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Research Article

Improving the Specificity of the Prostate-Specific Antigen Substrate Glutaryl-Hyp-Ala-Ser-Chg-Gln as a Promoiety

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To develop PSA peptide substrates with improved specificity and plasma stability from the known substrate sequence glutaryl-Hyp-Ala-Ser-Chg-Gln, systematic replacements of the *N*-terminal segment with D-retro-inverso-peptides were performed with the incorporation of 7-amino-4-methylcoumarin (7-AMC) after Gln for convenient fluorometric determination and ranking of the PSA substrate activity. The D-retro-inverso-peptide conjugates with P2-P5 D-amino acid substitutions were moderate but poorer PSA substrates as compared to the original peptide, suggesting that inversion of the amide bonds and/or incorporation of the additional atom as in the urea linker adversely affected PSA binding. However, P5 substitution of Hyp with Ser showed significant improvements in PSA cleavage rate; the resulting AMC conjugate, glutaryl-Ser-Ala-Ser-Chg-Gln-AMC (11), exhibited the fastest PSA cleavage rate of 351 pmol/min/100 nmol PSA. In addition, GABA←mGly-Ala-Ser-Chg-Gln-AMC (conjugate 6) was the second best PSA substrate and released 7-AMC at a rate of 225 pmol/min/100 nmol PSA as compared to 171 pmol/min/100 nmol PSA for the control conjugate glutaryl-Hyp-Ala-Ser-Chg-Gln-AMC. Incubations of selected AMC conjugates with mouse and human plasma revealed that GABA←D-Ser-ψ[NH-CO-NH]-Ala-Ser-Chg-Gln-AMC (5) and GABA←mGly-Ala-Ser-Chg-Gln-AMC (6) were most stable to non-PSA-mediated proteolysis. Our results suggest that the PSA specificity of glutaryl-Hyp-Ala-Ser-Chg-Gln is improved with Ser and mGly substitutions of Hyp at the P5.

Key words: 7-amino-4-methylcoumarin, D-retro-inverso-peptide, prostate-specific antigen

Abbreviations: 7-AMC, 7-amino-4-methylcoumarin; 7-AZD, 7-azido-4-methylcoumarin; CPM, counts per min; DCM, dichloromethane; FCC, flash column chromatography; GABA, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; IPCF, isopropyl chloroformate; LC-MS, liquid chromatography–mass spectrometry; mGly, malonyl analog of glycine; NaSeH, sodium hydrogen selenide; NMP, N-methyl-2-pyrrolidone; NMR, nuclear magnetic resonance; PSA, prostate-specific antigen; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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Prostate cancer is the most common type of cancer reported and the second leading cause of morbidity and death among American males next to lung cancer. According to the American Cancer Society, there will be around 233 000 newly diagnosed cases of prostate cancer and about 29 480 estimated deaths from prostate cancer in 2014 (1,2). Although hormonal treatment through androgen ablation may initially reduce tumor growth and metastasis rates, it is rarely curative because tumor cells become refractory to hormonal therapy within 1–2 years (3). Furthermore, the propensity of prostate cancer to progress through osteoblastic metastasis remains a clinical challenge. While alternative treatment modalities involving platinum compounds, radiopharmaceuticals and monoclonal antibodies have been explored recently (4–8), the clinical use of chemotherapeutics such as docetaxel, mitoxantrone, and doxorubicin is limited by systemic toxicity stemming from non-discriminatory drug exposure to normal tissues (9–11). To mitigate this issue, one of the approaches developed to improve the selectivity of chemotherapy toward tumor cells involves the targeted prodrug strategy (12–15). Secretion of active prostate-specific antigen (PSA) in the tumor microenvironment can be exploited for targeted activation of peptide prodrugs in cancer cells (15). PSA is a serine protease with chymotrypsin-like activity and is predominantly expressed in the prostate although present in other normal and tumor tissues at low concentrations (16–18). It exhibits high substrate specificity being the only endopeptidase known to cleave peptide substrates at the amide bond after Gln. Moreover, its enzymatic activity is restricted to the tumor microenvironment due to inactivation by α1-antichymotrypsin and α2-macroglobulin in the systemic circulation (19,20), which makes PSA an attractive target for tumor-selective prodrug activation. Several prodrugs consisting of

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PSA-specific peptide substrates coupled to cytotoxic drugs have been reported (21–23).

Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox (L-377202, prodrug I) is one example of a PSA-activated prodrug for prostate cancer that was advanced to phase I clinical studies (24). Based on the cleavage sequences of PSA's natural substrates, semenogelins I and II, the highly specific PSA substrate, glutaryl-Hyp-Ala-Ser-Chg-Gln was developed and coupled to the aminoglycoside of doxorubicin via a Ser-Leu linker (21,25). Following intravenous administration of prodrug I to mice, rats, dogs, and monkeys in preclinical studies, roughly 30–40% of the parent compound was metabolized to doxorubicin by non-PSA-mediated mechanisms (25). In tumor-bearing mice, the dose fraction converted to doxorubicin increased to 87% versus 28% in control mice (25). Following intravenous administration of prodrug I to 19 patients with advanced hormone-refractory prostate cancer at escalating doses (up to 315 mg/m^2), circulating levels of the active metabolites Leu-doxorubicin (AUC = 4.3 μM h, C_{max} = 5 μM) and doxorubicin (AUC = 1.2 μM h, C_{max} = 0.12 μM) were detected at the efficacious dose of 225 mg/m^2 (24,26,27). Dose-limiting neutropenia was also observed in 1 of 6 patients at this dose (24). The protease responsible for the non-specific hydrolysis of prodrug I was not identified. Although the observed toxicity was not correlated with systemic levels of active metabolites, the clinical development of prodrug I was not pursued beyond phase I studies most likely due to toxicity concerns following intravenous administration at the efficacious dose. Thus, we decided to further improve the PSA specificity of glutaryl-Hyp-Ala-Ser-Chg-Gln by introducing D-retro-inverso-peptide fragments in its sequence using urea and malonate (mGly) linkers. Although previous optimization studies suggest that amino acid substitution in P1-P4 for the sequence of prodrug I may compromise substrate specificity for PSA (25), we anticipated that D-retro-inverso-peptide fragment insertion will mitigate non-PSA-mediated hydrolysis while maintaining or improving PSA cleavage rate. This is based on the assumption that amino acid side-chain orientation is most significant to optimal PSA substrate binding. Sequence optimization was streamlined with the choice of a fluorescent tag that was readily coupled to a variety of peptides. The optimized sequences can then be coupled to cytotoxic drugs via appropriate linkers and evaluated. In this study, peptide conjugates of the fluorescent dye 7-amino-4-methylcoumarin (7-AMC) were synthesized as convenient surrogates for developing peptide substrates with improved PSA specificity because enzyme-mediated cleavage of AMC conjugates at the Gln-AMC bond results in quantifiable fluorescence increase.

Methods and Materials

Fmoc-protected amino acids were purchased from Chem-Impex (Wood Dale, IL, USA). Solvents were either ACS reagent or HPLC grade and used without further purification. Reactions were monitored by TLC and/or on an LC-MS system consisting of PE 200 Series autosampler and pumps (Perkin Elmer, Waltham, MA, USA) coupled to an LCQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA). Accurate mass values of final peptide conjugates were determined by direct inlet infusion of 10 μg/mL solutions using a LTQ Orbitrap (Thermo Scientific). Flash column chromatography (FCC) was performed on a Teledyne ISCO CombiFlash Companion Automated Flash Chromatographic System (Teledyne Technologies, Thousand Oaks, CA, USA) with prepacked silica gel columns. All ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker spectrometer at ambient temperature and calibrated using residual undeuterated solvents as the internal reference.

