Bioorganic & Medicinal Chemistry 24 (2016) 2697–2706

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design and synthesis of peptide conjugates of phosphoramide mustard as prodrugs activated by prostate-specific antigen

Xinghua Wu [a], Longqin Hu [a,b,*]

[a] Department of Medicinal Chemistry, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA
[b] The Cancer Institute of New Jersey, New Brunswick, NJ 08901, USA

ARTICLE INFO

Article history:
Received 18 February 2016
Revised 8 April 2016
Accepted 17 April 2016
Available online 19 April 2016

Keywords:
Phosphoramide mustard prodrug
Prostate-specific antigen
Site-specific activation
Self-immolative linker
Structure–activity relationship

ABSTRACT

A series of Glutaryl-Hyp-Ala-Ser-Chg-Gln-4-aminobenzyl phosphoramide mustard conjugates (**1a**–**e**) was designed and synthesized as potential prodrugs for site-specific activation by PSA in prostate cancer cells. All conjugates were found to be substrates of PSA with cleavage occurring between Gln and the *para*-aminobenzyl (PAB) linker. Structure–activity relationship studies on these conjugates indicated that introduction of electron-withdrawing fluorine(s) on the phenyl ring in the PAB linker uniformly improved the chemical stability of the conjugates while the position of substitution affected differently the self-immolative process of conjugates upon proteolysis. Introduction of a fluorine at *ortho* position to benzylic phosphoramide as in **1b** results in better stability of the conjugate prior to activation while maintaining its antiproliferative activity upon activation by PSA. The conjugate **1b** with 2-fluoro substitution was identified as a promising lead for further evaluation and optimization in the development of prostate cancer-targeted prodrugs.

1. Introduction

According to American Cancer Society, prostate cancer is the second most common cancer found in American men after skin cancer.[1] Current treatment options include surgery, radiation, hormone deprivation, and chemotherapy. After initial treatment, many patients may still progress to metastatic prostate cancer for which there is no effective therapy. Several recently approved drugs, such as abiraterone, enzalutamide, sipuleucel-T, docetaxel, cabazitaxel and Radium-223, are only providing an extended survival benefit of several months.[2–7] Thus, there is still an urgent need for new drugs that provide better antitumor selectivity and effectiveness.[8]

Prostate-specific drug design is an attractive approach to localize the cytotoxicity at the prostate tumor site.[8] Prostate-specific antigen (PSA) is among the most studied targets for the design of next generation of drugs against prostate cancer. PSA is an antigen with a serine protease activity and is over-expressed in the prostate tissue and prostate carcinoma.[9] The PSA in systemic circulation is enzymatically inactive due to formation of complexes with the protease inhibitors present in the blood, while its protease activity is confined to the prostate or prostate-derived cancer cells.[10,11] One approach to obtain PSA-targeted drugs is to use PSA's antigen feature: A PSA-specific antibody conjugated to a cytotoxic drug can be developed to improve cytotoxic selectivity that will be localized in the prostate cancer tissues.[12] Another approach is to use PSA's protease activity: A prodrug can be designed and activated only upon exposure to the enzymatically active PSA in the prostate cancer tissues, while the prodrug remains inactive in systemic circulation. Incorporation of PSA-specific peptides with various anticancer cytotoxic agents has been reported to exhibit selective cytotoxicity against PSA-expressing cancer cells.[8,13–22] Some dual-action prodrugs were also designed to increase uptake of drugs by tumor cells, like albumin-binding PSA-specific peptide drug conjugates[23,24] and PSA-specific peptide drug conjugates linked with an HER2-specific peptide.[25] In this paper, we describe our PSA-targeted prodrug approach to release cytotoxic phosphoramide mustard site-specifically in prostate tumor tissues by the proteolytic action of PSA.

2. Design and synthesis

By design, the PSA-specific peptide-drug conjugate remains inactive until it reaches the prostate-derived tumor tissues. Upon proteolytic cleavage by PSA, the cytotoxic drug is released locally in the microenvironment that surrounds prostate cancer cells. The anticancer selectivity is, therefore, achieved through this site-specific activation mechanism. PSA as an enzyme is sensitive to structural bulkiness at the cleavage site: Coupling the peptide

* Corresponding author. Tel.: +1 848 445 5291; fax: +1 732 445 6312.
E-mail address: LongHu@rutgers.edu (L. Hu).

http://dx.doi.org/10.1016/j.bmc.2016.04.035
0968-0896/

substrate directly with a bulky anticancer drug molecule, such as doxorubicin and paclitaxel, could lead to slow cleavage and thus insufficient activation by PSA. For better cleavage and activation by PSA, it is essential to insert a spacer between the cleavage site and the bulky anticancer drug. Thus, upon proteolytic cleavage by PSA, a spacer-drug conjugate is released in the prostate tumor tissue instead of the free drug.[26] The spacer-drug conjugate may still be in the form of a prodrug with no or low cytotoxicity, requiring a post PSA-cleavage process to restore the cytotoxicity of the parent drug. However, the potential problem is that the spacer-drug conjugate may enter into systemic circulation, and thus could be distributed to other tissues. The subsequent proteolysis of the spacer-drug conjugate by other proteases, however, can be non-specific, and the release of the active drug in the blood or other tissues could lead to undesired effects as a result of decreased selectivity.

Our approach was to select a small molecule cytotoxic agent that could be coupled to a PSA-specific peptide with or preferably without a spacer. We previously reported several PSA-specific peptide conjugates of 4-aminocyclophosphamide without a spacer, which showed good substrate activity toward PSA.[27] These conjugates, however, did not present satisfactory cytotoxicity toward PSA-secreting LNCaP prostate cancer cells. We rationalized that, after cleavage of the peptide by PSA, the immediately released 4-aminocyclophosphamide was less cytotoxic and needed to decompose to the phosphoramide mustard as the ultimate anti-proliferative agent. Our new strategy was then to insert a self-immolative linker, *para*-aminobenzyl (PAB), between a PSA-specific peptide and the phosphoramide mustard **3**. After proteolytic cleavage of the PSA-specific peptide followed by a spontaneous [1,6]-elimination process, the cytotoxic phosphoramide mustard **3** is released tracelessly in the prostate tumor tissues producing the desired alkylating activity as shown in Scheme 1. The substitution on the phenyl ring in the PAB linker could be used to modulate the stability of the prodrug, the rate of cleavage by PSA, and the rate of phosphoramide mustard release.

