



# Screening for new peptide substrates for the development of albumin binding anticancer pro-drugs that are cleaved by prostate-specific antigen (PSA) to improve the anti tumor efficacy

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## ABSTRACT

Several attempts have been made over the past decade to explore the concept of prodrug strategies that exploit PSA as a molecular target for the release of anticancer drugs in prostate tumors using various prostate specific antigen (PSA)-cleavable peptide linkers, but the desired antitumor and antimetastatic efficacy has not yet been fully achieved. We set out to look for new PSA-cleavable peptide substrates that could be cleaved more rapidly and efficiently than the previously used peptides. To look for the most susceptible PSA-cleavable peptide substrates, we used the so-called spot technology. With the following general formula, we designed 25 different fluorogenic heptapeptides; Cellulose-P5-P4-P3-P2-P1-P1'-P2' (Fluorophore). The increase of the fluorescence in the supernatant of the reaction mixture was monitored using a 96-well fluorometric plate reader with excitation of  $\lambda_{ex}$  485 nm and  $\lambda_{em}$  535 nm. Three sequences showed a high fluorogenic liberation after incubation with PSA, i.e., Arg-Arg-Leu-His-Tyr-Ser-Leu (7), Arg-Arg-Leu-Asn-Tyr-Ser-Leu (8) and Arg-Ser-Ser-Tyr-Arg-Ser-Leu (23). Future incorporation of these optimized substrates in the PSA-cleavable prodrug formulations could further optimize the cleavage pattern and so the release characteristics of these prodrugs to rapidly and efficiently liberate the free cytotoxic agents inside the tumor tissues.

## 1. Introduction

Prostate-specific antigen (PSA) is a 33 kDa single-chain glycoprotein [1] composed of 237 amino acid residues, four carbohydrate side chains, and multiple disulfide bonds [2–4]. The amino acid sequence of PSA is homologous to proteases of the kallikrein family and may be referred to as human glandular kallikrein-3 (hK-3) [5]. Whereas most of the other kallikreins have trypsin-like proteolytic activity, PSA is considered a chymotrypsin-like protease based on similarities with chymotrypsin in the specificity of the catalytic site and its preference for cleaving after hydrophobic residues in the P<sub>1</sub> position [6].

PSA is aptly named; in that it is specifically and exclusively produced by normal and malignant prostate epithelial cells. Its blood levels used as a screening test for prostate cancer and is not produced in any significant amounts by other normal tissue in the human male [7,8]. The increased secretion of PSA by prostate carcinoma cells coupled with the disruption of normal tissue architecture in the prostate or distal sites by prostate cancer cells results in the leakage of increased amounts of PSA

into circulation in a positive correlation with the stage of the disease [7, 9].

Interestingly, these PSA molecules have been reported to be secreted in an active form only in the extracellular fluid surrounding prostate carcinoma cells. In the circulation, PSA loses its enzymatic activity as a result of binding to abundant serum protease inhibitors  $\alpha_1$ -antichymotrypsin and  $\alpha_2$ -macroglobulin, which were reported to be at a  $10^4$ – to  $10^5$ -fold molar excess in the circulation of patients with prostate carcinoma [9–12].

Many strategies have previously been investigated to inhibit the protease activity of PSA to control the prostate tumor progression and metastasis but as a result of heterogeneity and autocatalysis, no success was achieved [3,13–16]. In our research, we had investigated the concept of prodrug strategies that exploit PSA as a molecular target for releasing anticancer drugs in prostate tumors. Consequently, and as a main goal of the present work, we set out to look for new optimized PSA-cleavable peptide substrates that could be cleaved more rapidly and efficiently.

