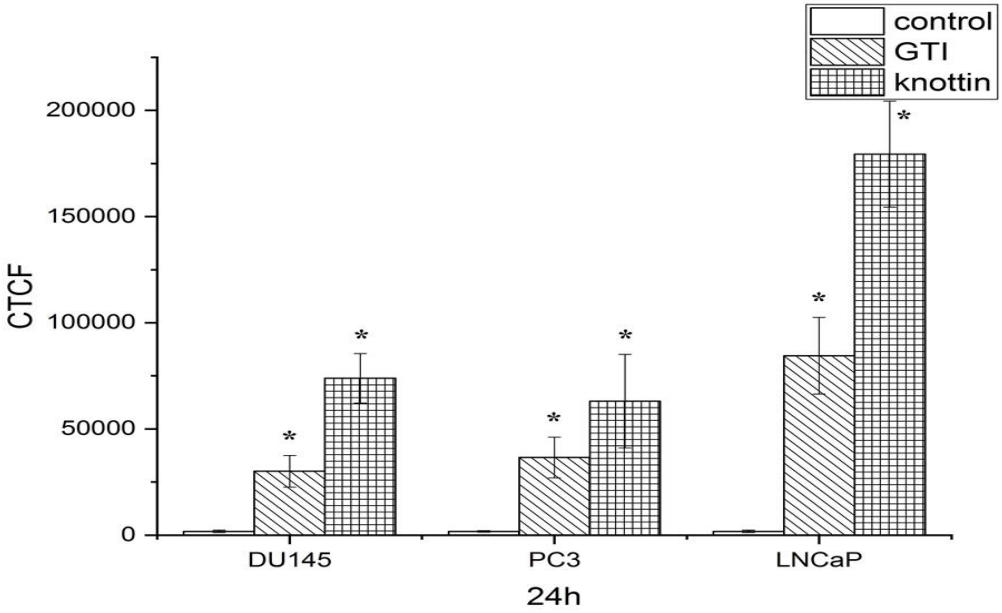


Figure 3. Fluorescence intensity of cell cultures 3 hours after the addition of PSMA-tropic fusion and original PSMA-tropic peptide.

Figure 3 shows the results of a fluorescence study of prostate cancer cell cultures 3 hours after the addition of the hybrid peptide and the original PSMA-tropic peptide. The highest fluorescence is observed in LNCaP culture cells to which the hybrid peptide was added. This means that this peptide binds better to the prostate-specific membrane antigen in a given cell culture. The original peptide binds better to the prostate-specific antigen on the PC3 cell culture, which can also be seen from Figure 3. The overall fluorescence level of the hybrid peptide, with the exception of LNCaP cells, is lower than that for the original peptide.



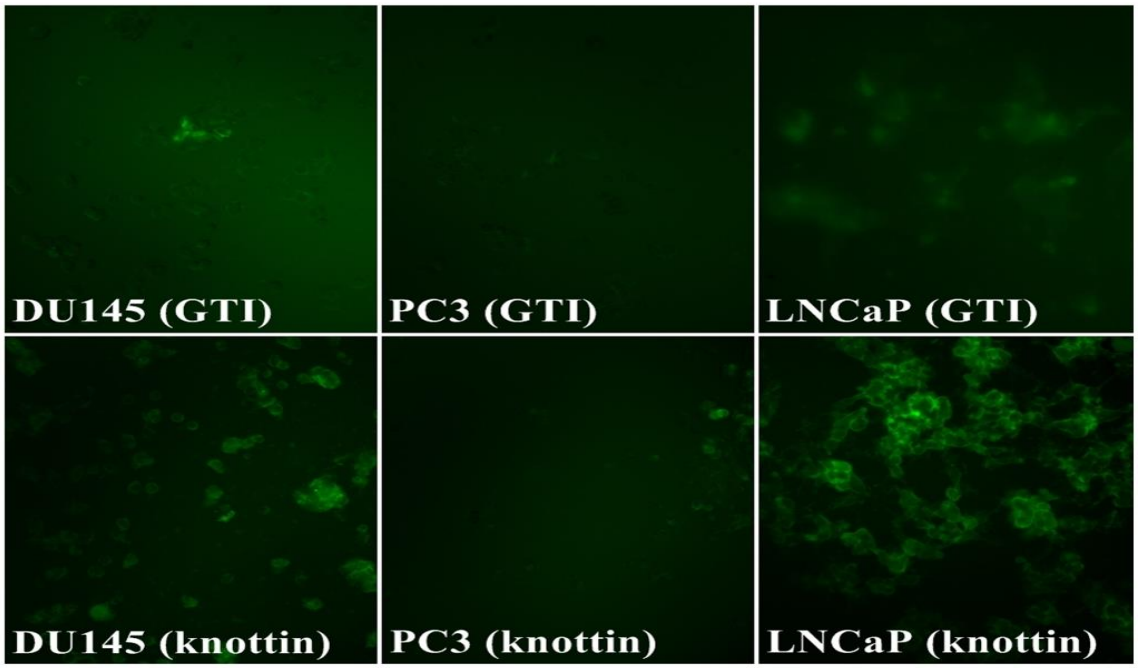


Figure 4. Fluorescence intensity of cell cultures 24 hours after the addition of PSMA-tropic hybrid and original PSMA-tropic peptide.

Figure 4 shows the results of a fluorescence study of prostate cancer cell cultures 24 hours after the addition of the hybrid peptide and the original PSMA-tropic peptide. The highest fluorescence is observed in LNCaP culture cells to which the hybrid peptide was added. This means that this peptide binds better to the prostate-specific membrane antigen in a given cell culture. The original peptide binds better to the prostate-specific antigen in the LNCaP cell culture, which can also be seen from Figure 4. The overall fluorescence level of the hybrid peptide is higher than that of the original peptide.

Studies show that the prostate-specific membrane antigen is most actively expressed in the cells of the LNCaP culture, which is consistent with the data on the increased

fluorescence of this culture obtained during the work. However, the same studies show that the cells of the DU145 and PC3 cultures are PSMA negative [27,28], and the ability of PSMA-tropic peptides to bind to the cells of these cultures is interesting and may give rise to new research.

PSMA-tropic fusion peptide showed better binding to prostate cancer cells than the original peptide. This can be associated with the aforementioned greater than that of conventional peptides, stability [20,21]. Previously, studies have already been carried out showing that peptides modified with a cysteine knot construct have a higher affinity for the target receptors of cancer cells than unmodified peptides [29,30].

In the course of the work, an increase in the amount of bound hybrid peptide with prostate cancer cells with an increase in incubation time has also been shown to correlate with the results of similar studies [31,32].

CONCLUSION

In the course of the work, a hybrid peptide was synthesized, consisting of the U5-Sth1a peptide containing a cysteine knot, into which the PSMA-tropic GTI peptide was inserted.

The resulting hybrid peptide showed a higher affinity for the prostate-specific membrane antigen than the original GTI peptide. It has been shown that the amount of hybrid peptide bound to the prostate-specific membrane antigen increases with an increase in the incubation time of cells with the peptide.

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