3. Results and discussion

3.1. Chemistry

In our previous work, we used three different PSA-specific peptides to couple with 4-aminocyclophosphamide.[27] The PSA proteolysis study showed that Glutaryl-Hyp-Ala-Ser-Chg-Gln-4-aminocyclophosphamide conjugate had the best PSA substrate activity. Therefore, we used this same peptide for our new approach for the traceless release of the phosphoramide mustard **3**. The structure–activity relationship (SAR) study was performed on the self-immolative PAB linker by introducing fluorine(s) to the aromatic ring. A novel synthetic methodology, selenocarboxylic acid/azide amidation,[28] was developed for the convergent synthesis of the peptide-Linker-phosphoramide mustard conjugates as shown in Scheme 2. This is because we needed to use a more stable intermediate like 4-azidobenzyl phosphoramide mustard **6** instead of the 4-aminobenzyl analog **2**, as the latter would spontaneously decompose and release the phosphoramide mustard **3** before it could be conjugated to an amino acid or peptide. We selected the conjugation of the first amino acid **7** to the azide **6** through selenocarboxylate/azide amidation, which gives the amino acid–PAB–phosphoramide mustard **8** upon deprotection. The last step in the synthesis is the fragment condensation of the remaining peptide with the amino acid–PAB–phosphoramide mustard **8** followed by any necessary deprotection to give the desired peptide–PAB–phosphoramide mustard conjugate **1**.

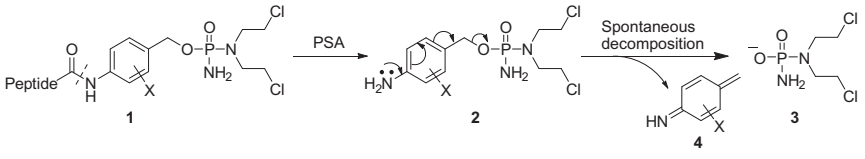
3.2. Synthesis of 4-azidobenzyl phosphoramide mustards 6a–e

The synthesis started from preparing various 4-azidaobenzyl alcohols **12a–e** as shown in Scheme 3. Briefly, the nitro group of **5b–d** was converted to the amino group by catalytic hydrogenation, affording nearly quantitative yields of the corresponding 4-aminobenzoic acids **10b–d**. 4-Aminobenzoic acid (**10a**) and 4-amino-2,3,5,6-tetrafluorobenzoic acid (**10e**) were commercially available. The amino group was then replaced by the azido group to afford 4-azidobenzoic acids **10a–e** in yields between 80% and 98%, following the standard diazotization/azido-displacement protocol. The benzoic acid group was converted to the HOBt activated ester and then selectively reduced by $NaBH_4$ in THF to benzyl alcohol without affecting the azido group, providing the corresponding alcohols **12a–e** in yields between 70% and 77%. The desired 4-azido-benzyl phosphoramides **6a–e** were synthesized via a 3-step one-pot procedure by first deprotonation of the 4-azidobenzyl alcohol with 1.0 equiv of *n*-BuLi at −78 °C followed by phosphorylation with bis-(2-chloroethyl)phosphoramidic dichloride and then ammonolysis with ammonia. The desired products were obtained in yields between 48% and 55%.

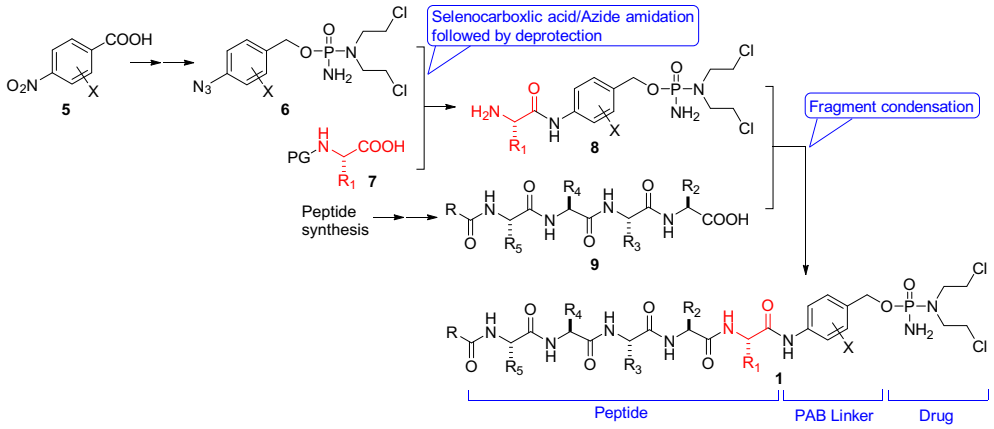
3.3. Synthesis of H-glutaminyl-4-aminobenzyl phosphoramide mustards 8a–e

We previously reported a one-pot selenocarboxylate/azide amidation procedure for direct coupling of a *N*-protected amino acid with an azide to form an amide.[28–31] Herein, the same selenocarboxylate/azide amidation methodology was used to synthesize N^{α}-protected glutaminyl-4-aminobenzyl phosphoramides. We first synthesized the Boc and Cbz protected analogs, namely, N^{α}-Boc-glutaminyl-4-aminobenzyl phosphoramide mustards and N^{α}-Cbz-glutaminyl-4-aminobenzyl phosphoramide mustards. However, the removal of Boc- and Cbz-protecting groups could not be done without affecting the phosphoryl functionality under the deprotection conditions tried. N^{α}-Fmoc-glutamine was not used because of its poor solubility in THF. N^{α}-Trifluoroacetyl glutamine (**7**) was soluble in THF and was readily prepared by the treatment of L-glutamine with *S*-ethyl trifluorothioacetate in 84% yield. Thus, N^{α}-TFA-glutamine was used to prepare H-glutaminyl-4-aminobenzyl phosphoramide mustards **8a–e** (Scheme 4).

