

Synthesis and Binding Profile Using Molecular Dynamics of New Building Blocks for PSMA Theranostics and the PSMA617-Cystine-Doxorubicin Therapeutic Bioconjugate Against Prostate Cancer

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Abstract

GCPII also known as Prostate Specific Membrane Antigen (PSMA), is overexpressed in prostate cancer (PCa) cells and provide a biomarker for tumor targeting PSMA receptor. The development of lysine-urea-glutamate pharmacophore based inhibitors targeting PSMA for theranostics applications led to PSMA11 and PSMA617. In PSMA11, this pharmacophore is attached via aminohexanoic acid (Ahx) spacer to a chelator while in PSMA617 the pharmacophore is connecting with the linker 2-naphthyl-L-Ala, trans-4-(aminomethyl)cyclohexanecarboxylic acid and then to chelator. Here, we synthesized: (a) a squaramide analog of lysine-urea-glutamic acid; (b) two new building blocks for PSMA theranostics in which lysine-urea-glutamic acid was attached with two (i) phenyl alanine residues to target arene binding site of PSMA receptor and (ii) amino hexanoic acid residues. (d) Inspired from the promising structure of PSMA617 we synthesized a new bioconjugate prodrug in which we replaced the chelator in PSMA617 with a cleavable cystine linker attached to the anticancer drug Doxorubicin. Induced fit docking and MD simulations explored the binding profile of the new molecules.

Keywords: arene binding site; bioconjugate; cleavable linker; cystine; docking calculations; doxorubicin; induced fit; molecular dynamics; Prostate Specific Membrane Antigen; PSMA11; PSMA617; squaramide; theranostics

Introduction

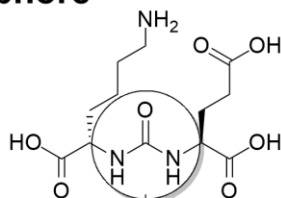
Most patients with metastatic disease initially respond to androgen deprivation therapy, taxane-based chemotherapies, immunotherapy, or radium-223, but each of these regimens provides only limited 2–4 months median survival benefit.¹ So far, no active treatment of PCa has shown superiority regarding survival rates. Strong side effects and development of resistances pressured researchers to search for alternatives. New pharmacological targets and therapies are under research giving hope to improve the survival and the quality of life of patients with PCa.²

In humans, glutamate carboxylpeptidase type II (GCPII), a zinc-dependent metallopeptidase, is expressed physiologically in the nervous system, small intestine, kidney, and prostate. Within the nervous system and small intestine, GCPII hydrolyzes its two recognized natural substrates, N-acetyl-aspartyl-glutamate (NAAG) and folyl-poly- γ -glutamates, participating directly in signal transmission via neural pathways and intestinal folate absorption, respectively. In the kidney and prostate, the physiological function remains unknown.³

GCPII is known as Prostate Specific Membrane Antigen (PSMA) since is up to 100-1000-fold overexpressed in prostatic adenocarcinoma vs benign prostatic tissue and undergoes internalization through clathrin-coated pits to be transported into lysosomes. Due to its overexpression in PCa cells, PSMA provides a biomarker for tumor targeting. Apart from its expression in PCa, PSMA has also been found to be expressed in several other tumors like renal carcinoma, breast cancer, nonsmall cell lung cancer (NSCLC), oral cancer and many others.² Although, histopathological studies have confirmed PSMA expression in salivary glands, duodenal mucosa, proximal renal tubular cells, and neuroendocrine cells in the colonic crypts, the ratio is substantially lower (100-1000 fold) in these tissues than in PCa lesions.⁴ Agents with the potential to be used as theranostics for PSMA targeted prostate cancer therapy and diagnosis have been developed.

The development of lysine-urea-glutamate (Lys-CO-Glu-OH) based inhibitors targeting PSMA for theranostic applications led to PSMA11 and PSMA617. PSMA11 was radiolabelled with ^{68}Ga to obtain [^{68}Ga]Ga-PSMA-11 (with international nonproprietary name gozetotide) for PET imaging towards the prostate cancerous lesions in men. PSMA-617 (vipivotide_tetaxetan) was radiolabelled with ^{68}Ga to obtain [^{68}Ga]Ga-PSMA-617 for PET imaging or with ^{177}Lu (beta particle therapy) to obtain [^{177}Lu]Lu-PSMA-617 for therapeutic applications. Both [^{68}Ga]Ga-PSMA-11 or [^{177}Lu]Lu-PSMA-617 were licenced by Novartis, approved for medical use in the European Union in December 2022, and sold under the brand Locametz and Pluvicto, as radiopharmaceutical medications used for diagnosis of prostate cancer or for the treatment of PSMA-positive metastatic castration-resistant prostate cancer (mCRPC). Both PSMA 11 and PSMA617 include the selective PSMA pharmacophore Lys-CO-Glu-OH and are consisting of three or four components, respectively. In PSMA11, Lys-CO-Glu-OH pharmacophore is attached via aminohexanoic acid (Ahx) spacer to the chelator HBED-CC (N,N'-bis-[2-hydroxy-5-(carboxyethyl)benzyl]ethylenediamine-N,N'-diacetic acid), see Scheme 1. PSMA617 or vipivotide tetaxetan includes the PSMA pharmacophore Lys-CO-Glu-OH which is connecting with the linker 2-naphthyl-L-Ala, trans-4-(aminomethyl)cyclohexanecarboxylic acid and then to the chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) which can bind both ^{68}Ga or ^{177}Lu and ^{225}Ac as theranostic radionuclides. The ^{177}Lu version of PSMA-617 was recently approved by FDA for PCa diagnosis.