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## 2. Materials and methods

### 2.1. Materials

In collaboration with *JPT Peptide Technologies GmbH* (Berlin, Germany), we designed 25 different fluorogenic heptapeptides with the following general formula; Cellulose-P<sub>5</sub>-P<sub>4</sub>-P<sub>3</sub>-P<sub>2</sub>-P<sub>1</sub>-P<sub>1</sub>'-P<sub>2</sub>'-(Fluorophore) as seen in (Table 1). The rationale of this design considered that the major physiologic substrates for PSA appear to be the gel-forming proteins in freshly ejaculated semen, semenogelin I and semenogelin II which are synthesized and secreted by the seminal vesicles. Active PSA in the seminal fluid cleaves preferentially after tyrosyl and glutaminyl peptide bonds to generate several low-molecular-weight soluble protein fragments [6,17,18]. On this basis of the PSA cleavage map for semenogelin I and semenogelin II, so much information became available about the amino acid sequence proximal to the PSA proteolytic sites as described in (Table 1). With this knowledge, we used the so-called spot technology [19–22], to guess or search for the most susceptible PSA-cleavable peptide substrates.

Each heptapeptide is attached from its C-terminus with Alexa-Fluor-488 as a fluorescent label, whereas the N-terminus is attached to Cellulose disc (Each cellulose disc contains approximately 57 nmol peptide) which is accurately fixed to a well in a 96-well plate (Table 2).

Among the designed 25 fluorogenic substrates there were two PSA-uncleavable heptapeptides, i.e., Arg-Ala-Ser-Tyr-DGln-Ser-Leu (1) and Gly-Gly-Gly-Gly-Gly-Ser-Leu (2) that were included in the study as a negative control. All the other remaining 23 heptapeptides are expected to be PSA cleavable substrates including our previously used heptapeptide substrate i.e., Arg-Ser-Ser-Tyr-Tyr-Ser-Leu (3) which was incorporated to serve as a positive control. Each of the 25 heptapeptides is prepared as a triplicate (repeated three times) and placed consequently in the 96-well plate. Specifically, we decided to use the amino acids Ser in P<sub>1</sub>' position and Leu in P<sub>2</sub>' position in all prepared 25 heptapeptides (Table 2), depending on some emerging evidences that have been concluded in our previous work, as well as by other investigators. Additionally, these amino acids have been observed to occupy these positions in the natural PSA cleavable substrates and were also used in these positions by other investigators to design some PSA-

**Table 2**

The fluorogenic heptapeptides that were designed and examined as PSA substrates.

Nr	Cellulose-P <sub>5</sub> -P <sub>4</sub> -P <sub>3</sub> -P <sub>2</sub> -P <sub>1</sub> -P <sub>1</sub> '-P <sub>2</sub> '-Alexa-Fluor-488
1	Arg-Ala-Ser-Tyr-DGln-Ser-Leu
2	Gly-Gly-Gly-Gly-Gly-Ser-Leu
3	Arg-Ser-Ser-Tyr-Tyr-Ser-Leu
4	Lys-Gly-Gln-His-Tyr-Ser-Leu
5	Ile-Ser-Ser-Gln-Tyr-Ser-Leu
6	Gln-Ser-Ser-Ile-Tyr-Ser-Leu
7	Arg-Arg-Leu-His-Tyr-Ser-Leu
8	Arg-Arg-Leu-Asn-Tyr-Ser-Leu
9	Arg-Ser-Ser-Ser-Tyr-Ser-Leu
10	Arg-Ser-Ser-Tyr-Gln-Ser-Leu
11	Lys-Ile-Ser-Tyr-Gln-Ser-Leu
12	Lys-Met-Ser-Tyr-Gln-Ser-Leu
13	Ser-Ser-Lys-Tyr-Gln-Ser-Leu
14	Ser-Ser-Lys-Val-Gln-Ser-Leu
15	Lys-Ser-Gln-Asn-Gln-Ser-Leu
16	Arg-Ile-Pro-Ser-Gln-Ser-Leu
17	Arg-Ile-Arg-Ser-Gln-Ser-Leu
18	Arg-Ser-Ser-Tyr-His-Ser-Leu
19	Lys-Ser-Lys-Gln-His-Ser-Leu
20	Arg-Ser-Ser-Gln-His-Ser-Leu
21	Arg-Ser-Ser-Ala-His-Ser-Leu
22	Arg-Ser-Ser-Leu-His-Ser-Leu
23	Arg-Ser-Ser-Tyr-Arg-Ser-Leu
24	Ser-Ser-Ser-Arg-Arg-Ser-Leu
25	Gly-Gln-Ser-Thr-Asn-Ser-Leu

cleavable substrates [19–22].