Synthesis of 7-Azido-4-methylcoumarin (7-AZD, 12)

7-azido-4-methylcoumarin was prepared as a yellow solid in 79% yield (234 mg) starting from 7-amino-4-methyl coumarin (257 mg) as previously described (28). ^{1}H NMR (DMSO-d$_6$, 400 MHz): δ 7.83 (d, 1H, J = 8.5 Hz), 7.2 (m, 1H), 7.18 (d, 1H, J = 2.1 Hz), 6.39 (s, 1H), 2.47 (s, 3H); ^{13}C NMR (DMSO-d$_6$, 100 MHz): δ 159.5, 154.0, 152.9, 143.3, 126.9, 116.7, 115.5, 113.2, 106.8, 18.0, MS (ESI+): *m/z* (intensity) 201.9 ([M + H]$^+$, 100%).

Synthesis of 7-(N^{α}-t-butyloxycarbonyl-L-glutaminyl) amino-4-methylcoumarin (Boc-Gln-AMC, 13)

Boc-Gln-AMC was synthesized using our previously reported amidation procedure between Boc-Gln selenocarboxylate and 7-AZD (28). Briefly, a fresh solution of NaHSe was obtained by reacting $NaBH_4$ and an isopropanolic suspension of Se as previously described (29). In parallel, the mixed anhydride of Boc-Gln-OH was prepared by adding a 1.0 M solution of IPCF in toluene (2.0 mL, 2.0 mmol) to a solution of Boc-Gln-OH (2.0 mmol) and N-methylpiperidine (244 μL, 2.0 mmol) in 20 mL of anhydrous THF at −15 °C under nitrogen and allowing the mixture to stir for 20 min at −15 °C. The selenocarboxylate was generated *in situ* by adding the freshly prepared NaHSe reagent above to the mixed anhydride via cannula over a period of 5 min and stirring the reaction mixture for an additional 30 min below −10 °C under nitrogen atmosphere. A solution of 7-AZD (1.62 mmol) in 2 mL of anhydrous THF was cannulated into the selenocarboxylate solution, and the reaction carried out at room temperature under nitrogen for 2 h. Organic solvents were removed; the residue was suspended in 15 mL of a saturated sodium bicarbonate solution and extracted with 45 mL (3×) of ethyl acetate. The organic extracts were combined, washed sequentially with water and brine, and dried over anhydrous sodium sulfate. After filtering out sodium sulfate, the crude mixture was dry-loaded onto silica gel and purified by FCC. The product (142 mg) was obtained as a

light-yellow solid in 84% yield. ^{1}H NMR (CD_3OD, 400 MHz): δ 7.70 (d, 1H, J = 2.0 Hz), 7.60 (d, 1H, J = 8.7 Hz), 7.39 (d, 1H, J = 8.2 Hz), 6.13 (s, 1H), 4.12 (s, 1H), 2.35 (s, 3H), 2.28 (t, 2H, J = 7.5 Hz), 2.02 (m, 1H), 1.87 (m, 1H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 177.8, 173.4, 163.2, 157.9, 155.4, 155.2, 149.9, 143.4, 126.7, 117.3, 117.2, 113.7, 108.1, 81.0, 56.5, 32.5, 29.1, 28.8, 18.5; MS (ESI+): *m/z* (intensity) 403.7 ([M + H]$^+$, 12%), 706.7 ([2M + H-Boc]+, 100%).

Synthesis of glutaryl-Hyp-Ala-Ser-Chg-Gln-AMC (1)

The solid-phase synthesis of Fm-glutaryl-Hyp-Ala-Ser-Chg-OH was carried out using standard solid-phase peptide synthesis procedures. Loading of Fmoc-Chg-OH on 1 g of Wang resin (0.35 mmol/g resin) yielded 148 mg of peptide which was used without further purification. In order to couple Gln-AMC to the peptide, Boc-Gln-AMC (60 mg, 0.15 mmol) was deprotected with 1 mL of TFA/DCM/H_2O (50:45:5) in 30 min at room temperature. Solvents were removed under a gentle stream of nitrogen; the residue was reconstituted in 400 μL of methanol and treated with Amberlyst A-26 (0.5 g, 0.4 mmol) to remove residual TFA. After filtering out Amberlyst A-26, methanol was removed and the light-yellow residue resuspended in 500 μL of NMP ready for the next step. Gln-AMC (17 mg, 0.042 mmol) was coupled to Fm-glutaryl-Hyp-Ala-Ser-Chg-OH (9.6 mg, 0.013 mmol) in 600 μL of NMP using HBTU (10 mg, 0.027 mmol) preactivation of the peptide for 30 min in the presence of 1 eq. diethylisopropylamine (DIEA) at room temperature, followed by the addition of Gln-AMC. The reaction was allowed to proceed for 16 h at room temperature, after which 10% piperidine deprotection was carried out followed by HPLC purification. The desired peptide–AMC conjugate, Glutaryl-Hyp-Ala-Ser-Chg-Gln-AMC (**1**), was obtained in 52% yield (5.9 mg) and was > 98% pure by LC-UV analysis. HRMS (ESI$^+$) *m/z* calc'd for $C_{39}H_{54}N_7O_{13}$ [M + H]$^+$: 828.3780, found: 828.3806.

Synthesis of N$^{\gamma}$-(9-Fluorenyloxycarbonyl)-γ-aminobutyric Acid (Fmoc-GABA, 14)

To a solution of GABA (2.00 g, 19.4 mmol, in 14 mL 10% $NaHCO_3$), Fmoc-OSu (4 g, 11.7 mmol, in 40 mL acetonitrile) was added drop-wise over a period of 2 h at room temperature. The mixture was allowed to stir at room temperature for an additional hour. Acetonitrile was removed under reduced pressure and the aqueous layer acidified to pH 1 with 10% HCl. The precipitate was washed with two 20-mL portions of water, 20 mL of ethyl acetate, and dried under reduced pressure. Fmoc-GABA was obtained as a white solid in 73% yield (2.8 g). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.89 (d, 2H, J = 7.4 Hz), 7.44 (d, 2H, J = 7.2 Hz), 7.42 (t, 2H, J = 7.5 Hz), 7.35 (s, 1H), 7.33 (t, 2H, J = 7.0 Hz), 4.30 (d, 2H, J = 7 Hz), 4.21 (t, 1H, J = 6.7 Hz), 3.01 (q, 2H, J = 5.6 Hz), 2.20 (t, 2H, J = 7.3 Hz), 1.63 (q, 2H, J = 7.1 Hz); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 142.6, 139.4, 137.4, 128.9, 127.2, 124.2, 121.3, 120.0, 109.6, 77.5, 61.8, 51.1, 31.6; MS (ESI+): *m/z* (intensity) 325.8 ([M+H]+, 100%).