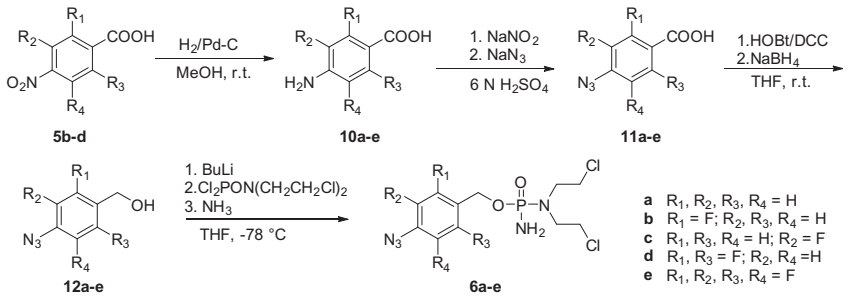
The synthesis of N^{α}-TFA-glutaminyl-4-aminobenzyl phosphoramides **13a–e** started from the selenocarboxylation of N^{α}-TFA-glutamine via the treatment of the mixed anhydride of N^{α}-TFA-glutamine with freshly prepared LiAlHSeH. The in situ generated N^{α}-TFA-glutaminyl selenocarboxylate was used directly to react with 4-azidobenzyl phosphoramide mustards **6a–e**, affording



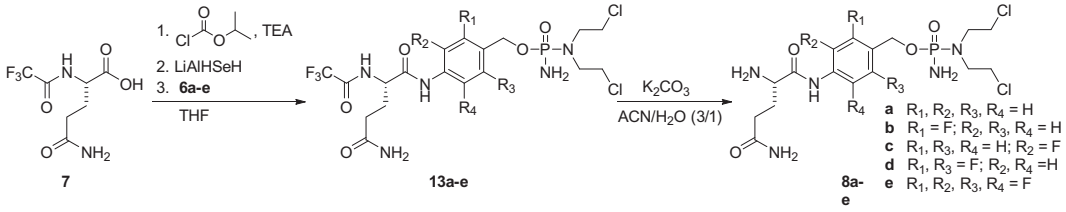
Scheme 1. Proposed activation of peptide–PAB–phosphoramide mustard conjugates by PSA proteolysis.



Scheme 2. Convergent synthesis of peptide-Linker-drug conjugates through selenocarboxylate/azide amidation methodology.



Scheme 3. Synthesis of 4-azido-benzyl phosphoramide mustards **6a–e**.

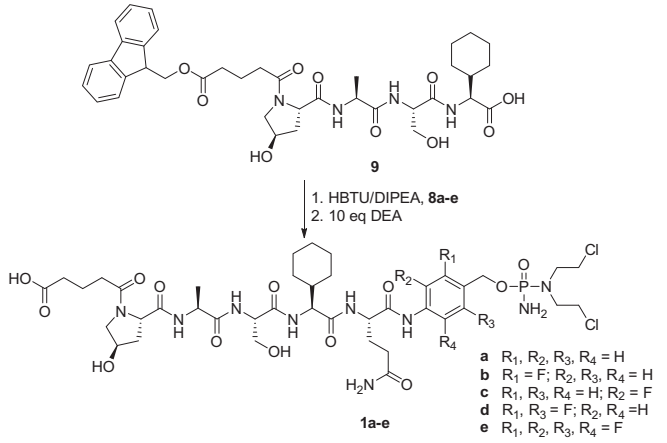


Scheme 4. Synthesis of H-glutaminyl-4-aminobenzyl phosphoramide mustards **8a–e**.

the desired products **13a–e** in yields between 20% and 87%. The TFA protecting group was smoothly removed by the treatment of N^{α}-TFA-glutamine conjugates **13a–e** with 4 equiv of K_2CO_3 in aqueous acetonitrile at room temperature. The resulting H-glutaminyl-4-aminobenzyl phosphoramide mustards **8a–e** were purified by flash column chromatography and their structures were confirmed by LC/MS and NMR.

3.4. Synthesis of Glutaryl-Hyp-Ala-Ser-Chg-Gln-4-aminobenzyl phosphoramide mustards 1a–e

The protected tetrapeptide, Fm-glutaryl-Hyp-Ala-Ser-Chg-OH (**9**), was synthesized on solid phase using Fmoc chemistry and Wang resin.[32] The tetrapeptide **9** was first activated by formation of the HOBt-activated ester using HBTU/DIPEA protocol, followed by amidation with the H-glutaminyl-4-aminobenzyl phosphoramide mustards **8a–e** in NMP (Scheme 5). The conjugated products were collected as white solids by precipitation from 5% aqueous $NaHCO_3$. The obtained conjugates were immediately dissolved in acetonitrile/methanol (50:50, v/v) and treated with 10 equiv of diethylamine at room temperature to remove the



Scheme 5. Synthesis of Glutaryl-Hyp-Ala-Ser-Chg-Gln-aminobenzyl phosphoramide mustards **1a–e**.

fluorenylmethyl protecting group of *N*-terminal glutarate. The desired peptide–PAB–phosphoramide mustard conjugates **1a–e** were purified in 28–40% yields by preparative HPLC.

3.5. PSA substrate activity of Glutaryl-Hyp-Ala-Ser-Chg-Gln-4-aminobenzyl phosphoramide mustards 1a–e

The peptidyl-4-aminobenzyl phosphoramide mustards **1a–e** were evaluated as substrates of PSA at an enzyme/substrate molar ratio of 1:100, while L-377,202, *N*-glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Doxorubicin, was used as a control. The stock solution of each substrate was prepared as a 10 mM solution in DMSO. The substrates were then incubated with 1% mol of PSA at 37 °C in 10 mM Tris/HCl buffer containing 0.01% Tween-20® at pH 8.0. Aliquots were withdrawn at various time intervals, quenched by acetonitrile (20%), and stored frozen prior to HPLC analysis.

All peptide-Linker-drug conjugates **1a–e** were cleaved by PSA in a time-dependent manner as observed by HPLC analysis (Fig. 1). The pentapeptide segment, Glutaryl-Hyp-Ala-Ser-Chg-Gln-OH, was confirmed by LC/MS, indicating that the proteolytic cleavage occurred after the glutamine residue. For the control substrate, L-377,202, the complete disappearance of the substrate was observed in about 2 h with a half-life of 30 min. For peptide-4-aminobenzyl phosphoramide mustards **1a–e**, the substrate concentration did not reach zero after 5-h incubation. This may be due to the inactivation of PSA by the released alkylating phosphoramide mustard **3** or the electrophilic quinonimine methides **4**.