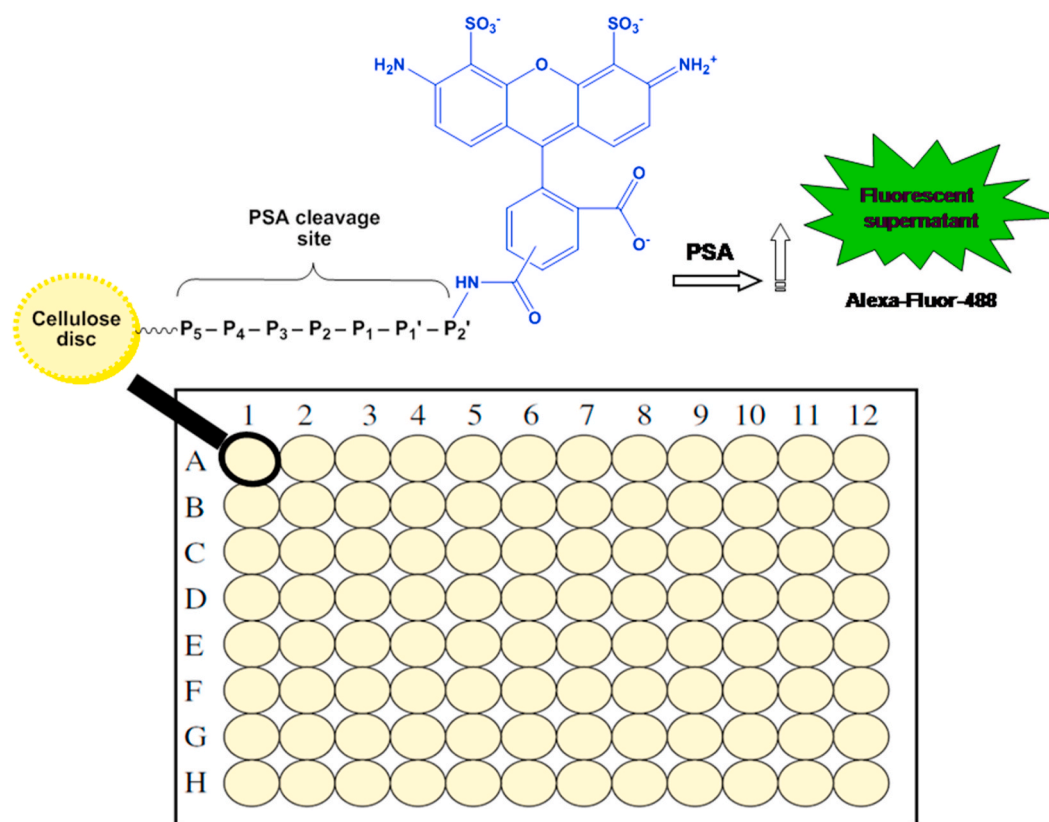
The peptides were synthesized on the cellulose discs, starting with a linker system at the C-terminus. The peptides ended at the N-terminus with a free Cysteine residue, where the fluorophore (in this case the Alexa-Fluor 488) was coupled as final step. The evidence of purity for the prepared peptides was provided with their characteristic UV-trace showed that the target mass was found at the peak at 2.8 min as shown in the analysis curve [Fig. 1S].