Loading of Fmoc-GABA on 2-chloro-trityl chloride resin

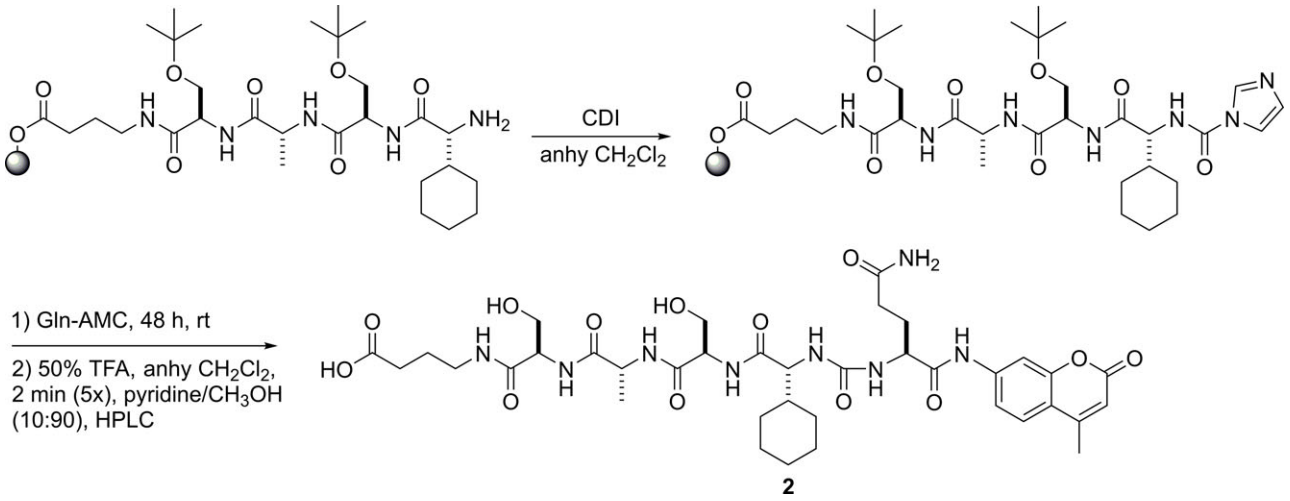
Attempts to load Fmoc-GABA on Wang resin failed using DIC, IPCF, or PyBOP in the presence of catalytic DMAP (0.1 eq.). Alternatively, Fmoc-GABA (1.2 g, 3.6 mmol) was effectively coupled to 2-chloro-trityl chloride resin (1 g, 1.7 mmol) in 10 mL of anhydrous DCM/NMP (9:1) in the presence of 4 eq. DIEA at room temperature for 3 h. The loading levels were determined to be 0.48 mmol/g resin by Fmoc-deprotection with 20% piperidine and UV analysis (A_{290}). The unreacted 2-chloro-trityl chloride on the resin was end-capped with 2 mL of methanol/DIEA (9:1) in 10 mL of anhydrous DCM under nitrogen for 60 min at room temperature, and the resin was ready for further coupling after 20% piperidine deprotection.

Synthesis of GABA←D-Ser←D-Ala←D-Ser←D-Chg-ψ[NH-CO-NH]-Gln-AMC (2)

GABA←D-Ser(*t*Bu)←D-Ala←D-Ser(*t*Bu)←D-Chg-H was generated on 2-chloro-trityl chloride resin (1.8 g, 0.48 mmol/g resin) starting with the GABA resin prepared above and following standard automated peptide synthesis procedures. Resin-GABA←D-Ser(*t*Bu)←D-Ala←D-Ser(*t*Bu)←D-Chg-H (53 mg, 0.026 mmol) was activated with CDI (22 mg, 0.14 mmol) in 200 μL of anhydrous DCM for 3 h at room temperature to generate the carbonyl-imidazole intermediate (Scheme 1). After washing the resin with three 1-mL portions of anhydrous DCM, coupling to H-Gln-AMC (43 mg, 0.14 mmol) was carried out in 600 μL of DCM/NMP (60:40) for 48 h at room temperature. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser←D-Ala←D-Ser←D-Chg-[NH-CO-NH]-Gln-AMC (**2**), in 6% yield (1.5 mg, 95% pure by LC-UV analysis). HRMS (ESI$^+$) *m/z* calc'd for $C_{37}H_{53}N_8O_{13}$ [M + H]$^+$: 817.3732, found: 817.3753.

Synthesis of H-Chg-Gln-AMC (16)

Fmoc-Chg-OH (104 mg, 0.27 mmol) was preactivated with HBTU (107 mg, 0.28 mmol) in the presence of 5 eq. DIEA in 200 μL of NMP for 1 h and then coupled to H-Gln-AMC (72 mg, 0.24 mmol) at room temperature for 2 h under nitrogen atmosphere. The crude mixture was suspended in 10 mL of saturated $NaHCO_3$ and extracted with 30 mL of DCM three times. The organic layer was dry-loaded onto silica gel and purified by FCC. The protected peptide intermediate (134 mg) was deprotected with 0.5 M TBAF and purified by HPLC to generate the desired peptide (*m/z* = 443.1) in 57% yield (60 mg).



Scheme 1: Synthesis of peptide–AMC conjugate 2.

Synthesis of GABA←D-Ser←D-Ala←D-Ser-ψ[NH-CO-NH]-Chg-Gln-AMC (3)

The amino end of resin-GABA←D-Ser(*t*Bu)←D-Ala←D-Ser(*t*Bu)-H (69 mg, 0.034 mmol) was preactivated with CDI (27 mg, 0.17 mmol) in 200 μL of anhydrous DCM at room temperature for 3 h and, after three 1-mL DCM washes, coupled to H-Chg-Gln-AMC in 600 μL of DCM/NMP (60:40) for 48 h at room temperature. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser←D-Ala←D-Ser-[NH-CO-NH]-Chg-Gln-AMC (**3**), in 10% yield (2.1 mg, > 99% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{37}H_{53}N_8O_{13}$ $[M + H]^+$: 817.3732, found: 817.3766.

Synthesis of H-Ser(tBu)-Chg-Gln-AMC (17)

Fmoc-Ser(*t*Bu)-OH (116 mg, 0.30 mmol) was preactivated with HBTU (120 mg, 0.32 mmol) in the presence of 5 eq. DIEA in 400 μL of NMP for 30 min and then coupled to H-Chg-Gln-AMC (119 mg, 0.27 mmol) at room temperature for 10 h under nitrogen atmosphere. The crude mixture was suspended in 10 mL of saturated $NaHCO_3$ and extracted with 30 mL of DCM three times. The organic layer was dry-loaded onto silica gel and purified by FCC. The protected peptide intermediate (53 mg) was deprotected with 0.5 M TBAF and purified by HPLC to generate the desired peptide (*m/z* = 586.0 in 28% yield (21 mg).

Synthesis of GABA←D-Ser←D-Ala-ψ[NH-CO-NH]-Ser-Chg-Gln-AMC (4)

Conjugate **4** was prepared by activating the amino end of resin-GABA←D-Ser(*t*Bu)←D-Ala-H (50 mg, 0.025 mmol) with CDI (23 mg, 0.14 mmol) in 200 μL of anhydrous DCM at room temperature for 3 h and, after three 1-mL DCM washes, coupled to H-Ser(*t*Bu)-Chg-Gln-AMC in 600 μL of DCM/NMP (60:40) for 24 h at room temperature. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser←D-Ala-[NH-CO-NH]-Ser-Chg-Gln-AMC (**4**), in 7.8% yield (1.6 mg, > 99% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{37}H_{53}N_8O_{13}$ $[M + H]^+$: 817.3732, found: 817.3754.

Synthesis of H-Ala-Ser($\Psi^{Me,Me}$ pro)-Chg-Gln-AMC (18)

Fmoc-Ala-Ser($\Psi^{Me,Me}$ pro)-OH (136 mg, 0.31) was preactivated with HBTU (105 mg, 0.27 mmol) in the presence of 5 eq. DIEA in 400 μL of NMP for 30 min and then coupled to H-Chg-Gln-AMC (97 mg, 0.22 mmol) at room temperature for 3 h under nitrogen atmosphere. One-pot Fmoc-deprotection was carried out with 10% DEA and NMP was removed with repeated hexane washes (5-mL portions, at least 10 times). The crude mixture was dissolved in 1 mL of 50% acetonitrile/water and purified by FCC. The peptide **18** (*m/z* = 641.1) was obtained in 66% yield (93 mg) and > 98% pure by LC-UV analysis.