**Basic Pharmacophore
Glu-Urea-Lys**



Squaramide analogue

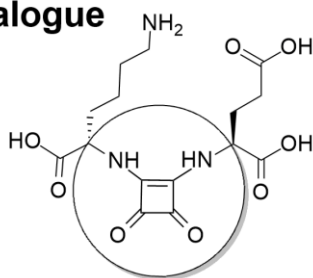


Figure 1. Squaramide analog of Lys-CO-Glu-OH pharmacophore.

Our interest for new Lys-CO-Glu-OH pharmacophores triggered us to synthesize a squaramide analog of Lys-CO-Glu-OH (Fig 1). Additionally, based on the structure of PSMA11, an increase of spacer linker with two aminohexanoic acids (**PSMA-Ahx2**) was tested. Finally, crystal structure analysis showed interactions with extremely low K_i of ARM-P's and natural substrate: folyl-poly- γ -glutamates as it is mentioned.^{5,6} Taking these data into consideration, we synthesized an analogue with a suitable spacer and two phenylalanine for enhancing arene binding interactions. (Figure 2).

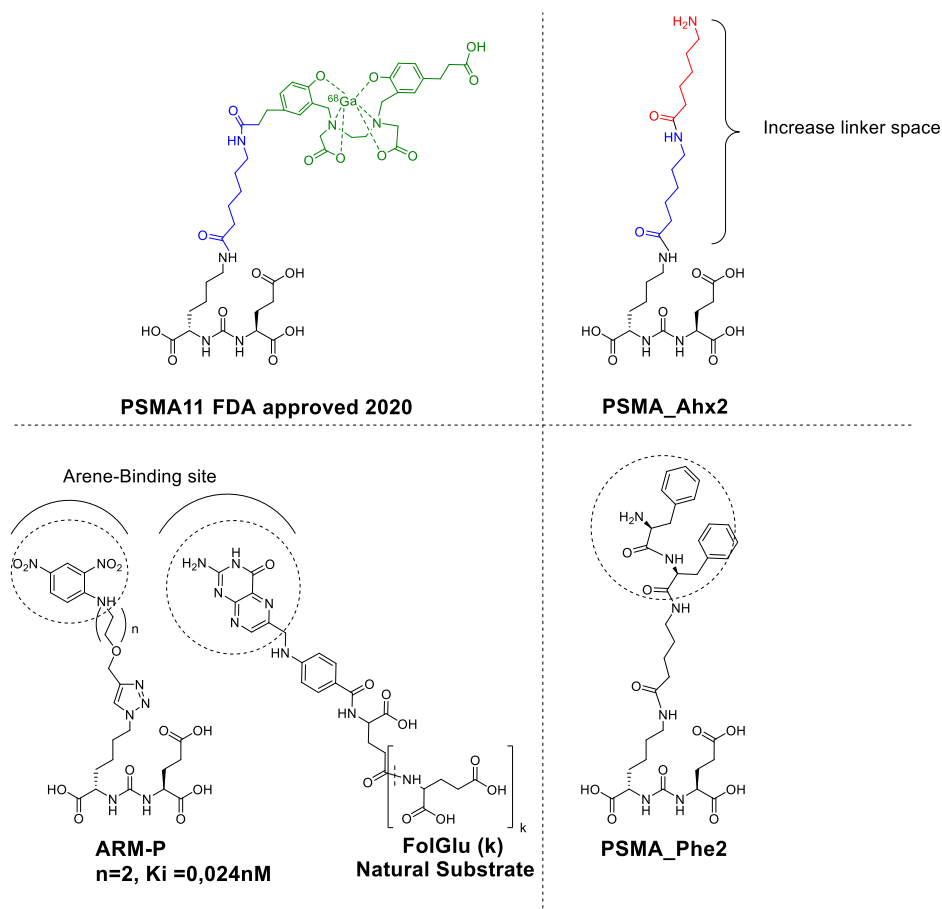


Figure 2. Design of the new **PSMA-Phe2** targeting arene binding site and of the new **PSMA-Ahx2** building block for synthesizing analogs of **PSMA11**.

In this work we took advantage of the promising structure of **PSMA617** and synthesized a new bioconjugate prodrug in which we replaced the chelator in **PSMA617** and connected the anticancer drug DOXorubicin (DOX) through a cleavable cystine linker to release the toxophore into the cell. The design principles of **PSMA617-cystine-DOX** are described in Figure 3.