Enzymatically active PSA was purchased from *Calbiochem* (Bad Soden, Germany). All other chemicals including methanol, Tris HCl buffer ingredients and solvents were of analytical grade and obtained from standard suppliers and were used without further purification. The

**Table 1**

Identified peptide sequences from the natural PSA substrates Semenogelin I and II (SG-I and SG-II), which are split by PSA [44].

P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	Cleavage site in Protein (P1)
Lys	Gly	Gln	His	Tyr	Ser	Gly	Gln	Lys	Gly	SG-I 25
Phe	Ser	Ile	Gln	Tyr	Thr	Tyr	His	Val	Asp	SG-I 44
Lys	Ser	Gln	Gln	Tyr	Asp	Leu	Asn	Ala	Leu	SG-I 62; SG-II 62
Ile	Ser	Ser	Gln	Tyr	Ser	Asn	Thr	Glu	Glu	SG-I 136
Glu	Leu	Leu	Val	Tyr	Asn	Lys	Asn	Gln	His	SG-I 240
Arg	Arg	Leu	His	Tyr	Gly	Glu	Asn	Gly	Val	SG-I 277, 337
Gln	Ser	Ser	Ile	Tyr	Ser	Gln	Thr	Glu	Glu	SG-I 292
Gln	Arg	Ser	Ile	Tyr	Ser	Gln	Thr	Glu	Glu	SG-I 352
Lys	Gly	Gln	His	Tyr	Phe	Gly	Gln	Lys	Asp	SG-II 25
Ser	Gln	Ser	Ser	Tyr	Val	Leu	Gln	Thr	Glu	SG-II 171
Arg	Arg	Leu	Asn	Tyr	Gly	Gly	Lys	Ser	Thr	SG-II 457
Lys	Gly	His	Tyr	Gln	Asn	Val	Val	Glu	Val	SG-I 198; SG-II 198
Ser	Ser	Lys	Val	Gln	Thr	Ser	Leu	Cys	Pro	SG-I 212
Lys	Ile	Ser	Tyr	Gln	Ser	Ser	Ser	Thr	Glu	SG-I 266, 326; SG-II 326, 386
Ser	Ser	Lys	Leu	Gln	Thr	Ser	Leu	His	Pro	SG-II 212
Lys	Ser	Gln	Asn	Gln	Val	Thr	Ile	His	Ser	SG-II 306
Arg	Ile	Pro	Ser	Gln	Ala	Gln	Glu	Tyr	Gly	SG-II 372
Lys	Met	Ser	Tyr	Gln	Ser	Ser	Ser	Thr	Glu	SG-II 446
Phe	Ser	Ile	Gln	His	Thr	Tyr	His	Val	Asp	SG-II 44
Lys	Ser	Lys	Gln	His	Leu	Gly	Gly	Ser	Gln	SG-II 76
Leu	His	Pro	Ala	His	Gln	Asp	Arg	Leu	Gln	SG-II 219
Glu	Arg	Gln	Leu	His	His	Gly	Glu	Lys	Ser	SG-II 276
Gly	Gln	Ser	Thr	Asn	Arg	Glu	Gln	Asp	Leu	SG-I 389
Glu	Arg	Arg	Leu	Asn	Ser	Gly	Glu	Lys	Asp	SG-II 396
Gly	Val	Gln	Lys	Asp	Val	Ser	Gln	Ser	Ser	SG-I 285, 345
Ser	Gln	Gln	Leu	Leu	His	Asn	Lys	Gln	Glu	SG-I 84
Ile	Thr	Ile	Pro	Ser	Gln	Glu	Gln	Glu	His	SG-I 311



**Fig. 1.** Diagrammatic illustration for the principle of assay and monitoring the fluorescence increase in the reaction supernatant after PSA cleavage of the fluorogenic heptapeptides substrates.

buffers used were vacuum-filtered through a 0.2  $\mu\text{m}$  membrane (Sartorius, Germany) and thoroughly degassed with ultrasound prior to use. A 96-well fluorometric plate reader with excitation of  $\lambda_{\text{ex}}$  485 nm and  $\lambda_{\text{em}}$  535 nm was used to measure the fluorescence.