3.6. Stability of Glutaryl-Hyp-Ala-Ser-Chg-Gln-4-aminobenzyl phosphoramide mustards 1a–e

Conjugates **1a–e** were incubated at 37 °C in 10 mM Tris/HCl buffer containing 0.01% TWEEN-20® at pH 8.0. Aliquots were withdrawn at various time intervals and stored frozen prior to HPLC analysis (Fig. 2). The half-lives were calculated based on the disappearance of the conjugates and summarized in Table 1: The conjugate **1a** had the shortest half-life of 46 h; Introduction of fluorine at the 2-, 2,6-, and 2,3,5,6-position of phenyl ring increased the stability of the peptide-Linker-drug conjugates (**1b**, **1d**, **1e**) by about 2, 3 and 4 folds, respectively. Introduction of fluorine at the 3-position of phenyl ring increased the stability of **1c** by about 10 folds. The degradation presumably goes through the hydrolysis of the P–O bond or the 1,6-elimination process, shown in Scheme 6. To understand the degradation pathway, 4-nitrobenzyl phosphoramide mustard as the control was incubated in the same buffer at 37 °C. After 3 days of incubation, there was no detectable degradation of 4-nitrobenzyl phosphoramide mustard, indicating that the phosphoryloxy functionality was stable toward hydrolysis under the same conditions. Therefore, the degradation of conjugates was most likely due to the 1,6-elimination. Introduction of electron-withdrawing fluorine atom(s) decreased the electron density of the aromatic ring and thus slowed down the 1,6-elimination process, resulting in enhanced stability of the fluoro-substituted peptide-Linker-drug conjugates. It is clear that the 1,6-elimination process is facilitated by the electron density of the aromatic ring: The rate of 1,6-elimination increases with increasing electron density of aromatic ring in the PAB linker. This is consistent with our observation that reduction of 4-nitrobenzyl phosphoramide mustard leads to elimination and release of phosphoramide mustard.[33,34]

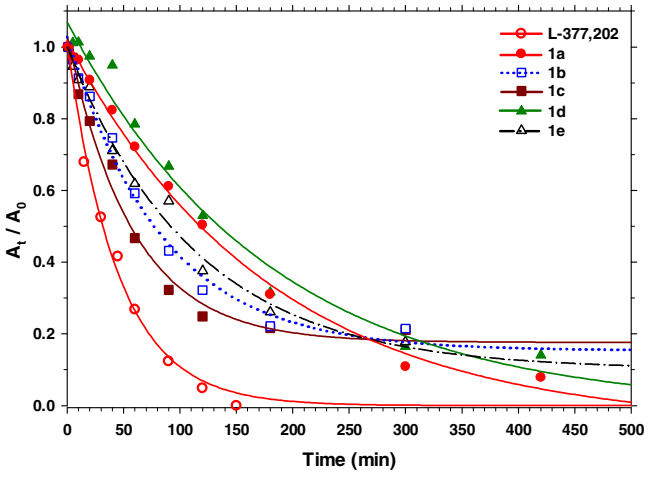


Figure 1. The disappearance of peptide-4-aminobenzyl phosphoramide conjugates **1a–e** (0.16 mM) in the presence of PSA in 50 mM Tris/HCl buffer, pH 8.0 containing 10 mM $CaCl_2$ and 0.1% Tween-20 at 37 °C.

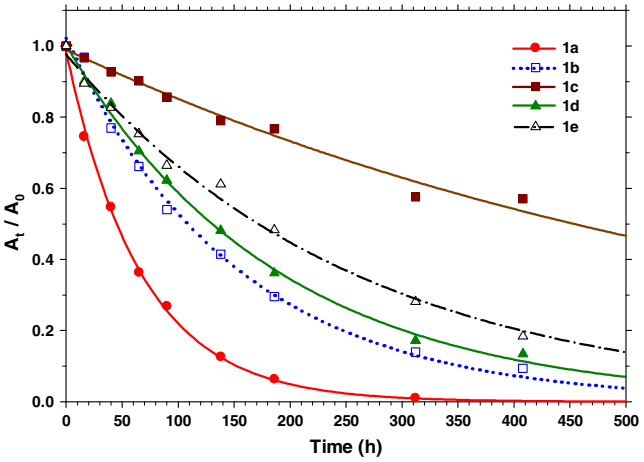


Figure 2. Stability of peptide-4-aminobenzyl phosphoramide conjugates **1a–e** in 50 mM Tris/HCl buffer, pH 8.0 containing 10 mM $CaCl_2$ and 0.1% Tween-20 at 37 °C.

Table 1
Stability, PSA cleavage, and antiproliferative activity of the peptide-conjugates

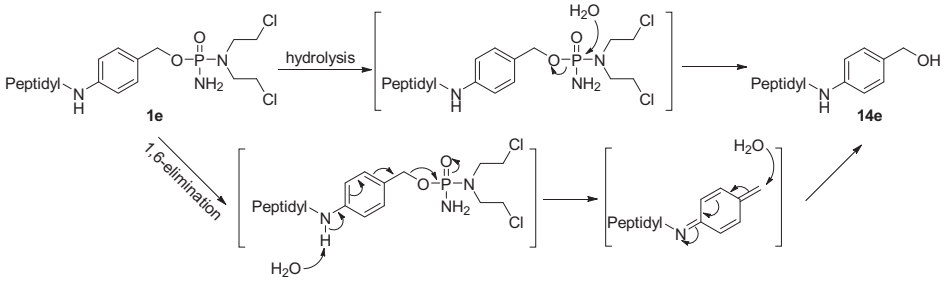
Prodrugs	$t_{1/2}$[a] (h)		IC_{50}[b] (μM)	
	PSA^-	PSA^+	DU145 PSA^-	LNCaP PSA^+
L-377,202	NA	0.6	>100	1.1
1a	46	2.1	35	11.0
1b (2-F)	105	0.93	>100	5.3
1c (3-F)	465	0.65	>100	29.0
1d (2,6-diF)	131	1.7	>100	20.0
1e (2,3,5,6-tetraF)	178	1.1	>100	>100

[a] Stability and substrate activity of conjugates in 50 mM Tris Buffer, pH 8, and 37 °C. Each conjugate (100 μM) was incubated in 50 mM Tris/HCl buffer, pH 8.0 containing 10 mM $CaCl_2$ and 0.1% Tween-20 at 37 °C.

[b] PSA-producing LNCaP and non-PSA-producing DU145 cell lines were treated with conjugates at concentrations ranging from 0.01 to 100 μM for 72 h and cell viability determined using the MTT assay.

3.7. Antiproliferative activity of Glutaryl-Hyp-Ala-Ser-Chg-Gln-4-aminobenzyl phosphoramide mustards 1a–e

The antiproliferative activities of peptidyl-4-aminobenzyl phosphoramide mustards (**1a–e**) were evaluated using the standard MTT assay in LNCaP cells that express PSA and DU145 cells that do not express PSA with L-377,202 used as a positive control. The IC_{50} values were calculated and summarized in Table 1. DU145 cell line was not sensitive to L-377,202 and conjugates **1b–e**. The conjugate **1a**, however, was cytotoxic towards DU145 cells with an IC_{50} of 35 μM, presumably due to its chemical instability leading to the release of phosphoramide mustard: this is consistent with its relatively shorter half-life of 46 h in the buffer; and after 72 h of incubation under cell culture conditions prior to the



Scheme 6. Proposed degradation pathways of peptide-Linker-drug conjugate **1e**.