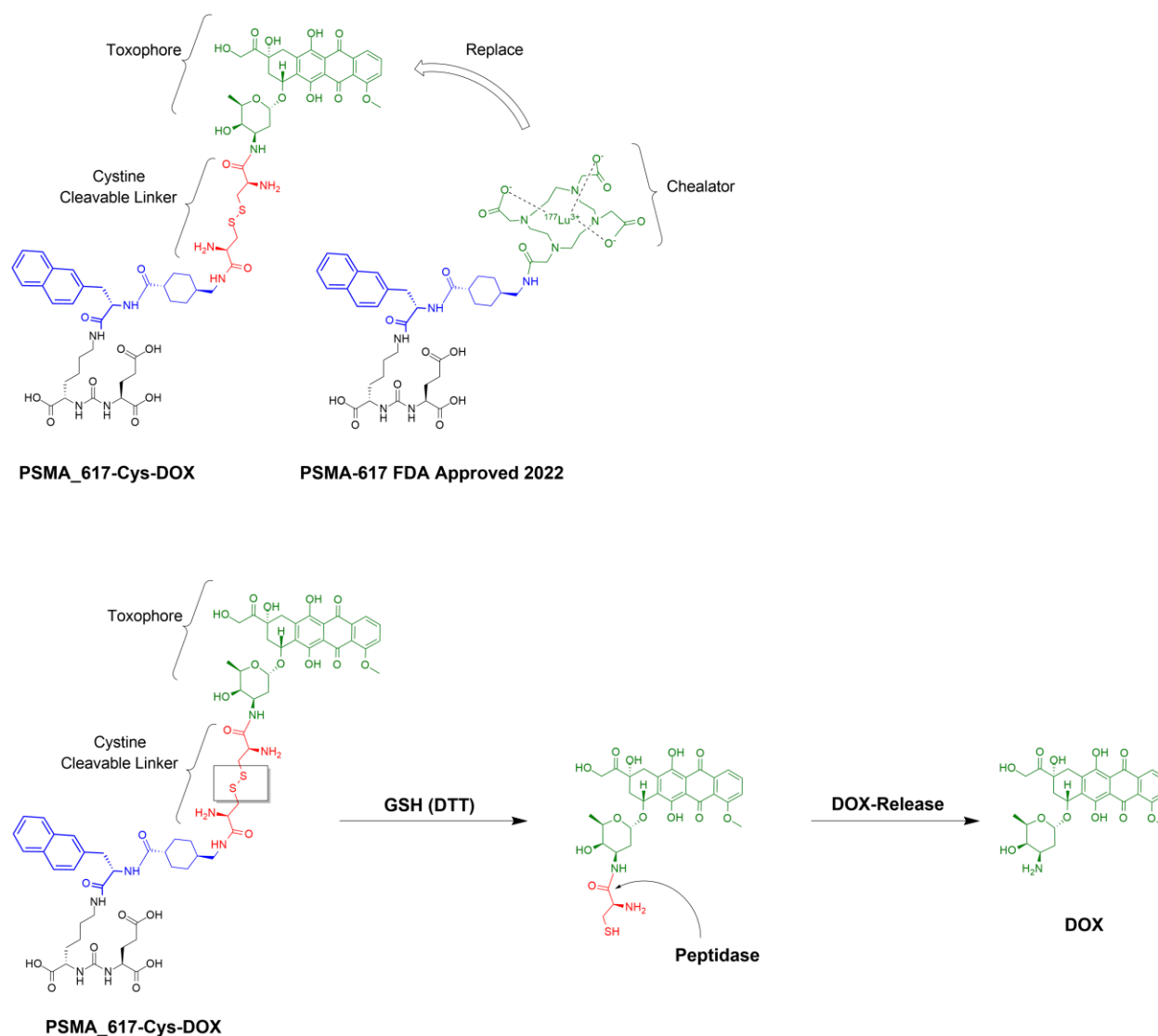
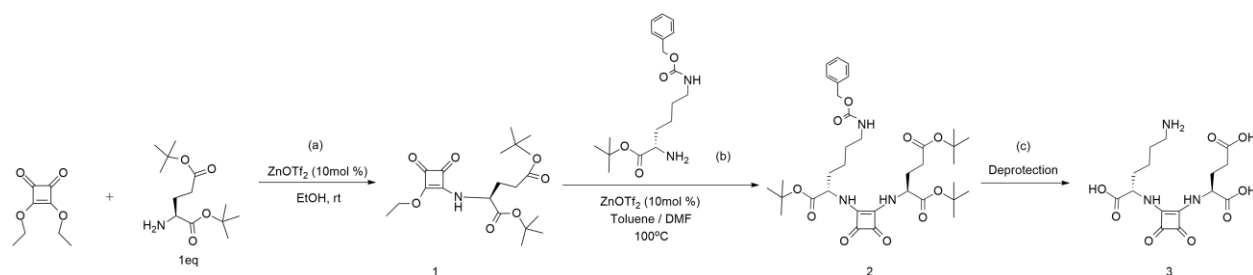


Figure 3. Design strategy of the new **PSMA617-cystine-DOX** bioconjugate as therapeutic agent against PCa.