## 2.2. Enzymatic fluorogenic assay

The principle of assay depends on monitoring the increase of the fluorescence in the supernatant of the reaction mixture as a result of the fluorophore liberation after the cleavage of the fluorogenic heptapeptides substrates with PSA (Fig. 1).

Accordingly, each spot was rinsed with methanol for 5 min to solubilize the peptides. Subsequently, it was washed 4 times with 250  $\mu\text{L}$  of 50 mM Tris buffer pH 7.5 with gentle agitation in each step. Afterwards, 250  $\mu\text{L}$  of enzymatically active PSA solution (20  $\mu\text{g}/\text{ml}$ ) was added to each well. A 50  $\mu\text{L}$  aliquot was transferred directly from each well to its corresponding well in a new 96-well plate containing 50  $\mu\text{L}$  of 50 mM Tris buffer pH 7.5. After mixing, the fluorescence of the new plate was measured to be considered as a 0-time fluorescence level (blank) using a 96-well fluorometric plate reader with excitation of  $\lambda_{\text{ex}}$  485 nm and  $\lambda_{\text{em}}$  535 nm. The reaction mixture was then sealed with a plastic seal and the reaction was allowed to proceed at room temperature without agitation. At 1-h intervals, an aliquot of 50  $\mu\text{L}$  from the reaction supernatant was transferred to a new 96-well plate, diluted and mixed with 50  $\mu\text{L}$  of 50 mM Tris buffer pH 7.5 [23], and its fluorescence was measured as described above. Analysis is performed at JPT using HPLC-MS at 220 nm, linear gradient, 5–95% acetonitrile in water +0.05% TFA.

## 2.3. Statistical analysis

The results were determined from the initial linear increase of fluorescence till 5 h and were expressed as the mean fluorescence change per

hour ( $\Delta F/h$ ) after its analyses by GraphPad Prism version 5.

## 3. Results

The obtained cleavage results revealed that among the examined 25 fluorogenic heptapeptide substrates, there were three sequences that showed the highest fluorogenic liberation after incubation with PSA (Fig. 2).

These heptapeptides are Arg-Arg-Leu-His-Tyr-Ser-Leu (7), Arg-Arg-Leu-Asn-Tyr-Ser-Leu (8) and Arg-Ser-Ser-Tyr-Arg-Ser-Leu (23). Of note that, this pronounced increase in the rate of fluorescence release in these three heptapeptides was, however, higher than that of our previously used substrate, i.e., Arg-Ser-Ser-Tyr-Tyr-Ser-Leu (3). Additionally, and in a clear conformation for the PSA cleavage liability, the two heptapeptides which were included as negative controls, i.e., Arg-Ala-Ser-Tyr-DGln-Ser-Leu (1) and Gly-Gly-Gly-Gly-Gly-Ser-Leu (2), did not show any significant increase in the fluorescence rate over the taken period. This can indicate that the three heptapeptides with the highest increase in the fluorescence rate could resemble the best substrates for PSA among the all examined heptapeptides.

## 4. Discussion

Based on unique biology of PSA (a serine protease), several studies have investigated prodrug strategies that exploit PSA as a molecular target for releasing anticancer drugs from enzymatically cleavable prodrug formulations inside the prostate tumors [12,24–27]. PSA proteolysis of extracellular matrix proteins such as fibronectin and laminin can stimulate the detachment of tumour cells and promote tumour invasion and metastasis, ultimately contributing to the spread and progression of prostate cancer [28,29]. The inhibition and/or consumption of PSA at the tumour site can therefore be a useful method for curing prostate