MTT assay, over 60% of **1a** had already decomposed and released the cytotoxic phosphoramide mustard. All peptidyl-4-aminobenzyl phosphoramide mustard conjugates **1a–e** were cytotoxic towards the PSA-secreting LNCaP cell line with the 2-fluoro substituted conjugate **1b** showing the highest cytotoxicity against LNCaP cells (IC_{50} = 5.3 μM), followed by **1a** (IC_{50} = 11.0 μM), **1d** (IC_{50} = 20.0 - μM), and **1c** (IC_{50} = 29 μM).

Compared to **1a**, the 3-fluoro substituted conjugate **1c** showed about 3-fold decreased cytotoxicity towards PSA-expressing LNCaP cells; the 2,6-difluoro substituted conjugate **1d** showed about 2-fold decreased cytotoxicity; and the 2,3,5,6-tetrafluoro substituted conjugate **1e** did not show cytotoxicity at the highest assay concentration of 100 μM. These results suggest that the rate of self-immolative 1,6-elimination process decreased because of the decreased electron density of the PAB linker caused by the inductive effects of electron-withdrawing fluorine(s). The lack of cytotoxicity of **1e** was because the 1,6-elimination process was completely halted by the introduction of the four electron-withdrawing fluorine atoms, and thus the release of phosphoramide mustard was blocked. To demonstrate our hypothesis, 4-azido-2,3,5,6-tetrafluorobenzyl phosphoramide mustard (**6e**) was reduced to the corresponding amine by catalytic hydrogenation. As expected, there was no 1,6-elimination occurring after reduced to 4-amino-2,3,5,6-tetrafluorobenzyl phosphoramide mustard that was isolated as a stable product. However, the cytotoxicity of 2-fluoro substituted conjugate **1b** was improved by 2 folds. We postulated that the *ortho*-fluorine of **1b** facilitated the cleavage of benzylic C–O bond through a 1,6-elimination process via the resonance effect of fluorine. Both the chemical stability of the conjugates and the release of phosphoramide mustard from the conjugates upon proteolysis were impacted by the self-immolative PAB linker through the electron-withdrawing inductive effect as well as the electron-donating resonance effect of fluorine substitutions.

4. Conclusions

We designed, synthesized and evaluated a series of peptidyl-4-aminobenzyl phosphoramide mustard conjugates as prodrugs for site-specific activation by PSA in prostate cancer cells. The design principle is based on proteolytic cleavage of the PSA-specific peptide to activate the self-immolative 4-aminobenzyl (PAB) linker that releases cytotoxic phosphoramide mustard via a 1,6-elimination process. All conjugates were found to be the substrates of PSA. The proteolytic cleavage by PSA occurred between Gln and the PAB linker. In vitro antiproliferative activity assay of these conjugates showed promising cytotoxicity and selectivity. Structure–activity relationship studies on these conjugates indicated that introducing electron-withdrawing fluorine(s) on the phenyl ring in the PAB linker improved the stabilities of the conjugates. However, introducing a fluorine atom at the *ortho* position to benzylic phosphoramide was crucial to maintain the conjugate's cytotoxicity. Introduction of a fluorine atom at the *meta* position led to significant decrease in cytotoxicity. Decreasing electron density of the PAB linker would improve the stability of the conjugate but adversely affect the release of cytotoxic phosphoramide mustard from the conjugates upon proteolysis. The conjugate **1b** was identified as a promising lead for further evaluation and optimization, representing a balance of the desired chemical stability, PSA substrate activity, antiproliferative activity, and selectivity.

5. Experimental section

5.1. General methods

Moisture-sensitive reactions were performed in flame-dried glassware under a positive pressure of nitrogen or argon. Air and moisture-sensitive materials were transferred by syringe or cannula under an argon atmosphere. Except for redistillation prior to use, solvents were either ACS reagent grade or HPLC grade. Unless otherwise stated, all reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using 0.25 mm Whatman precoated silica gel plates and/or LC–MS. Flash column chromatography (FCC) was conducted on a Teledyne ISCO Combi-Flash Companion Automated Flash Chromatographic System (Teledyne Technologies, Thousand Oaks, CA) using pre-packed silica gel columns. TLC plates were visualized using either 7% (w/w) ethanolic phosphomolybdic acid or 1% (w/w) aqueous potassium permanganate containing 1% (w/w) $NaHCO_3$. Yield refers to chromatographically and spectroscopically (^{1}H NMR) homogeneous material, unless otherwise noted.

Infrared spectra were recorded with Perkin–Elmer model 1600 series FTIR spectrometer using polystyrene as an external standard. Infrared absorbance is reported in reciprocal centimeters (cm^{-1}) with broad signals denoted by br. ^{1}H NMR spectra were recorded on Varian Gemini 200, 300, or 400 MHz spectrometers as indicated at ambient temperature and calibrated using residual undeuterated solvents as the internal reference. ^{13}C NMR spectra were recorded at 50 MHz on a Varian Gemini 200 MHz spectrometer or 75 MHz on a Varian Gemini 300 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to $CDCl_3$ (δ 7.27 ppm for ^{1}H and 77.2 ppm for ^{13}C) or CD_3OD (δ 3.31 ppm for ^{1}H and 49.2 for ^{13}C). Coupling constants (J values) are given in hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad. High resolution mass spectral (HRMS) data were obtained from the University of Kansas Mass Spectrometry Laboratory (Lawrence, KS).

Purified PSA (99%, 1.37 mg protein/ml) was purchased from Fitzgerald (North Acton, MA). Cell growth medium was prepared by adding L-glutamine (2 mM), fetal bovine serum (10%), penicillin G (100 units/mL) and streptomycin sulfate (100 units/mL) to phenol red-containing RPMI 1640.