Results and Discussion

Chemistry

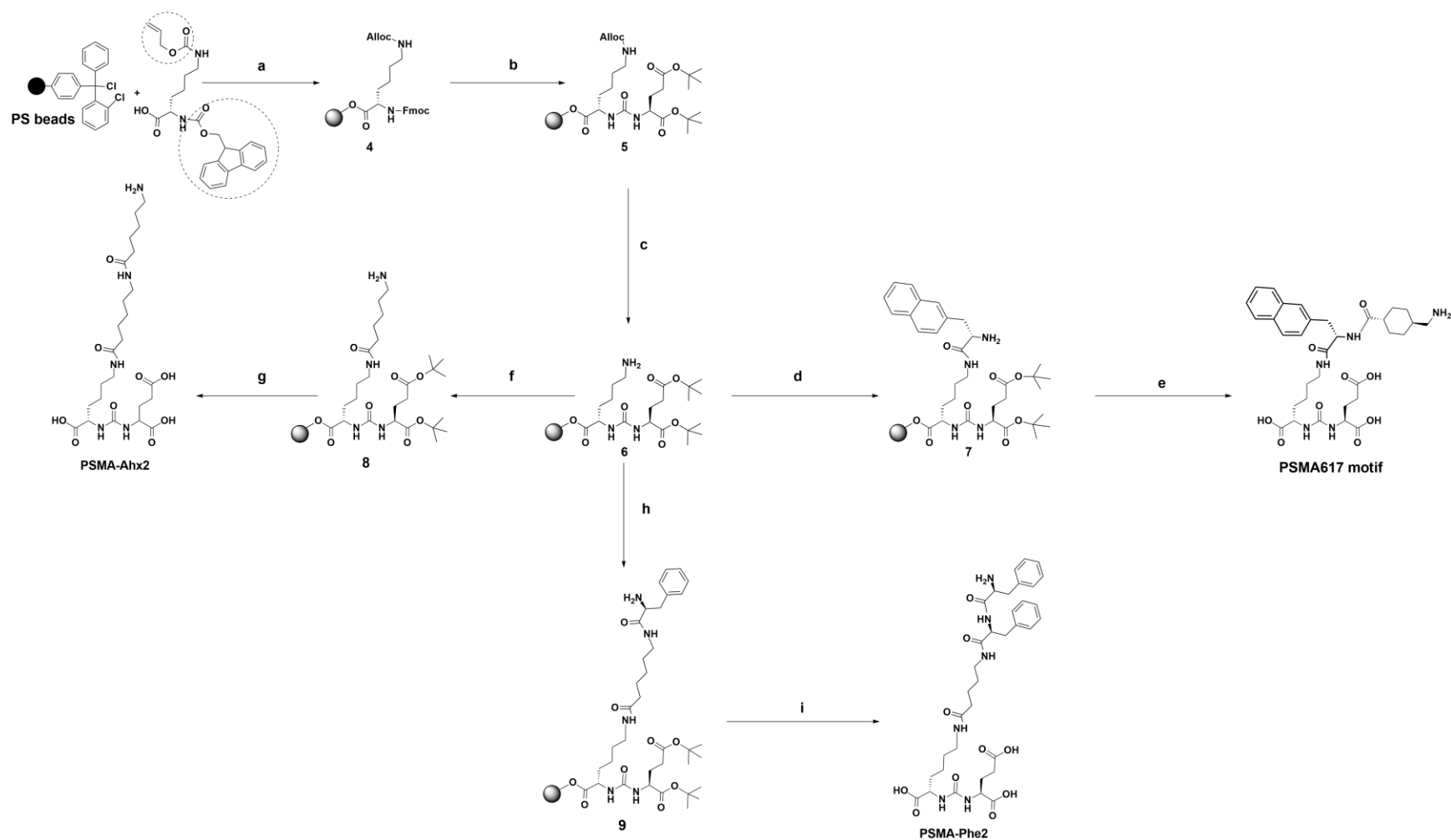
The synthesis of squaramide analog of Lys-CO-Glu-OH pharmacophore was accomplished according to previously applied methods (Scheme 2).⁷ Firstly, 3,4 Diethoxycyclobut-3-ene-1,2 dione and di-tert-butyl glutamate reacted leading to the mono-squaramide. Then protected Lysine was coupled as a following using ZnOTf_2 and DIPEA at 100°C with a yield (53%). Deprotection of compound **2** produced squaramide **3** described in Scheme 1.



Scheme 1. (a) ZnOTf₂, DIPEA, ethanol, rt; (b) ZnOTf₂, DIPEA, toluene: DMF 19:1 at 100 °C; (c) i. H₂ Pd/C, ii. TFA

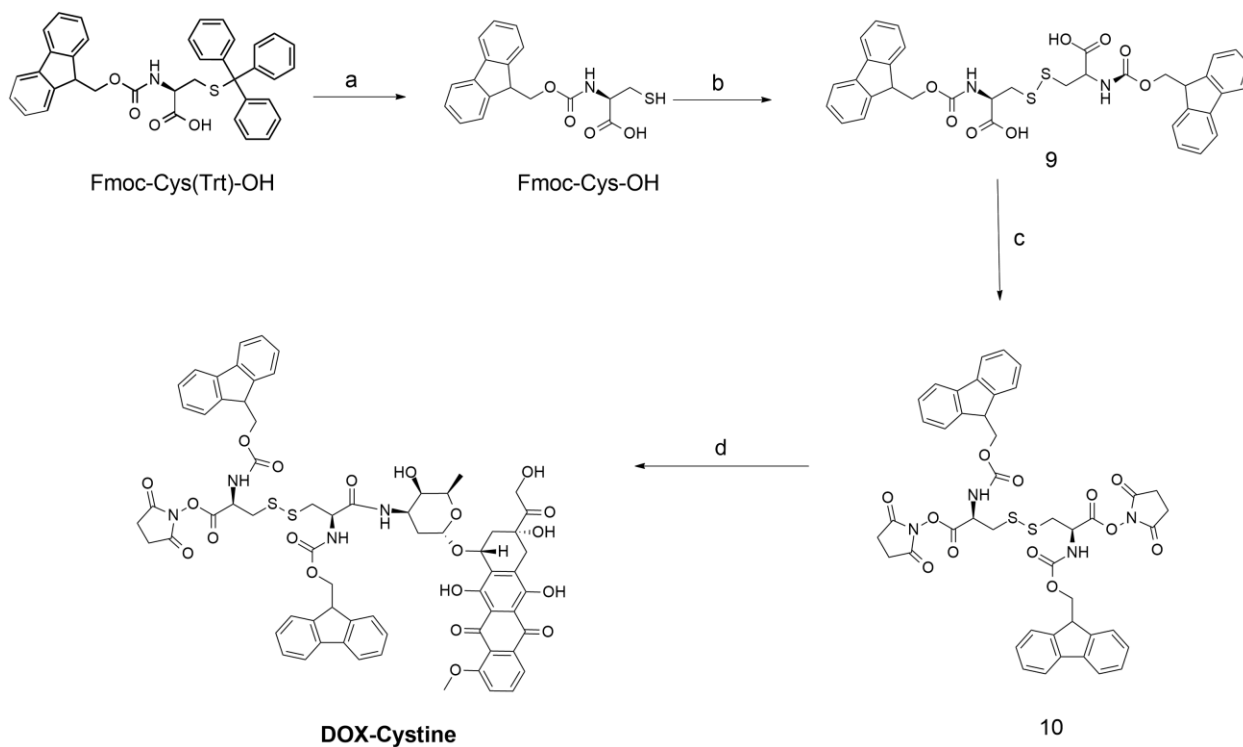
The synthesis of **PSMA-Ahx2**, **PSMA-Phe2** and **PSMA-617** peptidomimetic moieties are described in Scheme 2.