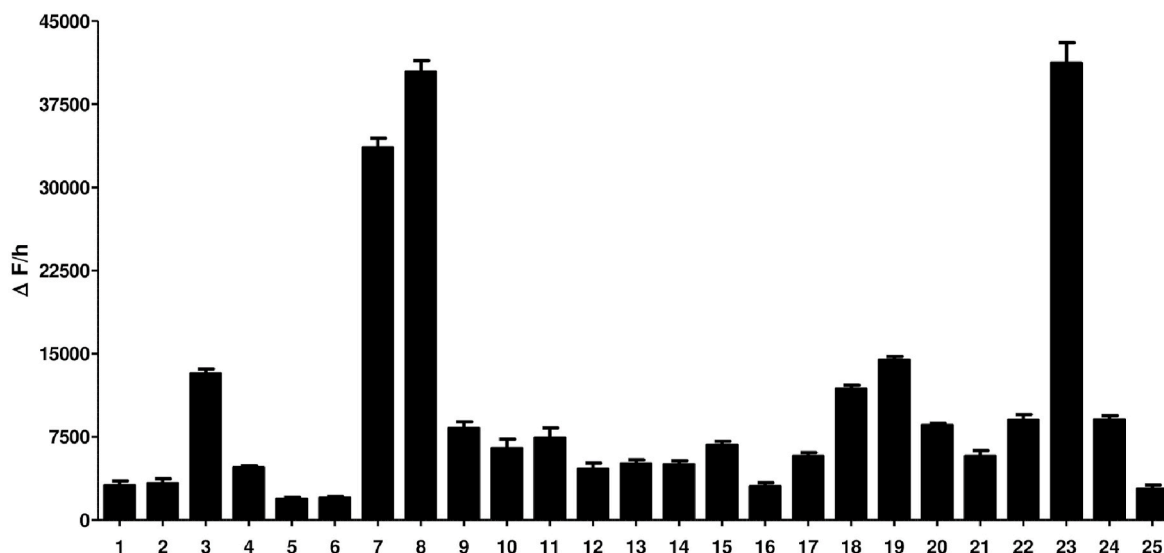


Fig. 2. The mean fluorescence change per hour ( $\Delta F/h$ ) of each individual fluorogenic heptapeptide substrates after PSA cleavage. Data are presented as the mean  $\pm$  SEM ( $n = 3$ ).

cancer.

PSA as a molecular target for releasing anticancer drugs is based mainly on the conjugation of the conventional anticancer drugs with a wide spectrum of low- and high-molecular-weight carriers including sugars, growth factors, vitamins, peptides, antibodies, polysaccharides, lectins, serum proteins, and synthetic polymers through a linker molecule that incorporates a pre-determined breaking point to ensure a tumor-specific release of the drug payload [30].

Denmeade research group introduced a relevant example of the low-molecular weight prodrug strategies that exploit PSA as a molecular target for the release of anticancer drugs from prodrug formulations when they produced an inactive doxorubicin prodrug that was synthesized by coupling the doxorubicin primary amine to the COOH-terminal carboxyl of a seven-amino acid peptide carrier [i.e., Mu-His-Ser-Ser-Lys-Leu-Gln-Leu (Mu = morpholinocarbonyl)] which was recorded to be precisely hydrolysable by PSA [1,31]. The resulting prodrug was shown to be efficiently activated by human prostate cancer cell lines producing PSA (such as PC-82 and LNCaP cells) to release the active cytotoxin L-leucyl-doxorubicin, whereas the prodrug had no cytotoxic effect on PSA-nonproducing TSU human prostate cancer cells *in vitro* [1].