Synthesis of GABA←D-Ser-ψ[NH-CO-NH]-Ala-Ser-Chg-Gln-AMC (5)

Conjugate **5** was prepared by activating the amino end of resin-GABA←D-Ser(*t*Bu)-H (54 mg, 0.025 mmol) with CDI (20 mg, 0.12 mmol) in 200 μL of anhydrous DCM at room temperature for 3 h and, after three 1 mL DCM washes, coupled to H-Ala-Ser($\Psi^{Me,Me}$ pro)-Chg-Gln-AMC (50 mg, 0.078 mmol) in 600 μL of DCM/NMP (60:40) for 24 h at room temperature. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser-[NH-CO-NH]-Ala-Ser-Chg-Gln-AMC (**5**), in 7.8% yield

(2.6 mg, > 98% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{37}H_{53}N_8O_{13}$ $[M + H]^+$: 817.3732, found: 817.3754.

Synthesis of t-butyl fluorenylmethyl malonate (15)

Malonic acid (283 mg, 2.7 mmol) and fluorenyl methanol (213 mg, 1.1 mmol) were dissolved in 2 mL of acetonitrile at room temperature under nitrogen atmosphere. EDC (531 mg, 2.8 mmol) was added, and the reaction was carried out at room temperature for 30 min under nitrogen atmosphere. After removing acetonitrile under reduced pressure, the residue was redissolved in 50 mL of DCM and extracted with two 20-mL portions of saturated $NaHCO_3$. The aqueous layers were combined, acidified to pH 1, and extracted with 35 mL of DCM three times. The organic layers were combined, washed with water, brine and dried over sodium sulfate. DCM was removed under reduced pressure, and the product recovered as an off-white solid in 57% yield (176 mg, > 99% pure by LC-UV analysis). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.80 (d, 2H, J = 7.4 Hz), 7.61 (d, 2H, J = 6.8 Hz), 7.44 (t, 2H, J = 8.0 Hz), 7.35 (t, 2H, J = 8.3 Hz), 4.52 (d, 2H, J = 7.2 Hz), 4.27 (t, 1H, J = 7.2 Hz), 3.55 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.9, 166.8, 143.5, 140.7, 127.7, 127.1, 125.2, 120.1, 66.4, 46.3, 41.6.

Synthesis of HO-mGly-Ala-Ser($\Psi^{Me,Me}$ pro)-Chg-Gln-AMC (16)

Fluorenylmethyl malonate (14 mg, 0.050 mmol), H-Ala-Ser($\Psi^{Me,Me}$ pro)-Chg-Gln-AMC (20 mg, 0.031 mmol), and EDC (13 mg, 0.068 mmol) were dissolved in 400 μL of NMP in the presence of 5 eq. DIEA at room temperature under nitrogen atmosphere. DMAP (9 mg, 0.07 mmol) was added, and the reaction was carried out for 16 h at room temperature under nitrogen atmosphere. The reaction mixture was washed with 2 mL of hexane (five times) to remove NMP, reconstituted in 1 mL of 50% acetonitrile/water and purified by HPLC. The peptide **16** (*m/z* = 641.1) was obtained in 62% yield (14 mg) and was > 98% pure by LC-UV analysis.

Synthesis of GABA←mGly-Ala-Ser-Chg-Gln-AMC (6)

HO-mGly-Ala-Ser($\Psi^{Me,Me}$ pro)-Chg-Gln-AMC (12 mg, 0.017 mmol) was activated with EDC (9 mg, 0.05 mmol)/DMAP (6 mg, 0.05 mmol) in the presence of DIEA (5 eq.) in 400 μL of NMP and coupled to GABA resin (20 mg, 0.0094 mmol) for 48 h at room temperature. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←mGly-Ala-Ser-Chg-Gln-AMC (**6**), in 32% yield (2.5 mg, > 98% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{36}H_{50}N_7O_{12}$ $[M + H]^+$: 772.3517, found: 772.3520.

Synthesis of GABA←D-Ser←mGly-Ser-Chg-Gln-AMC (7)

Fluorenylmethyl malonate (8.2 mg, 0.029 mmol), H-Ser-Chg-Gln-AMC (14 mg, 0.024 mmol), and EDC (12 mg, 0.063 mmol) were dissolved in 400 μL of NMP in the presence of DIEA (5 eq.) at room temperature under nitrogen atmosphere. DMAP (7.6 mg, 0.063 mmol) was added, and the reaction carried out at room temperature for 16 h under nitrogen atmosphere. The reaction mixture was washed with 2 mL of hexane (five times), reconstituted in 400 μL of NMP, and used for the second coupling step without further purification. Additional portions of EDC (25 mg, 0.13 mol) and DMAP (12 mg, 0.098 mmol) were added to couple the peptide to resin-GABA←D-Ser(*t*Bu)-H (20 mg, 0.01 mmol) at room temperature for 16 h in the presence of 5 eq. DIEA. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser←mGly-Ser-Chg-Gln-AMC (**7**), in 32% yield (2.3 mg, > 98% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{36}H_{50}N_7O_{13}$ $[M + H]^+$: 788.3467, found: 788.3454.

Synthesis of GABA←D-Lys←mGly-Ser-Chg-Gln-AMC (8)

Fluorenylmethyl malonate (20 mg, 0.071 mmol), H-Ser-Chg-Gln-AMC (27 mg, 0.046 mmol), and EDC (22 mg, 0.12 mmol) were dissolved in 400 μL of NMP at room temperature in the presence of 5 eq. DIEA under nitrogen atmosphere. DMAP (14 mg, 0.12 mmol) was added, and the reaction was carried out at room temperature for 16 h under nitrogen atmosphere. The reaction mixture was washed with 2 mL of hexane (five times), reconstituted in 400 μL of NMP, and used for the second coupling step without further purification. Additional portions of EDC (44 mg, 0.23 mol) and DMAP (28 mg, 0.13 mmol) were added to couple the peptide to resin-GABA←D-Lys(Boc)-H (20 mg, 0.01 mmol) at room temperature for 24 h in the presence of 5 eq. DIEA. The protected peptide conjugate was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Lys←mGly-Ser-Chg-Gln-AMC (**8**), in 11% yield (1.1 mg, > 98% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{39}H_{57}N_8O_{12}$ $[M + H]^+$: 829.4096, found: 829.4092.

Synthesis of GABA←D-Ser←D-Ala←mGly-Chg-Gln-AMC (9)

Fluorenylmethyl malonate (16 mg, 0.057 mmol), H-Chg-Gln-AMC (21 mg, 0.047 mmol), and EDC (23 mg, 0.12 mmol) were dissolved in 400 μL of NMP at room temperature in the presence of DIEA (5 eq.) under nitrogen atmosphere. DMAP (13 mg, 0.11 mmol) was added, and the reaction was carried out at room temperature for 16 h

under nitrogen atmosphere. The reaction mixture was washed with 2 mL of hexane (five times), reconstituted in 400 μL of NMP, and used for the second coupling step without further purification. Additional portions of EDC (45 mg, 0.24 mol) and DMAP (31 mg, 0.25 mmol) were added to couple the peptide to resin-GABA←D-Ser(*t*Bu)←D-Ala-H (26 mg, 0.013 mmol) at room temperature for 24 h in the presence of 5 eq. DIEA. The protected peptide conjugate was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser←D-Ala←mGly-Chg-Gln-AMC (**9**), in 20% yield (2.0 mg, > 95% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{36}H_{50}N_7O_{12}$ $[M + H]^+$: 772.3517, found: 772.3536.