The synthesis of Lys-CO-Glu-OH connected with two amino hexanoic acid or two phenyl alanines connected the latter with two aminohexanoic acids (**PSMA-Ahx2**) or two phenyl alanines (**PSMA-Phe2**). **PSMA617** moiety was synthesized according to standard Fmoc solid phase peptide synthesis (spps) protocols we previously applied.⁸ In particular, synthesis of intermediates **1**, **2** and pharmacophore **3** was accomplished using spps on a 2-chloro-trytyl-resin and rink amide resin, respectively (Scheme 4). Amino acid coupling was carried out using Oxyma pure, DIC, DIPEA according to standard Fmoc peptide synthesis protocols we previously described.^{8,9,10} Amine **3** was coupled using Oxyma, DIC, DIPEA affording the protected amine **4**. Fmoc-deprotection and amino acid coupling again of **5** using Oxyma, DIC, DIPEA produced amine **6**. The **PSMA617** moiety was produced with a good yield (37%) by Fmoc deprotection and cleavage from the resin with TFA/TIPS/H₂O, which was purified with RP-HPLC and analyzed with MALDI-MS.



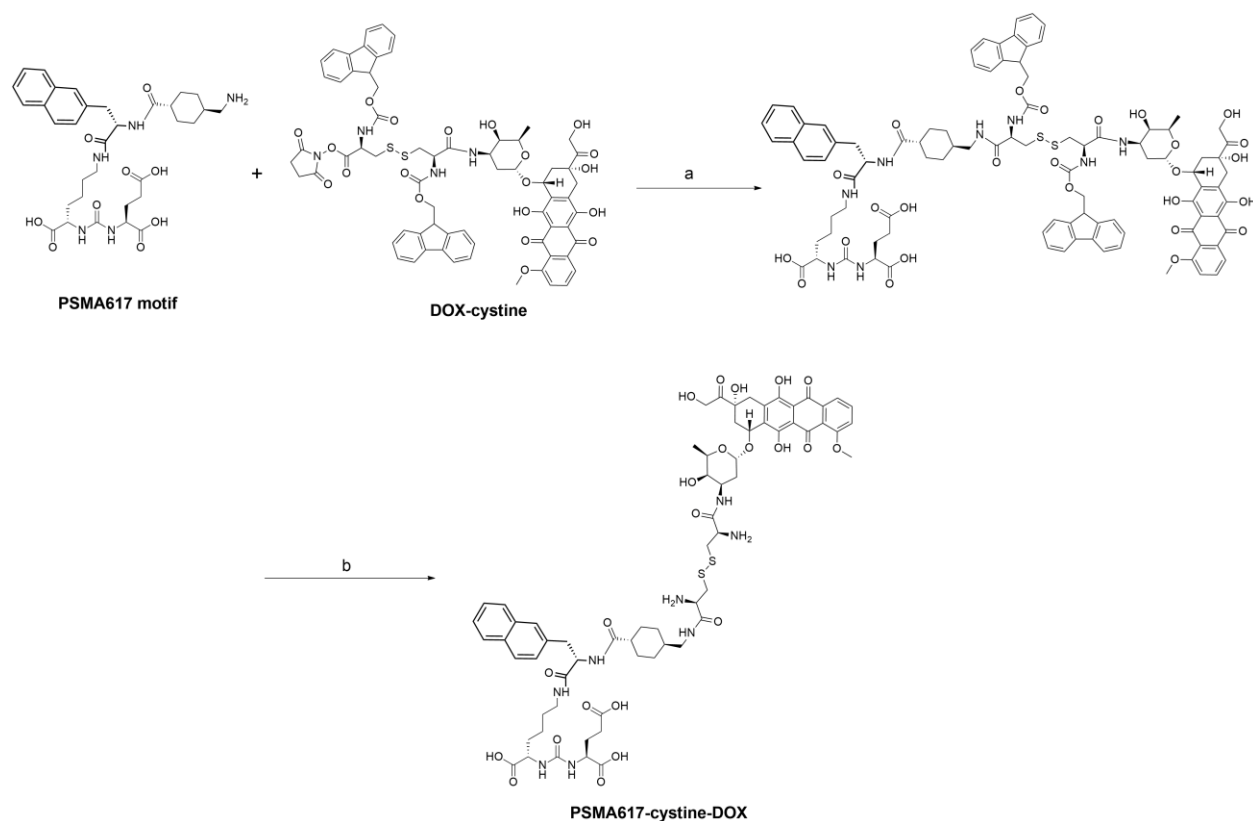
Scheme 2. Spps of **PSMA617**: (a) DCM(dry), DIPEA; (b) triphosgene, DIPEA, DCM (°C); (c) Pd(PPh₃)₄, morpholine, DCM (dry); (d) i. Fmoc-3-(2-naphthyl)-L-alanine, DIPEA, DIC, Oxyma pure, ii. 20% piperidine, DMF; (e) i. trans-4-(Fmoc-aminomethyl) cyclohexanecarboxylic acid DIPEA, DIC, Oxyma pure, ii. Fmoc deprotection: 20% piperidine, DMF, iii. TFA/TIPS/H₂O 95:2.5:2.5 (v/v/v); (f) i. Fmoc-aminohexanoic acid, DIPEA, DIC, Oxyma pure, ii. 20% piperidine, DMF, (g) i. Fmoc-aminohexanoic acid, DIPEA, DIC, Oxyma pure, ii. 20% piperidine, DMF, iii. TFA/TIPS/H₂O 95:2.5:2.5 (v/v/v).