In principle, in addition to the active cytotoxic agent, an enzymatically cleavable prodrug contains a peptide linker substrate that guarantees the subsequent release of the active compound within the tumour interstitium [30]. We and others have produced several prodrug formulations that integrate PSA-cleavable peptide substrates based on some available information from the proteolytic cleavage map for PSA's physiological substrate, human semenogelin I and II [24,26,32,33]. In these prodrugs, the most commonly used PSA-cleavable peptide linkers were the N-glutaryl-(4-hydroxypropyl)AlaSer-cyclohexaglycyl-GlnSerLeu-COOH [27], Mu-His-Ser-Ser-Lys-Leu-Gln-Leu [1], and our previously used heptapeptide substrate, i.e., Arg-Ser-Ser-Tyr-Tyr-Ser-Leu [34,35]. Despite the relatively promising results of these studies, further optimization of the PSA-cleavable peptide substrates remains important to achieve the maximum improved cleavage pattern and thus achieve the desired antitumor and antimetastatic efficacy of the corresponding prodrug in order to allow complete tumour remission. The lack of ideal optimization can be attributed to the fact that the main PSA-cleavage products of these prodrugs represent the drug mono-peptides or dipeptides that still need further cleavage to release the active drug as a final product over time [24, 33,35].

In the current study, as an option, we set out to look for new optimized PSA-cleavable peptide substrates that could be cleaved more

quickly and efficiently than the peptides previously mentioned. The incorporation of these optimized substrates into prodrug formulations could further optimize the pattern of PSA cleavage and thus the release properties of these prodrugs to release the free cytotoxic agents within the tumour tissues quickly and efficiently and thus maximally improve their antitumor and antimetastatic efficacy.

Our study findings showed that after incubation with PSA, three sequences had high fluorogenic release, i.e., Arg-Arg-Leu-His-Tyr-Ser-Leu (7), Arg-Arg-Leu-Asn-Tyr-Ser-Leu (8) and Arg-Ser-Ser-Tyr-Arg-Ser-Leu (23).

Incubation studies with PSA showed that the albumin-bound form of the two drugs was quickly split, but at the P1-P1' scissile bond, releasing the drug-dipeptides, i.e. H-Ser-Leu-PABC-doxorubicin and H-Ser-Leu-PABC-paclitaxel, in the same previous manner, which were further degraded to release the free drug as a final cleavage product within few hours in prostate tumor tissue homogenates as well as in PSA-positive LNCaP LN cell lysates [33–36]. These data indicate that the N-glutaryl-(4-hydroxypropyl) AlaSer-cyclohexaglycyl-GlnSerLeu-CO<sub>2</sub>HH [26], Mu-His-Ser-Ser-Lys-Leu-Gln-Leu [1], and our previously used heptapeptide substrate, i.e., Arg-Ser-Ser-Tyr-Tyr-Ser-Leu [33–35], were the most commonly used PSA-cleavable peptide linkers in previous studies to develop PSA-cleavable prodrugs. It is apparent that neither of these PSA-cleavable peptide binders has been sufficiently satisfactory to achieve the maximum optimized cleavage pattern of the corresponding prodrug, so the desired antitumor and antimetastatic efficacy has not yet been fully achieved.

In conclusion, there were at least three sequences of the various fluorogenic heptapeptide substrates tested in the current study that showed the highest fluorogenic release after PSA incubation, i.e., Arg-Arg-Leu-His-Tyr-Ser-Leu (7), Arg-Arg-Leu-Asn-Tyr-Ser-Leu (8) and Arg-Ser-Ser-Tyr-Arg-Ser-Leu (23). These three sequences may be considered, according to these findings, to be promising candidates for the future production of the corresponding PSA-cleavable prodrugs integrating these substrates.

#### Informed consent

This manuscript does not contain any studies with human subjects or animals performed by any of the authors.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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This study had no funding from any resource.

## Authors' contributions

Study concept and design: BEME and MHH; Data analysis and writing manuscript: BEME and MHH; All authors revised and approved the final version of the manuscript.

## Statements

In this work we used the so-called Alexa-spot technology to look for new peptide substrates for the development of albumin binding anti-cancer pro-drugs that are cleaved by prostate-specific antigen (PSA) to improve the anti tumor efficacy.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2021.100966>.

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