Moisture-sensitive reactions were performed in oven-dried glassware under a positive pressure of argon or nitrogen. Air and moisture sensitive materials were transferred via syringe or cannula under argon or nitrogen atmosphere. Solvents were either ACS reagent grade or HPLC grade and used directly without further purification unless otherwise stated: THF and dichloromethane were dried by pressure filtration under nitrogen through activated alumina; *N*,*N*-dimethylformamide was dried and redistilled over calcium hydride. Reagents purchased were ACS grade or better and used without further purification unless otherwise stated: the concentration of *n*-BuLi in hexane was measured by using diphenylacetic acid protocol before use. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using Whatman polymer-backed F_{254} silica gel plates and/or Shimadzu 2010 LC–MS system. Flash column chromatography was performed using silica gel (Merck 230–400 mesh) or using a Teledyne ISCO CombiFlash Companion Automated Flash Chromatographic System with prepacked silica gel columns. Yields were based on the chromatographically pure compounds. Melting points were determined on a Mel-Temp capillary apparatus and are uncorrected. Infrared spectra were recorded with Thermo-Nicolet Avatar 360 FTR spectrometer and the absorbance is reported in reciprocal centimeters (cm^{-1}). All ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer at ambient temperature and calibrated using residual undeuterated solvents as the internal reference. The following abbreviations were used to indicate the multiplicities: s = singlet; d = doublet; dd = doublets of doublet; dt = doublets of triplet; t = triplet; td = triplets of doublet; q = quartet; m = multiplet; br = broad.

The reactions were monitored using Schimadzu LCMS-2010 system equipped with a Chromolith SpeedROD RP-18e column (50 × 4.6 mm). Solvent A was 0.1% HCOOH/H_2O and solvent B was 0.1% HCOOH/CH_3CN. The gradient was 10–90% of solvent B over 10 min with a flow rate at 1 mL/min. The kinetic studies were performed on a Waters symmetry C_{18} column (3.5 μm, 4.6 × 150 mm) at 1 mL/min with a gradient of 10–80% of acetonitrile containing 0.1% trifluoroacetic acid over 15 min on a HP1090 system. The peptide–aminoarylmethylphosphoramide mustards were purified using Gilson automatic preparative HPLC system equipped with C_{18} column (250 × 20 mm) at 12 mL/min with a gradient of 30–90% of acetonitrile containing 0.1% trifluoroacetic acid over 10 min.

5.2. Solid-phase synthesis of peptide

The peptide, Fm-glutaryl-Hyp-Ala-Ser-Chg-OH, was synthesized using Fmoc chemistry on 4-hydroxymethylphenoxy (WANG-type HMP) resin purchased from Advanced Chemtech. N^{α}-Fmoc protected L-amino acids and coupling reagents were purchased from Advanced Chemtech. Side-chain protection was Ser(*t*-Bu) and Lys(Cl-Z). First amino acid was attached to the resin via its C-terminal using HOBt/DMAP/DIC protocol. All N^{α}-Fmoc protected L-amino acids were used as 3-fold excess amount for each coupling in NMP. Following completion of the assembly on the resin support, the Fmoc protecting group was removed via the standard 20% piperidine/NMP protocol followed by washing with NMP 5 times and introduction of N-terminal capping group using acetic anhydride. Deprotection and cleavage of the peptide from the resin support were performed using 90% TFA/CH_2Cl_2. The benzyl ester and fluorenylmethyl ester (OFm) were not affected under these conditions. After removal of solvents under reduced pressure, the peptide was purified by preparative-HPLC on reversed phase C18 column. A linear gradient was used from 10% solvent A to 90% solvent B with a flow rate of 12 mL/min, where solvent A was 0.1% TFA/H_2O and solvent B was 0.1% TFA/CH_3CN. The UV detection wavelength was set at 220 nm. Homogeneous fractions containing the desired product were pooled and lyophilized to afford the peptide as a white powder. The purity and identity were confirmed by HPLC and LC/MS. The purity of all intermediates was >90% and the purity of final target prodrug conjugates was >95%.

5.3. Synthesis of fluorinated 4-aminobenzoic acids 10b–d

To a solution of **5b–d** (1.0 mmol) in methanol (20 mL) was added 10% Pd–C (10 mg, 0.01 mmol). The resulting mixture was stirred at room temperature under a hydrogen balloon for 16 h and filtered through a Celite 545 pad. The filtrate was concentrated to dryness to afford the corresponding aminobenzoic acid **10b–d** that was used directly for the next step without purification.

5.4. Synthesis of fluorinated 4-azidobenzoic acids 11a–e

To an ice-cold suspension of **10a–e** (1.0 mmol) in 6 N H_2SO_4 (20 mL) was added dropwise an aqueous solution of sodium nitrite (83 mg, 1.2 mmol) over 10 min. The resulting mixture was stirred at 0 °C for additional 30 min. An aqueous solution of sodium azide (98 mg, 1.5 mmol) was added dropwise to the above mixture. The reaction was then carried out at room temperature for 1 h. The reaction mixture was extracted with EtOAc (3 × 30 mL). The combined EtOAc phase was washed with water and brine, and dried over Na_2SO_4. After removal of Na_2SO_4 via filtration, the filtrate was concentrated to dryness. The crude product was purified by flash column chromatography (hexane to 50% EtOAc/hexane) to afford the desired azidobenzoic acid **11a–e**.

5.4.1. 4-Azido-benzoic acid (11a)

A white solid (163 mg, 98%); ^1H NMR (200 MHz, DMSO-d_6): δ 7.96 (d, 2H, J = 8.4 Hz), 7.17 (d, 2H, J = 8.4 Hz); ^{13}C NMR (50 MHz, DMSO-d_6): 167.5, 143.4, 131.9, 128.7, 119.7; MS (ESI^-): m/z (intensity), 162.0 ($[M-H]^-$, 100%).

5.4.2. 4-Azido-2-fluorobenzoic acid (11b)

A white solid (163 mg, 83%); ^1H NMR (200 MHz, $CDCl_3$): δ 7.78 (dd, 1H, J = 8.6 Hz, J = 8.4 Hz), 6.71 (d, 1H, dd, 1H, J = 8.4 Hz, J = 2.2 Hz), 6.62 (dd, 1H, J = 11.4 Hz, J = 2.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): 164.0, 162.8 (d, J = 250.0 Hz), 146.2 (d, J = 10.0 Hz), 133.6, 114.2 (d, J = 3.0 Hz), 107.3 (d, J = 26.0 Hz). MS (ESI^-): m/z (intensity), 180.1 ($[M-H]^-$, 100%).