Trityl deprotection of Fmoc-Cys(Trt)-OH and S-S bond coupling with H_2O_2 , NaI, produced the disulfide analog of cystine **9**. Activation of the two carboxylic acids with NHS and EDCI followed resulting in compound **10**. Finally, Doxorubicin was coupled, resulting in compound **DOX-cystine**.



Scheme 3. Synthesis of **DOX-cystine**: (a) TFA, TIPS, DCM; (b) H_2O_2 30%, NaI; (c) N-hydroxysuccinimide, EDCI, DMF, (l) Doxorubicin, DIPEA, DMF.

PSMA617-cystine-DOX was finally synthesized after the coupling of amine **PSMA-617 motif** and of already activated carboxylic acid **DOX-cystine** in DMF. The resulting bioconjugate after fmoc deprotection was purified with RP-HPLC and analyzed with MALDI-MS.



Scheme 4. Synthesis of **PSMA617-Cys-DOX** bioconjugate: (a) PSMA617 moiety, DOX-Cys, DIPEA, DMF; (b) 20% piperidine, DMF.

Experimental Part

Squaramide analog of Glu-Urea-Lys

3,4 Diethoxycyclobut-3-ene-1,2 dione (0,29mmol) and L-Glu(OtBu)-OtBu/HCl (0,29mmol) were both dissolved in one round flask of 0,87ml Ethanol. Then ZnOTf_2 (0,029), and DIPEA (0,58mmol) were added and left overnight. The reaction is checked by TLC system 50:1 DCM:MeOH. The next day a yellow solution was evaporated and was purified by flash column chromatography with mobile phase DCM, and then EtOAc. Yield: 65% Product **1** ^1H NMR (400 MHz, CDCl_3) δ 4.77 (s, 2H), 3.13 (q, $J = 7.2$ Hz, 1H), 2.45 – 2.01 (m, 4H), 1.48 (d, $J = 19.9$ Hz, 18H) and ESI-MS: Calcd for $[\text{M}+\text{H}]^+ = 383,19\text{Da}$ found 383,19. Product **1** (0,05mmol), Lys-Cbz-OtBu (0,055mmol), ZnOTf_2 (0,005mmol) and DIPEA (0,11mmol) were all dissolved in 250 μl Toluene: DMF (19:1) at 100°C for 12 hours. The solvents were evaporated and extracted with DCM-Brine three times. The organic phases were mixed together and evaporated. Product **2** was purified by flash column chromatography with stationary phase Silica gel and as a mobile phase was used (7:3 Cyclohexane: EtOAc, and then 1: 1 cyclohexane: EtOAc. The crude material was loaded with 7:3 EtOAc : Cyclohexane, and gave product **2**. Yield 53%, ^1H NMR (400 MHz, CDCl_3) δ 7.34 (s, 5H), 5.11 (s, 2H), 4.89 (d, $J = 22.5$ Hz, 2H), 4.71 (s, 1H), 3.30 – 3.09 (m, 2H), 2.47 – 2.27 (m, 3H), 2.16 – 1.83 (m, 5H), 1.47 (s, 18H), 1.43 (s, 9H), 0.95 – 0.76 (m, 4H) και ESI-MS: Calcd for $[\text{M}-\text{H}]^- = 672,36\text{Da}$ found 671,83. Compound **2** (20 mg, 0,03mmol) was dissolved

in anhydrous MeOH (1,5ml) and added slowly along the walls of a round bottom flask containing dry 10% Pd/C (5mg, 20% by mass). H₂ was introduced and allowed to stir for 13h at RT. To the reaction mixture was added dry, untreated celite and stirred for 5 min. The reaction mixture was filtered and concentrated under pressure. Yield: 97%. Without further purification, TFA was added for 3 hours at RT, producing squaramide **3** which was purified by RP-HPLC and characterised by ¹H-NMR (D₂O, 400 MHz) δ 4.07 (m, 2H), 2.98 (m, 2H), 2.36 (m, 2H), 2.08-2.00 (m, 1H), 1.93-1.60 (m, 5H), 1.41 (m, 2H) and **TOFF-MS**: Calcd for [M+H]⁺ = 372,13Da found 372,19Da.