Synthesis of GABA←D-Ser←D-Ala←D-Ser←mGly-Gln-AMC (10)

Fluorenylmethyl malonate (25 mg, 0.089 mmol), H-Gln-AMC (21 mg, 0.047 mmol), and EDC (20 mg, 0.10 mmol) were dissolved at room temperature in 400 μL of NMP in the presence of 5 eq. DIEA under nitrogen atmosphere. DMAP (11 mg, 0.90 mmol) was added, and the reaction was carried out at room temperature for 16 h under nitrogen atmosphere. The reaction mixture was washed with 2 mL of hexane (five times), reconstituted in 400 μL of NMP, and used for the second coupling step without further purification. Additional portions of EDC (67 mg, 0.35 mol) and DMAP (8 mg, 0.07 mmol) were added to couple the peptide to resin-GABA←D-Ser(*t*Bu)←D-Ala←D-Ser(*t*Bu)-H (26 mg, 0.013 mmol) at room temperature for 48 h in the presence of 5 eq. DIEA. The protected peptide was cleaved off the resin with 5% TFA/DCM. After HPLC purification, treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC conjugate, GABA←D-Ser←D-Ala←D-Ser←mGly-Gln-AMC (**10**), in 21% yield (1.5 mg, > 99% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{31}H_{42}N_7O_{13}$ $[M + H]^+$: 720.2841, found: 720.2869.

Synthesis of glutaryl-Ser-Ala-Ser-Chg-Gln-AMC (11)

Fmoc-Ser(*t*Bu)-OH (8.8 mg, 0.023 mmol) was preactivated with HBTU (7.7 mg, 0.020 mmol) in the presence of 5 eq. DIEA in 200 μL of NMP for 30 min at room temperature under nitrogen atmosphere and then coupled to H-Ala-Ser ($\Psi^{Me,Me}$ pro)-Chg-Gln-AMC (9.6 mg, 0.15 mmol) for 2 h. Fmoc-deprotection was carried out without purification by adding 20 μL of DEA to the reaction mixture and allowing the reaction to proceed for 90 min at room temperature. The reaction mixture was washed with 2 mL of hexane (five times), dried under reduced pressure (to remove residual DEA), and reconstituted in 200 μL of NMP. The reconstituted mixture was reacted with glutaric anhydride (5.1 mg, 0.045 mmol) for 2 h at room temperature under nitrogen atmosphere. Solvents were removed under reduced pressure and the protected peptide conjugate purified by HPLC. Treatment with 50% TFA/DCM at room temperature for 2 h afforded the desired peptide–AMC, Glutaryl-Ser-Ala-Ser-Chg-Gln-AMC (**11**), in 37% yield (93.9 mg, > 98% pure by LC-UV analysis). HRMS (ESI^+) *m/z* calc'd for $C_{37}H_{52}N_7O_{13}$ $[M + H]^+$: 802.3623, found: 802.3656.

PSA and blood stability assays

Measurement of peptide concentration by UV and the PSA enzyme assay

Peptide concentrations were determined by measuring the UV absorbance at 324 nm of 20 μM solutions prepared based on weight in methanol from 10 mM DMSO stock solutions. Using the predetermined extinction coefficient of Boc-Gln-AMC at 324 nm (12 800/M/cm), peptide concentrations were calculated and adjusted accordingly to prepare 10 μM solutions in 50 mM Tris buffer, pH 8 containing 2 mM $CaCl_2$ and 0.1% Tween-20. Each peptide solution (4 μL) was added to 36 μL of Tris buffer (final concentration 1 μM) in triplicate wells of a white Costar 384-well plate (Corning, Inc., Corning, NY) and centrifuged at 1600 × *g* for 1 min. Reactions were initiated with the addition of 4 μL aliquots of a 1.1 μM solution of human PSA (final concentration 100 nM), and time-points recorded over a period of 3 h. The stability of peptide conjugates in 50 mM Tris buffer was evaluated by measuring the fluorescence of 1 μM solutions throughout the course of the assay. Buffer fluorescence was measured by adding 40 μL aliquots of 50 mM Tris buffer to separate wells in triplicate. All wells were read using a 335/460 nm (ex/em) filters on a Victor 3V 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). PSA cleavage rates were calculated by taking the slopes of the linear portions of fluorescence counts–time curves and using a fluorescence standard curve generated with known amounts of 7-AMC.

Measurement of non-PSA-mediated proteolysis in plasma

Non-PSA-mediated hydrolysis of peptide conjugates was determined following a procedure similar to the PSA enzyme assay described above. Briefly, each peptide solution (4 μL) was added to 36 μL of phosphate buffer, human, or mouse plasma (final peptide concentration 1 μM) in a white Costar 384-well plate (Corning, Inc., Corning, NY, USA). The stability of peptide conjugates in 100 mM phosphate buffer, pH 7.4 was evaluated by measuring the fluorescence of 1 μM solutions throughout the course of the assay. Sample evaporation rate was also measured by adding mixtures of 36 μL of plasma and 4 μL of 100 mM phosphate to separate wells in triplicate and recording fluorescence increase over time. All wells were read using a 335/460 nm (ex/em) filters on a Victor 3V 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences) over a period of 24 h.

Results and Discussion

Prodrug I featuring the peptide sequence glutaryl-Hyp-Ala-Ser-Chg-Gln, a highly specific PSA substrate, coupled to doxorubicin via a Ser-Leu linker, was evaluated in clinical studies as a PSA-activated prodrug for the treatment of advanced prostate cancer (24). However, its development was discontinued probably due to toxicity concerns stemming from significant systemic levels of the active metabolite Leu-doxorubicin as well as free doxorubicin following prodrug I administration (25). Subsequent metabolism studies of prodrug I suggested that *in vivo* non-PSA-mediated hydrolysis may be responsible for the extra-prostatic release of doxorubicin (24,25,30). In the present study, we further optimized the aforementioned peptide sequence to improve PSA specificity. Sequence optimization was carried out using the convenient release of fluorescent 7-AMC from peptide conjugates following PSA cleavage of the Gln-AMC bond. Thus, we synthesized a series of partial D-retro-inverso-peptide conjugates which were evaluated as PSA substrates.

Our general strategy for optimizing the sequence glutaryl-Hyp-Ala-Ser-Chg-Gln involved systematic D-amino acid substitution in P2-P5 using either urea or malonate (mGly) linkage to generate various 7-AMC peptide conjugates which were evaluated as PSA substrates (Figure 1). The D-amino acid C-terminal ends of the peptide conjugates **2**–**10** were capped with γ-aminobutyric acid (GABA) in lieu of glutaryl, and Hyp was replaced by Ser. For consistency and the convenience of capping P5 D-amino acids through amide bond formation when a linkage is inserted between P3 and P5, glutaryl was replaced by GABA. In addition, Ser was inserted in P5 to remove the conformational restriction introduced by Hyp. Where urea linkage was used, peptides were synthesized by a combination of solid-phase and solution-phase chemistry using an Fmoc strategy. Urea linkages (AMC conjugates **2**–**5**) were inserted into the peptide sequence of Ser-Ala-Ser-Chg-Gln by first activating the amino end of the appropriate resin-bound peptide intermediate with carbonyldiimidazole (CDI) at room temperature, followed by coupling of the corresponding AMC peptide conjugate. The synthesis of conjugate **2** is shown in Scheme 1 as an example. Due to challenges encountered with the HPLC purification of final peptide conjugates, the *t*-