5.4.3. 4-Azido-3-fluorobenzoic acid (11c)

A white solid (163 mg, 90%); ^1H NMR (200 MHz, CD_3OD): δ 7.77 (d, 1H, J = 7.8 Hz), 7.69 (d, 1H, J = 11.4 Hz), 7.10 (dd, 1H, J = 7.8 Hz, J = 7.2 Hz); ^{13}C NMR (50 MHz, CD_3OD): 167.5, 154.9 (d, J = 250.0 Hz), 133.3 (d, J = 10.0 Hz), 129.1 (d, J = 6.0 Hz), 127.2 (d, J = 3.0 Hz), 121.3, 118.4 (d, J = 20.0 Hz); IR (KBr): 2146, 1691, 1432, 1298 cm^{-1}; MS (ESI^-): m/z (intensity), 180.1 ($[M-H]^-$, 100%).

5.4.4. 4-Azido-2,6-difluorobenzoic acid (11c)

A white solid (163 mg, 83%); ^1H NMR (200 MHz, Acetone-d_6): δ 6.87 (d, 2H, J = 9.2 Hz); ^{13}C NMR (50 MHz, Acetone-d_6): 171.9, 162.1 (dd, J = 255.0 Hz, J = 8.4 Hz), 161.4, 146.0 (t, J = 12.9 Hz), 108.2 (t, J = 19.8 Hz); IR (KBr): 3432, 2119, 1738, 1636, 1312, 1280 cm^{-1}; MS (ESI^-): m/z (intensity), 154.0 ($[M-COOH]^-$, 100%).

5.4.5. 4-Azido-2,3,5,6-tetrafluorobenzoic acid (11e)

A yellow solid (188 mg, 80%); ^1H NMR (200 MHz, $CDCl_3$): δ 10.34 (br s, –COOH); ^{13}C NMR (50 MHz, $CDCl_3$): 164.3, 146.0 (dm, J = 265.0 Hz), 140.7 (dm, J = 265.0 Hz), 124.8 (t. J = 13.0 Hz), 106. (t, J = 13.0 Hz), 51.7; IR (film): 3388, 2123, 1652, 1489, 1241, 1002, 945 cm^{-1}. MS (ESI^-): m/z (intensity), 234.0 ($[M-H]^-$, 100%).

5.5. Synthesis of fluorinated 4-azido-benzyl alcohols 12a–e

To a solution of **12a–e** (1.0 mmol) in THF (10 mL) was added HOBt (135 mg, 1.0 mmol) and DCC (227 mg, 1.1 mmol). The resulting mixture was stirred at room temperature for 1 h. The white precipitates were filtered off and rinsed with THF (2 × 5 mL). The filtrate was added dropwise to a suspension of $NaBH_4$ (38 mg, 1.0 mmol) in THF (10 mL) over 30 min. The reaction was carried out at room temperature for additional 1.5 h and quenched by adding 10 ml 1.0 N HCl. After evaporation of THF under reduced pressure, the remaining aqueous solution was extracted EtOAc (3 × 20 mL). The combined EtOAc phase was washed with water and brine, and dried over Na_2SO_4. After removal of Na_2SO_4 via filtration, the filtrate was concentrated to dryness. The crude product was purified by flash column chromatography (hexane to 50% EtOAc/hexane) to afford the desired alcohol **12a–e**.

5.5.1. 4-Azido-benzyl alcohol (12a)

A yellow solid (115 mg, 77%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.31 (d, J = 8.4 Hz), 7.00 (d, J = 8.4 Hz), 4.61 (s, 2H), 2.81 (br s, –OH); ^{13}C NMR (50 MHz, $CDCl_3$): 139.4, 137.7, 128.6, 119.2, 64.6; IR (film): 3332, 2107, 1506, 1287 cm^{-1}.

5.5.2. 4-Azido-2-fluorobenzyl alcohol (12b)

Yellow oil (124 mg, 74%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.27 (t, 1H, J = 8.0 Hz), 6.73 (dd, 1H, J = 8.0 Hz, J = 2.2 Hz), 6.63 (dd, 1H, J = 10.6 Hz, J = 2.2 Hz), 4.58 (s, 2H), 3.63 (br s, –OH); ^{13}C NMR (50 MHz, $CDCl_3$): 160.7 (d, J = 250.0 Hz), 140.9 (d, J = 10.0 Hz), 130.2 (d, J = 6.0 Hz), 124.2 (d, J = 15.0 Hz), 114.5 (d, J = 3.0 Hz), 106.2 (d, J = 25.0 Hz), 58.1 (d, J = 4.0 Hz); IR (film): 3321, 2116, 1621, 1583, 1502, 1301, 1212 cm^{-1}.

5.5.3. 4-Azido-3-fluorobenzyl alcohol (12c)

Yellow oil (117 mg, 70%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.94–7.08 (m, 3H), 4.57 (s, 2H), 2.59 (br s, –OH); ^{13}C NMR (50 MHz, $CDCl_3$): 154.7 (d, J = 250.0 Hz), 139.2 (d, J = 6.0 Hz), 126.8 (d, J = 12.0 Hz), 122.9 (d, J = 4.0 Hz), 120.9, 115.0 (d, J = 20.0 Hz), 63.8 (d, J = 1.0 Hz); IR (film): 3334, 2134, 2096, 1508, 1315 cm^{-1}.

5.5.4. 4-Azido-2,6-difluorobenzyl alcohol (12d)

A white solid (131 mg, 71%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.56 (d, 2H, J = 7.6 Hz), 4.66 (s, 2H), 2.39 (br s, –OH); ^{13}C NMR (50 MHz, $CDCl_3$): 162.1 (dd, J = 250.0 Hz, J = 10.0 Hz), 142.5 (t, J = 13.0 Hz), 113.0 (t, J = 20.0 Hz), 102.6 (d, J = 30.0 Hz), 52.4 (t, J = 4.0 Hz); IR (film): 3330, 2116, 1641, 1587, 1441, 1237, 1060, 1026 cm^{-1}.

5.5.5. 4-Azido-2,3,5,6-tetrafluorobenzyl alcohol (12e)

A white solid (155 mg, 70%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 4.71 (s, 2H), 2.85 (br s, –OH); ^{13}C NMR (50 MHz, $CDCl_3$): 144.6 (dm, J = 245.0 Hz), 139.7 (dm, J = 245.0 Hz), 119.2 (t. J = 20.0), 113.3 (t, J = 20.0 Hz), 51.7; IR (film): 3388, 2123, 1652, 1489, 1241, 1002, 945 cm^{-1}.