PSMA617 motif

In a syringe of 10ml for solid phase peptide synthesis resin (Chlorotriyl chloride, 100-200 mesh, 0,6mmol) Fmoc-Lys-(Alloc)-OH (0,6mmol) dissolved in 2ml of Dry DCM and DIPEA (2,4mmol) is added over 4 hours. The product **4** synthesized was treated with 4-methyl-piperidine for Fmoc deprotection. Then the isocyanate of the glutamul moiety was generated in situ by adding a mixture of mmol of bis(tert-butyl) L-glutamate hydrochloride and 1.5 mL of N-ethyl-diisopropylamine (DIPEA) in 200 mL of dry DCM to a solution of 1 mmol triphosgene in 10 mL of dry DCM at 0 °C over 4 h. After agitation of the reaction mixture for one further hour at 25 °C, 0.5 mmol of **4** with a free α-NH₂ amine, was added in one portion (in 4 mL DCM) and reacted for 16 h with gentle agitation leading to compound **5**. The resin was filtered off and the allyloxy-protecting group was removed using 92,4 mg tetrakis-(triphenyl)palladium(0) and 791μL morpholine in 2mL DCM for 3 h resulting in compound **6**. The following coupling was performed using 0,68 mmol of the Fmoc-3-(2-naphthyl)-L-alanine moiety, 0,68 mmol of Oxyma pure and DIC and 100mg of resin **6** in a final volume of 1,4 ml DMF. Intermediate product by Fmoc deprotection by 4-methylpiperidine and DMF gave product **7**, checked with kaiser test. The following step is the coupling of Trans-4-(Fmoc-aminomethyl) cyclohexanecarboxylic acid (0,6mmol) activated by Oxyma pure (0,6mmol) and DIC (0,6mmol) again, dissolved in 5ml of DMF and reacted with resin **7**. Intermediate Fmoc-product was deprotected again by (4-methylpiperidine-DMF) and finally **PSMA617 motif** was cleaved from the resin by cleavage mixture 2ml of 95% TFA, 2,5% water, 2,5% TIPS for 3 hours. The crude product was purified by RP-HPLC and characterised by ¹H NMR (400 MHz, MeOD) δ 7.80 (t, *J* = 8.9 Hz, 3H), 7.68 (s, 1H), 7.47 – 7.37 (m, 3H), 4.70 – 4.66 (m, 1H), 4.30 (dd, *J* = 8.6, 5.1 Hz, 1H), 4.17 (dd, *J* = 8.7, 4.6 Hz, 1H), 3.29 – 3.03 (m, 5H), 2.74 (d, *J* = 7.1 Hz, 2H), 2.45 – 2.37 (m, 2H), 2.25 – 2.09 (m, 3H), 1.95 – 1.25 (m, 17H), 1.09 – 0.95 (m, 3H).

Yield: 37%

DOX-cystine

Fmoc-Cys(Trt)-OH (6mmol) is suspended in 50ml DCM. TFA (0,1mol) and TIPS (14,6mmol) is added causing the deprotection of trityl group. After 2 hours (checked with TLC 1:1 Hexane/Ethyl Acetate) the reaction was over and TFA was evaporated completely after the addition of Et₂O twice. Finally was moved to buchner and washed many times with Hexane. The Fmoc-Cys-OH (5,6mmol, yield 93%) was used in the further step without further purification. Fmoc-Cys-OH (5,6mmol) was dissolved in 12ml EtOAc and NaI (0,28 mmol) was added. Then slowly at 0°C, H₂O₂ 30% (5,6mmol). The reaction was left for 3 hours rt, checking it with TLC (1:1 Hexane/Ethyl