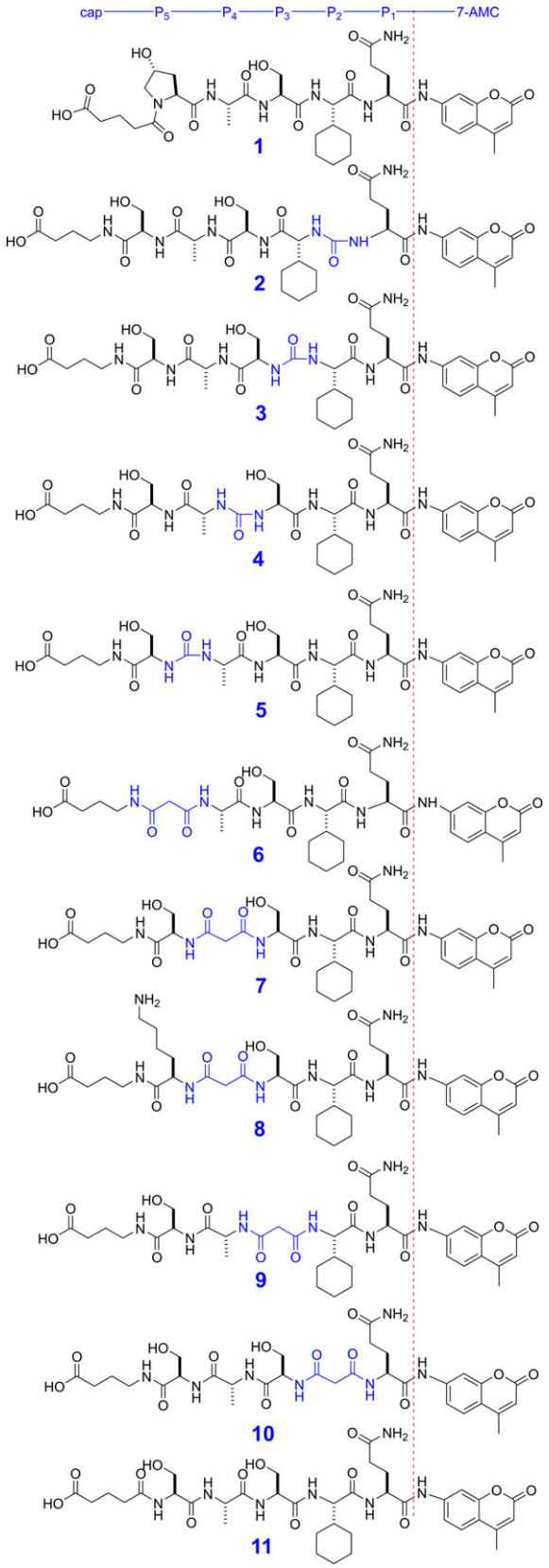
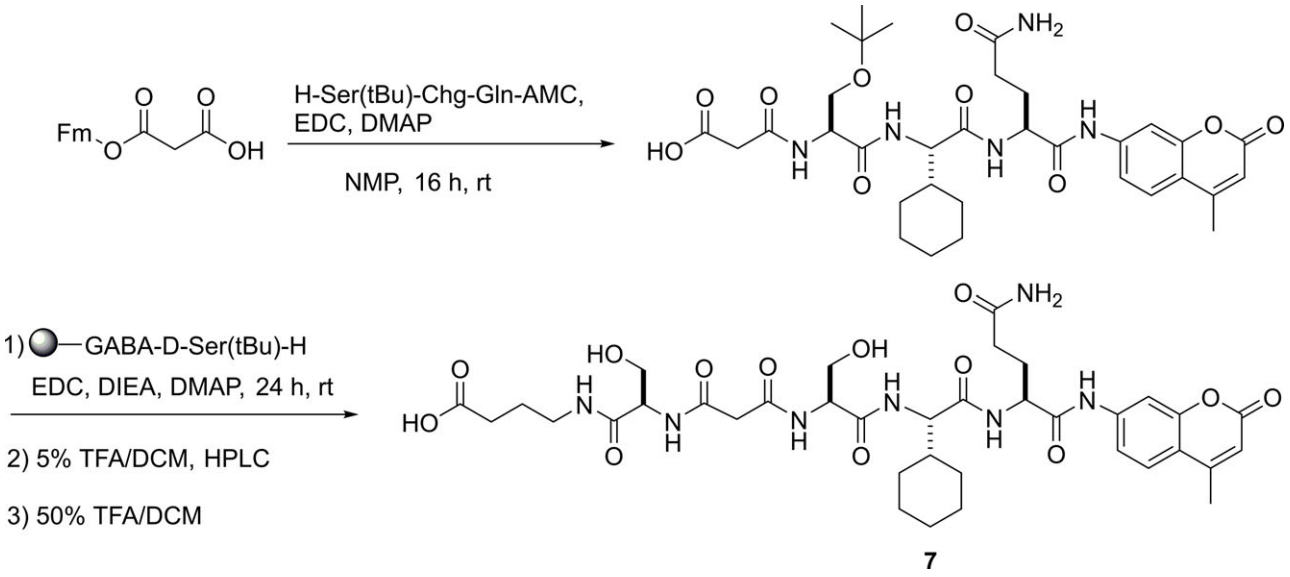


Figure 1: Structures of peptide–AMC conjugates designed and synthesized. Glutaryl-Hyp-Ala-Ser-Chg-Gln-AMC (**1**), GABA←D-Ser←D-Ala←D-Ser←D-Chg-ψ[NH-CO-NH]-Gln-AMC (**2**), GABA←D-Ser←D-Ala←D-Ser-ψ[NH-CO-NH]-Chg-Gln-AMC (**3**), GABA←D-Ser←D-Ala-ψ[NH-CO-NH]-Ser-Chg-Gln-AMC (**4**), GABA←D-Ser-ψ[NH-CO-NH]-Ala-Ser-Chg-Gln-AMC (**5**), GABA←mGly-Ala-Ser-Chg-Gln-AMC (**6**), GABA←D-Ser←mGly-Ser-Chg-Gln-AMC (**7**), GABA←D-Lys←mGly-Ser-Chg-Gln-AMC (**8**), GABA←D-Ser←D-Ala←mGly-Chg-Gln-AMC (**9**), GABA←D-Ser←D-Ala←D-Ser←mGly-Gln-AMC (**10**), Glutaryl-Ser-Ala-Ser-Chg-Gln-AMC (**11**).



Scheme 2: Synthesis of peptide–AMC conjugate 7.

butyl-protected conjugates were purified to homogeneity prior to TFA deprotection. To introduce the malonate linkage featured in the sequences of conjugates **6**–**10**, several conditions were explored. For example, attempts to couple the peptide, Ser(*t*Bu)-Chg-Gln-AMC to resin-GABA←D-Ser (*t*Bu)-H to generate conjugate **7** were unsuccessful when malonic acid was first activated with HBTU (or EDC) and reacted with resin-GABA←D-Ser(*t*Bu)-H. The lack of reactivity of activated malonic acid toward the amino group of resin-bound peptides was surprising because malonic acid could be effectively activated and trapped with benzylamine in solution. The acylation of sterically hindered alcohols through the ketene intermediate of malonic acid in the presence of dehydrating agents was previously reported (31). In our case, the OBt-activated ester, acyl urea, or ketene intermediate generated from malonic acid was anticipated to react more readily with amino groups as compared to sterically hindered alcohols. However, as the ketene intermediate is reactive and short-lived, its coupling with the amino group on solid phase may not be the major pathway. For example, the ketene could be trapped by EDC through a [4+2] cycloaddition mechanism similar to DCC (31). Optimized conditions involved activation of the fluorenylmethyl malonate mono-ester with EDC in the presence of 2.5 eq. DMAP to afford the mono-amide which was spontaneously deprotected upon prolonged exposure to DMAP (Scheme 2). Conjugates **6**, **8**–**10** were synthesized following this approach. Peptide segments were directly coupled to Gln-AMC to synthesize the final AMC conjugates. The Gln-AMC intermediate was obtained using our previously reported methodology for the convenient preparation of aminoacyl-AMC peptide conjugates (28) because standard coupling methods using HBTU, IPCF, DCC, or PyBOP afforded low product yields (often < 10%). Boc-Gln-AMC was generated in excellent yield (84%) by the amidation of the selenocarboxylate of Boc-Gln with 7-azido-4-methylcoumarin (AZD), followed by TFA-detprotection of the Boc-group at room temperature. As TFA can be readily activated by HBTU in subsequent steps and couple to Gln-AMC, residual TFA was removed using Amberlyst A-26.

