Synthesis and Binding Profile Using Simulations of New Building Blocks for PSMA Theranostics 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 and (ii) amino hexanoic acid residues. Induced fit docking explored the binding profile of the new molecules. Biological experiments will provide data on the significance of the new molecules.

Keywords: bioconjugate; docking calculations; induced fit; 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 benef. [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 diagnonis have been developed.

The development of lysine-urea-glutamate (Lys-CO-Glu-OH) based inhibitors targeting PSMA for theranostics 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_tetraxetan) 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 tetraxetan 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 theranostics radionuclides. The ^{177}Lu version of PSMA-617 was recently approved by FDA for PCa diagnosis.

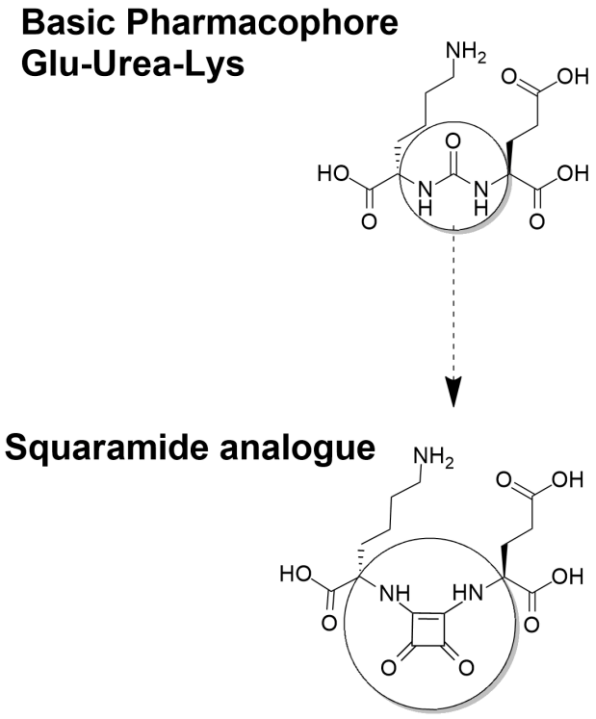


Figure 1. Squaramide analog of Lys-CO-Glu-OH pharmacophore.

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 Ki of ARM-P's and natural substrate: folyl-poly-γ-glutamates as it was reported. [5,6] Taking these data into consideration, we synthesized an analogue with a suitable spacer and two phenylalanine for enhancing binding interactions with PSMA receptor (Figure 2).