Acetate, 100µl TFA). In the yellow-brown suspension occurred, water solution of Na₂S₂O₃ was added under good stirring until it becomes a colourless solution. After the end of the reaction, extraction was done with water and Ethyl Acetate three times and the organic phases were combined. Finally, followed by salt Na₂SO₄ and further concentration by the evaporator. Yield: 95% **9**: ¹H NMR (400 MHz, Acetone) δ 7.84 (d, *J* = 6.5 Hz, 2H), 7.71 (d, *J* = 7.6 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 4.40 – 4.28 (m, 3H), 4.24 (t, *J* = 7.2 Hz, 2H), 3.30 (dd, *J* = 13.9, 8.9 Hz, 1H), 3.20 (dd, *J* = 13.9, 8.9 Hz, 1H). ¹³C NMR (400 MHz, CDCl₃) δ 167.95, 141.41, 132.59, 131.04, 128.95, 120.12, 68.34, 38.89, 30.51, 29.84, 29.07. ESI-MS: Calcd for (M-H)=683,15, Found 683,15. In a solution of **9** (0,23mmol) in 5ml DMF, Oxyma (0,7mmol) and EDCI (0,7mmol) were added. After 5 min, NHS (0,7mmol) was added in rt for 3 hours. After the end of the reaction, extraction was done with water and Ethyl Acetate three times and the organic phases were combined. Finally, followed by salt Na₂SO₄ addition and further concentration by the evaporator. It was purified by flash column chromatography with the system Hexane: Ethyl Acetate 9:1. Yield: 40% **10**: ¹H NMR (400 MHz, CDCl₃) δ 7.78 (d, *J* = 7.6 Hz, 2H), 7.59 (d, *J* = 7.4 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 7.5 Hz, 2H), 4.50 (d, *J* = 6.8 Hz, 2H), 4.26 (t, *J* = 6.8 Hz, 1H), 2.96 (s, 1H), 2.89 (d, *J* = 2.2 Hz, 5H). Product **9** (0,041mmol) and DOXorubicin (0,037mmol) was diluted in 4ml DMF and DIPEA (0,1mmol) overnight at rt. The next day the crude material was loaded as it was (DMF solution) and purified by liquid chromatography using Silica gel as a stationary phase and 9:1 Hexane-Ethyl Acetate as a mobile phase. Product **DOX-Cystine** was isolated with a Yield: 53% ¹H NMR: (400 MHz, CDCl₃) δ 13.99 (s, 1H), 13.25 (s, 1H), 8.04 (d, *J* = 7.8 Hz, 1H), 7.81 – 7.70 (m, 4H), 7.64 – 7.48 (m, 4H), 7.45 – 7.22 (m, 12H), 6.54 (d, *J* = 8.1 Hz, 1H), 6.02 (s, 1H), 5.54 (s, 1H), 5.32 (s, 1H), 5.13 (s, 1H), 4.78 (s, 2H), 4.46 (s, 1H), 4.39 (d, *J* = 7.0 Hz, 2H), 4.21 (d, *J* = 7.0 Hz, 4H), 4.08 (s, 4H), 3.69 (s, 1H), 3.47 (t, *J* = 7.0 Hz, 4H), 3.31 (d, *J* = 19.2 Hz, 1H), 3.04 (d, *J* = 19.0 Hz, 2H), 2.72 (s, 4H), 2.43 – 2.32 (m, 3H), 2.24 – 2.03 (m, 4H), 1.97 – 1.76 (m, 3H), 1.42 (d, *J* = 4.2 Hz, 1H), 1.31 (d, *J* = 7.0 Hz, 3H), 1.25 (s, 2H), 1.21 (t, *J* = 7.0 Hz, 6H).

PSMA-Phe2

In a syringe of 5ml for solid phase peptide synthesis, 50mg of resin Lys-CO-Glu (**6**) was coupled with 0,34mmol of Fmoc-L-Phenylalanine, 0,34 mmol of Oxyma pure and DIC in a final volume of 0,7ml DMF. Intermediate Fmoc-product was deprotected by 4-methylpiperidine and DMF giving product ??. The same procedure was executed twice, and finally cleaved from resin by cleavage mixture 2ml of 95% TFA, 2,5% water, 2,5% TIPS for 3 hours. The crude product was purified by RP-HPLC and characterised by ¹H NMR (400 MHz, MeOD) δ 7.36 – 7.24 (m, 9H), 7.23 – 7.18 (m, 1H), 4.62 (t, *J* = 7.6 Hz, 1H), 4.30 – 4.22 (m, 2H), 4.13 – 4.05 (m, 1H), 3.29 – 3.04 (m, 6H), 2.99 (s, 4H), 2.86 (d, *J* = 0.6 Hz, 1H), 2.44 – 2.30 (m, 2H), 2.15 (q, *J* = 7.0 Hz, 3H), 1.97 – 1.76 (m, 2H), 1.70 – 1.35 (m, 10H), 1.26 (q, *J* = 7.7 Hz, 2H), 1.10 (d, *J* = 6.6 Hz, 1H) and **TOFF-MS**: Calcd for [M-H⁺]⁺ = 727,36Da found 727,85Da.

Yield: 17%

PSMA-Ahx2

In a syringe of 5ml for solid phase peptide synthesis, 50mg of resin Lys-CO-Glu (**6**) was coupled with 0,34mmol of Fmoc-Aminohexanoic acid, 0,34 mmol of Oxyma pure and DIC in a final

column of 0,7ml DMF. Intermediate Fmoc-product was deprotected by 4-methylpiperidine and DMF giving product ???. The same procedure was executed twice, and finally cleaved from resin by cleavage mixture 2ml of 95% TFA, 2,5% water, 2,5% TIPS for 3 hours. Crude product was purified by RP-HPLC and characterised by NMR and **TOFF-MS**: Calcd for $[M+H]^+$ =546,31Da found 546,73Da. Yield: 22%

PSMA617-cystine-DOX

DOX-Cystine (0,013mmol) and PSMA_617 motif (0,013mmol) were diluted 17mg in 0,5ml DMF and DIPEA (0,026mmol). The reaction was left overnight and purified by RP-HPLC and characterised by **TOFF-MS**: Calcd for $[M+3K^++H^+]^+$ =1520,39Da found 1520,80Da.

Time (min)	H ₂ O	MeCN
0	100	0
26	20	80
27-32	0	100
32-35	100	0

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Author Information

AK and CL conceived the project. EM synthesized squaramide analog of lysine-urea-glutamate pharmacophore, the PSMA-Phe2, PSMA617-cystine-Doxorubicin. GL synthesized PSMA-Ahx2. GL performed the simulations. HPLC work was carried out in MH lab. EM and AK wrote the paper and AK revised it.

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TOC Graphic