To evaluate their PSA cleavage rates, all AMC conjugates were incubated with human PSA and fluorescence measured over a period of 3 h (Table 1 and Figure 2). Using the standard curve generated from known amounts of free 7-AMC, the rate of 7-AMC liberated upon PSA-mediated cleavage of the Gln-AMC was determined from the slope of the linear portions of the fluorescence counts–time curve for each conjugate; fluorescence increase was insignificant for Boc-Gln-AMC up to a concentration of 1 μM (data not shown). From the urea-containing AMC conjugates synthesized, conjugate **5** exhibited modest substrate specificity for PSA with a 7-AMC formation rate of 92 pmol/min/100 nmol PSA. As shown in Table 1, AMC conjugates **2**–**4** and **8**–**10** were poor substrates for PSA and did not generate any significant amount of fluorescence.

The results in Table 1 and Figure 2 indicate that, although amino acid side-chain orientation was maintained with D-amino acid substitution (as compared to the original peptide sequence of prodrug I), introducing urea in place of any amide bond between P1 and P5 may have disrupted critical peptide substrate binding interactions with PSA such as backbone H-bonding and side-chain interactions. Our findings support previous optimization studies which suggested that further amino acid substitution in P1-P4 for the optimized sequence of prodrug I may not be well tolerated and thereby compromise substrate specificity for PSA (25). Our observations are consistent with Ménez and colleagues analysis of key PSA substrate binding interactions

Table 1: PSA-mediated hydrolysis of AMC peptide conjugates

Entry	Peptide–AMC conjugate	7-AMC Fluorescence (counts/min)	PSA cleavage rate (pmol/min/100 nmol PSA)
1	Glutaryl-Hyp-Ala-Ser-Chg-Gln-AMC (**1**)	156	171
2	GABA←D-Ser←D-Ala←D-Ser←D-Chg-ψ[NH-CO-NH]-Gln-AMC (**2**)	<1	<1
3	GABA←D-Ser←D-Ala←D-Ser-ψ[NH-CO-NH]-Chg-Gln-AMC (**3**)	<1	<1
4	GABA←D-Ser←D-Ala-ψ[NH-CO-NH]-Ser-Chg-Gln-AMC (**4**)	<1	<1
5	GABA←D-Ser-ψ[NH-CO-NH]-Ala-Ser-Chg-Gln-AMC (**5**)	84	92
6	GABA←mGly-Ala-Ser-Chg-Gln-AMC (**6**)	205	225
7	GABA←D-Ser←mGly-Ser-Chg-Gln-AMC (**7**)	37	41
8	GABA←D-Lys←mGly-Ser-Chg-Gln-AMC (**8**)	<1	<1
9	GABA←D-Ser←D-Ala←mGly-Chg-Gln-AMC (**9**)	<1	<1
10	GABA←D-Ser←D-Ala←D-Ser←mGly-Gln-AMC (**10**)	<1	<1
11	Glutaryl-Ser-Ala-Ser-Chg-Gln-AMC (**11**)	320	351

Conditions: Each peptide conjugate (1 μM) was subject to human PSA (100 nM) in 50 mM Tris buffer, pH 8 containing 2 mM $CaCl_2$ and 0.1% Tween-20 in triplicate. Fluorescence was measured using a 335/460 (ex/em) filter over a period of 3 h. PSA cleavage rates were computed by taking the slopes of the linear portions of fluorescence counts–time curves (see Figure 2) and using a standard curve generated with known amounts of 7-AMC.

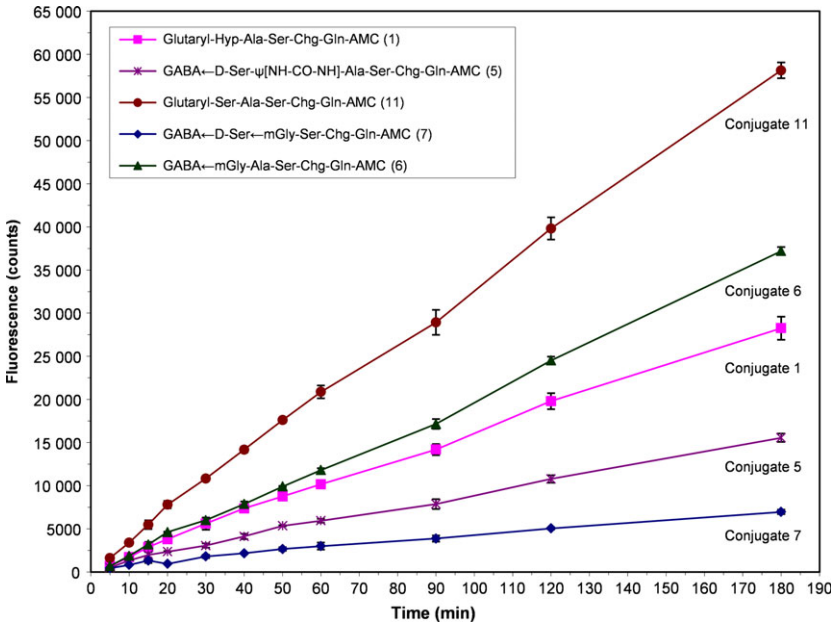


Figure 2: PSA-mediated hydrolysis of AMC peptide conjugates (PSA substrates).

using the first reported crystal structure of PSA in complex with the monoclonal antibody 8G8F5 and the fluorogenic semenogelin I-derived substrate Mu-Lys-Gly-Ile-Ser-Ser-Gln-Tyr-AFC. Their findings suggest tight binding interactions in the P1-P4 of the substrate within the active site of PSA (32); thus it is not surprising that modifications in this region of the substrate sequence translate to significant changes in binding affinity. We also explored the strategic insertion of malonate in place of one amino acid between D- and L-amino acids in the peptide sequence of prodrug I. Although P2-P5 substitution decreased PSA cleavage rate, it was gratifying to find that peptide conjugate **6** showed a significant improvement over conjugate **1** (representative sequence of prodrug I). Furthermore, AMC conjugate **6** exhibited the second fastest cleavage rate of 225 pmol/min/100 nmol PSA (Table 1, Figure 2). Introduction of urea linkages in the peptide sequence of AMC conjugate **1** increased the peptide chain length by one carbon atom, whereas malonate preserves the original sequence length but deleted the side chain of amino acid it replaces. Moreover, the malonate linkage imparts less rotational restriction to the peptide chain overall compared to urea.

The lower rotational conformation restriction is demonstrated with AMC conjugate **6** as the malonate linkage is moved farther away from P1; only modest effects on PSA cleavage rate were observed with the corresponding urea-containing AMC conjugate **5** (Figure 2). The fastest cleavage rate of 351 pmol/min/100 nmol PSA was observed with AMC conjugate **11**. It is possible that the bend and rigidity introduced by Hyp at P5 in conjugate **1** compromised optimal substrate binding. Moreover, for conjugate **11**, the hydroxyl of Ser may have contributed to additional stabilizing H-bonding interactions in the active site of PSA. Overall, we confirmed that PSA specificity was lost with modifications in P2-P5 as suggested by previous substrate optimization studies. Additionally, we showed that PSA cleavage rate was maintained or improved by up to 2-fold with the P5 substitutions.