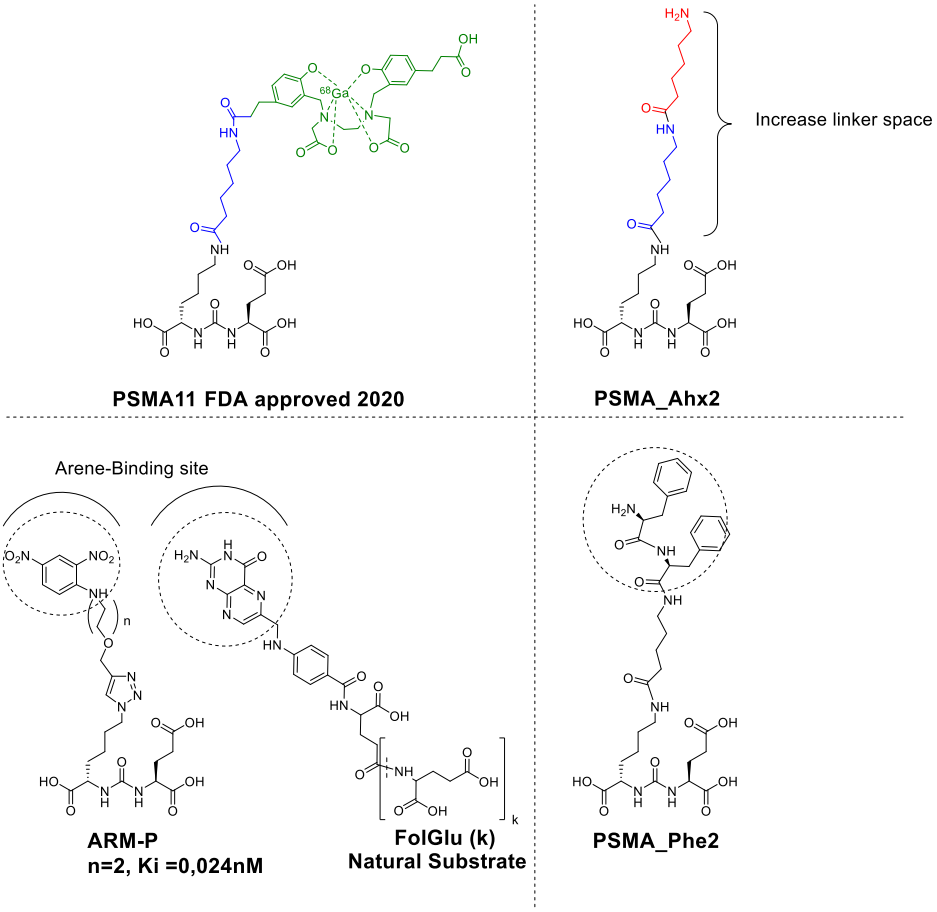
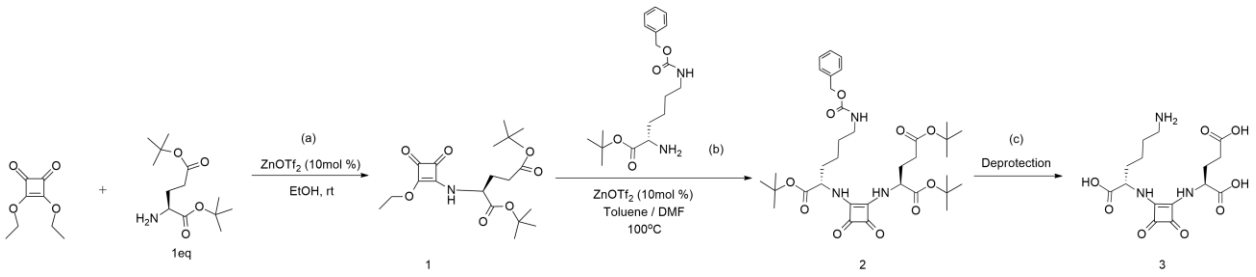


Figure 2. Design of the new **PSMA-Phe2** and of the new **PSMA-Ahx2** building blocks as analogs of **PSMA11** targeting PSMA.