To further assess the PSA specificity of the peptide sequences generated, we measured conjugate stability in mouse and human plasma (Table 2, Figure 3). The selected peptide conjugates were examined in mouse plasma because prodrugs generated from the peptide substrate with the highest PSA specificity could further be evaluated in nu/nu mouse PK/PD studies. Peptide conjugates with PSA cleavage rates > 1 pmol/min/100 nmol PSA were incubated with mouse or human plasma for 24 h and 7-AMC formation monitored by fluorescence. Although 7-AMC formation was insignificant during the first 3 h of incubation, Gln-AMC hydrolysis increased to detectable levels (up to 10 nM) at 24 h, suggesting that non-PSA-mediated hydrolysis did occur. The apparent delay in 7-AMC formation observed in Figure 4 is due to the fact that non-specific endopeptidase cleavage must first occur at scissile bonds other than the Gln-AMC before hydrolysis of the Gln-AMC bond via subsequent aminopeptidase action. Furthermore, all peptide conjugates exhibited greater non-PSA-mediated hydrolysis in mouse plasma than in human plasma (Figures 3 and 4) suggesting the involvement of multiple proteases or isoforms of the same protease in conjugate instability.

Conjugates **5** and **6** were most stable in mouse plasma indicating that urea and malonate substitutions in P4-P5 effectively mitigated hydrolysis mediated by a yet unidentified protease at scissile bond between P4 and P5. P5 substitution appears to block cleavage by this protease, the major contributor to non-PSA-mediated hydrolysis for conjugates **1**, **7**, and **11** in mouse plasma.

Table 2: Non-PSA-mediated hydrolysis of AMC peptide conjugates in mouse and human plasma at 24 h

Entry	Peptide–AMC conjugate	7-AMC generated in mouse plasma (nM)	7-AMC generated in human plasma (nM)
1	Glutaryl-Hyp-Ala-Ser-Chg-Gln-AMC (**1**)	9.7	1.1
2	GABA←D-Ser-ψ[NH-CO-NH]-Ala-Ser-Chg-Gln-AMC (**5**)	0	1.1
3	GABA←mGly-Ala-Ser-Chg-Gln-AMC (**6**)	4.7	1.4
4	GABA←D-Ser←mGly-Ser-Chg-Gln-AMC (**7**)	8.1	0.98
5	Glutaryl-Ser-Ala-Ser-Chg-Gln-AMC (**11**)	2.4	0.82

Conditions: Each peptide conjugate (1 μM) was incubated in mouse or human plasma. Fluorescence was measured using a 335/460 (ex/em) filter over a period of 24 h (see Figure 3).

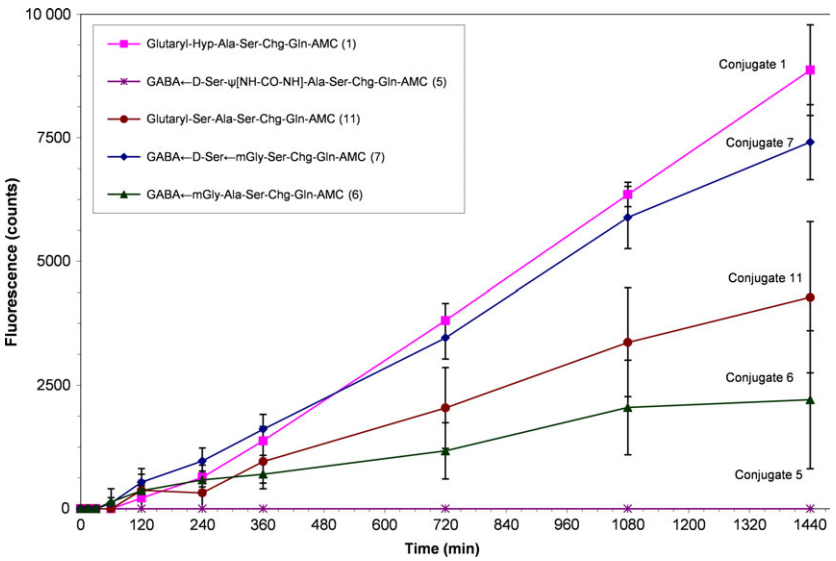


Figure 3: Stability of AMC peptide conjugates in mouse plasma.

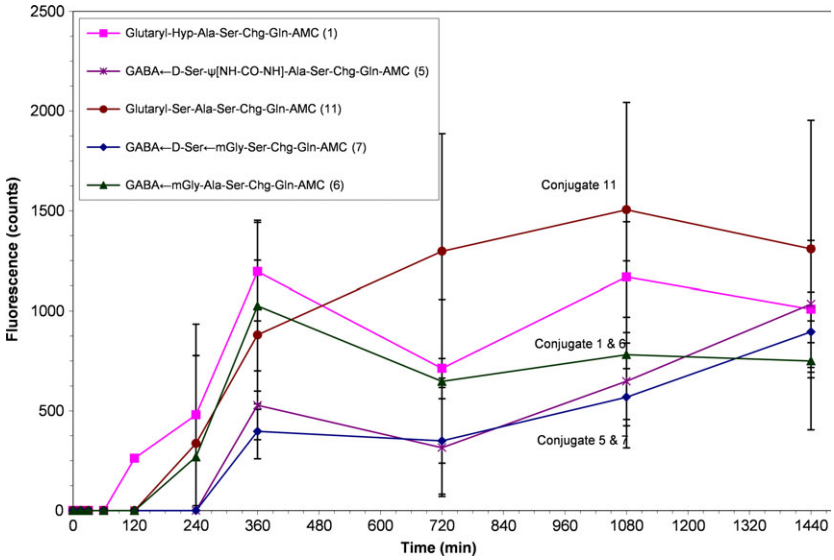


Figure 4: Stability of AMC peptide conjugates in human plasma.

Although conjugate **5** was completely stable in mouse plasma over 24 h, it did exhibit minor instability in human plasma. Moreover, non-PSA-mediated hydrolysis appears to be comparable for all conjugates in human plasma (Figure 4). Nevertheless, differences in hydrolysis rate for conjugates **5** and **6** in mouse plasma suggest that at least two additional proteases with different cleavage maps may be responsible for the residual hydrolysis observed for conjugate **5** in human plasma and conjugate **6** in both matrices. The protease(s) responsible for the non-PSA-mediated hydrolysis of the sequences examined herein and their PSA specificity are being further investigated in additional studies by coupling the optimized sequence GABA←mGly-Ala-Ser-Chg-Gln to select cytotoxic agents. It should be noted that the extent of hydrolysis of conjugate **6** in mouse plasma (2.42 nM eq. 7-AMC over 24 h) was much lower as compared to conjugate **1** (9.74 nM eq. AMC over 24 h), further suggesting that modifications at P5 also improved peptide stability.

In summary, we carried out D-amino acid substitutions between P1 and P4 and incorporated either urea or malonic acid linkage between the D- and L-amino acids to improve the specificity of the PSA substrate, glutaryl-Hyp-Ala-Ser-Chg-Gln. Our results suggest that modifications between P1 and P4 would adversely affect PSA specificity while substitutions at P5 improved PSA cleavage rate and resistance to non-PSA-mediated hydrolysis. To assess the applicability of the optimized sequences to peptide prodrug design, the PSA specificity of the optimized sequences (conjugates **6** and **11**) is being further investigated in additional studies. To further evaluate the utility of our newly designed sequences, glutaryl-Ser-Ala-Ser-Chg-Gln and GABA←mGly-Ala-Ser-Chg-Gln, as promoieties with improved PSA cleavage rate and stability to non-PSA-mediated metabolism, they will be coupled to doxorubicin or phosphoramide mustard using appropriate linkers. The resulting prodrugs will be assessed for their human PSA cleavage rate, PSA-mediated cytotoxicity and resistance to hydrolysis in biological matrices lacking active PSA.

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Conflict of Interest

The authors declare that they have no conflict of interest that would influence their objectivity.

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