Results and Discussion

Chemistry

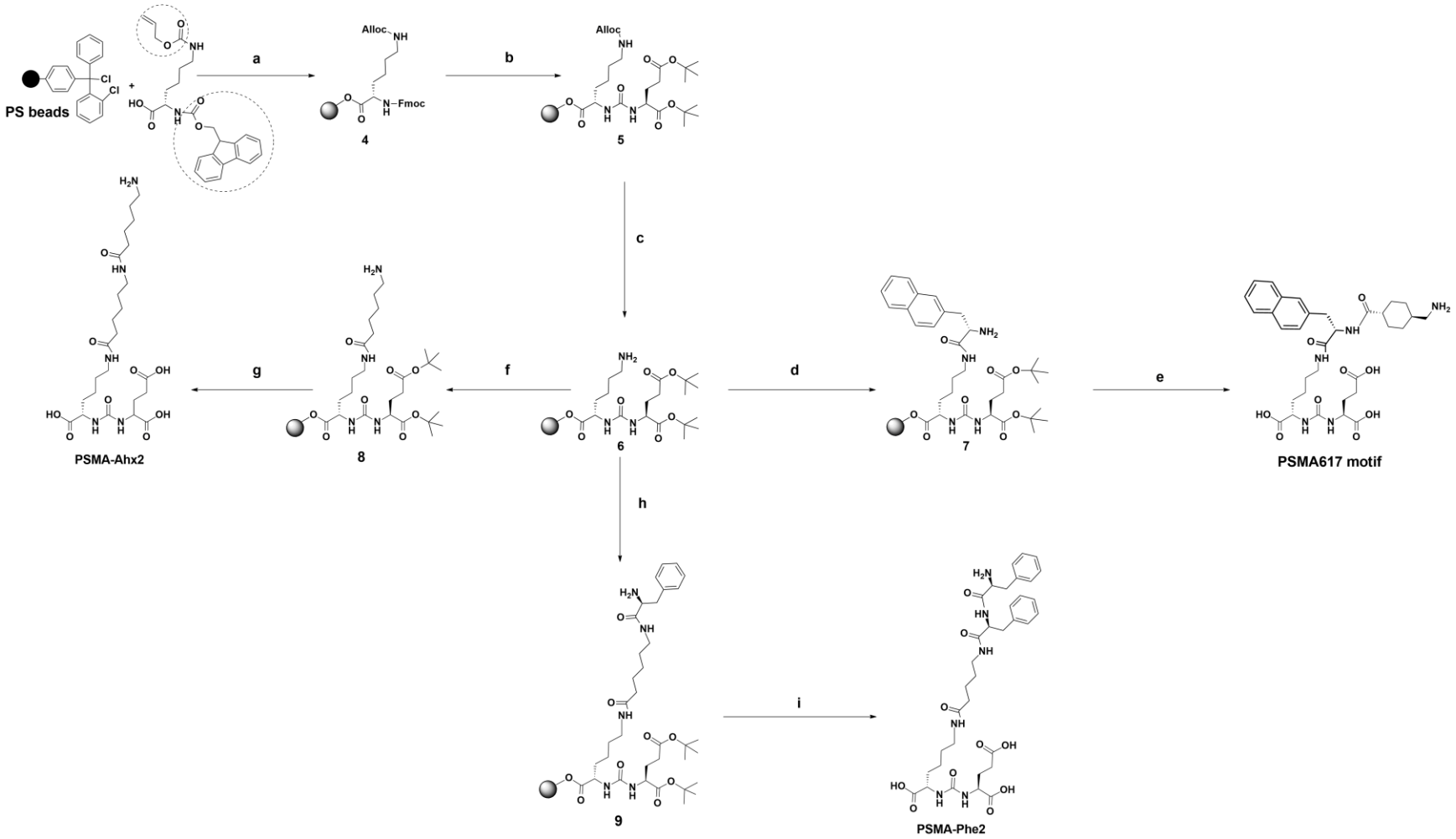
The synthesis of squaramide analog of Lys-CO-Glu-OH pharmacophore was accomplished according to previously applied methods (Scheme 2). [7] Firstly, 3,4 Diethoxycyclobut-3-ene-1,2 dione and di-tert-butyl glutamate reacted leading to the mono-squaramide. Then protected Lysine was coupled as a following using $ZnOTf_2$ and DIPEA at 100^0C with a yield (53%). Deprotection of compound **2** produced squaramide **3** descibed in Scheme 1.



Scheme 1. (a) $ZnOTf_2$, DIPEA, ethanol, rt; (b) $ZnOTf_2$, DIPEA, toluene: DMF 19:1 at 100 ^{0}C; (c) i. H_2 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/H_2O. The peptidomimetic moieties **PSMA-Ahx2**, **PSMA-Phe2** and **PSMA-617** were purified with RP-HPLC and analyzed with MALDI-MS.



Scheme 2. Spps of **PSMA617**: (a) DCM(dry), DIPEA; (b) triphosgene, DIPEA, DCM (°C); (c) $Pd(PPh_3)_4$, morpholine, DCM (dry); (d) i. Fmoc-3-(2-napthyl)-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/H_2O 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/H_2O 95:2.5:2.5 (v/v/v).

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 Ethnanol. 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** ^{1}H 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 $[M+H]^+$ =383,19Da found 383,19. Product **1** (0,05mmol), Lys-Cbz-OtBu (0,055mmol), $ZnOTf_2$ (0,005mmol) and DIPEA (0,11mmol) were all dissolved in 250μl Toluene: DMF (19:1) at 100^0C 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%, ^{1}H 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 $[M-H]^-$ =672,36Da 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). H_2 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 ^{1}H-NMR (D_2O, 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 (Chlorotrityl 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 mixrutre of mmol of bis(tert- butyl) L-glutamate hydrochloride and 1.5 mL of N-ethyldiisopropylamine (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-napthyl)-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 ^{1}H 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%

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 colume 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 ^{1}H 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 $[M\text{-}H^{+}]^{-}$ =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 colume 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%

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Author Information

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

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