5.6. Synthesis of fluorinated 4-azido-benzyl phosphoramide mustards 6a–e

To a solution of alcohol **12a–e** (5.0 mmol) in anhydrous THF (25 mL) was added a solution of BuLi in cyclohexane (2.0 M, 2.75 mL) at −78 °C. After 20 min, the above solution was transferred to a pre-cooled solution of bis(2-chloroethyl)phosphoramidic dichloride (1.43 g, 5.5 mmol) in THF (25 mL) at −78 °C via cannula. The resulting mixture was stirred at −78 °C for 5 h followed by bubbling with ammonia for 10 min. The reaction mixture was allowed to gradually warm up to room temperature over 2 h. After removal of THF via distillation under reduced pressure, the residue was suspended in saturated aqueous sodium bicarbonate (50 mL) followed by extraction with dichloromethane (3 × 50 mL). The combined organic phase was washed with water (50 mL) and saturated brine (50 mL), and dried over Na_2SO_4. After filtration off Na_2SO_4, the filtrate was concentrated to dryness under reduced pressure. The crude product was purified by flash column chromatography (dichloromethane to 5% methanol in dichloromethane) to afford the desired product **6a–e**.

5.6.1. 4-Azido-benzyl phosphoramide mustard (6a)

A yellow semi-solid (0.965 g, 55%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.45 (d, 2H, J = 8.4 Hz), 7.09 (d, 2H, J = 8.4 Hz), 4.97 (d, 2H, J = 7.4 Hz), 3.61–3.69 (m, 4H), 3.33–3.48 (m, 4H); ^{13}C NMR (50 MHz, CD_3OD): 139.5, 133.3 (d, J = 8.0 Hz), 128.6, 118.2, 65.7 (d, J = 5.0 Hz), 48.8 (d, J = 5.0 Hz), 41.2; IR (film): 3415, 1655, 1437, 1407, 1315, 1022, 953 cm^{-1}; MS (ESI$^+$): m/z (intensity), 352.1 ([M+H]$^+$, 100%), 354.1 ([M+H]$^+$ + 2, 65%), 356.1 ([M+H]$^+$ + 4, 10%), 393.2 ([M+H]$^+$ + CH_3CN, 20%), 395.2 ([M+H]$^+$ + 2 + CH_3CN, 13%), 397.2 ([M+H]$^+$ + 4 + CH_3CN, 2%).

5.6.2. 4-Azido-2-fluorobenzyl phosphoramide mustard (6b)

A yellow semi-solid (0.959 g, 52%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.24 (t, 1H, J = 8.0 Hz), 6.63 (dd, 1H, J = 2.2 Hz, J = 8.0 Hz), 6.54 (dd, 1H, J = 2.2 Hz, J = 8.0 Hz), 4.79 (d, 2H, J = 7.2 Hz), 3.28–3.45 (m, 4H), 3.13–3.26 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$): 161.2 (d, J = 248.9 Hz), 142.2 (d, J = 9.9 Hz), 131.5 (d, J = 5.4 Hz), 120.2 (dd, J = 15.0 Hz, J = 8.0 Hz), 114.8 (d, J = 3.4 Hz), 106.6 (d, J = 25.0 Hz), 60.7 (d, J = 4.1 Hz), 49.1 (d, J = 5.0 Hz), 42.4; IR (film): 3431, 2118, 1621, 1505, 1303, 1214, 1091, 1012, 980 cm^{-1}; MS (ESI$^+$): m/z (intensity), 370.1 ([M+H]$^+$, 100%), 372.1 ([M+H]$^+$ + 2, 65%), 374.1 ([M+H]$^+$ + 4, 10%), 411.1 ([M+H]$^+$ + CH_3CN, 20%), 413.1 ([M+H]$^+$ + 2 + CH_3CN, 13%), 415.1 ([M+H]$^+$ + 4 + CH_3CN, 2%).

5.6.3. 4-Azido-3-fluorobenzyl phosphoramide mustard (6c)

A yellow semi-solid (0.959 g, 52%); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.06–7.25 (m, 3H), 5.00 (dd, 2H, J = 7.8 Hz, J = 4.0 Hz), 3.66–3.73 (m, 4H), 3.43–3.55 (m, 4H); ^{13}C NMR (50 MHz, $CDCl_3$): 154.3 (d, J = 248.5 Hz), 134.7 (t, J = 7.5 Hz), 127.4 (d, J = 10.0 Hz), 123.7 (d, J = 3.4 Hz), 120.8, 115.7 (d, J = 19.7 Hz), 65.5 (d, J = 3.1 Hz), 48.9 (d, J = 4.6 Hz), 42.3; IR (film): 3424, 2134, 2099, 1643, 1509, 1218 cm^{-1}; MS (ESI$^+$): m/z (intensity), 370.10 ([M+H]$^+$, 100%), 372.10 ([M+H]$^+$ + 2, 65%), 374.10 ([M+H]$^+$ + 4, 10%), 411.1 ([M+H]$^+$ + CH_3CN, 30%), 413.1 ([M+H]$^+$ + 2 + CH_3CN, 20%), 415.1 ([M+H]$^+$ + 4 + CH_3CN, 3%).

5.6.4. 4-Azido-2,6-difluorobenzyl phosphoramide mustard (6d)

A light yellow solid (0.970 g, 50%); ^{1}H NMR (200 MHz, CD_3OD): δ 6.72 (d, 2H, J = 8.4 Hz), 4.95 (d, 2H, J = 7.0 Hz), 3.52–3.60 (m, 4H), 3.26–3.39 (m, 4H); ^{13}C NMR (50 MHz, CD_3OD): 163.6 (dd, J = 250.0 Hz, J = 9.5 Hz), 145.2 (t, J = 13.3 Hz), 110.5 (td, J = 20.0 Hz, J = 8.4 Hz), 103.9 (dd, J = 29.6 Hz, J = 2.0 Hz), 55.4 (dd, J = 8.0 Hz, J = 3.8 Hz), 50.6 (d, J = 4.6 Hz), 43.0; MS (ESI$^+$): m/z (intensity), 388.2 ([M+H]$^+$, 100%), 390.2 ([M+H]$^+$ + 2, 65%), 392.2 ([M+H]$^+$ + 4, 10%), 429.3 ([M+H]$^+$ + CH_3CN, 20%), 431.3 ([M+H]$^+$ + 2 + CH_3CN, 13%), 433.3 ([M+H]$^+$ + 4 + CH_3CN, 2%).

5.6.5. 4-Azido-2,3,5,6-tetrafluorobenzyl phosphoramide mustard (6e)

A dark green solid (1.02 g, 48%); ^{1}H NMR (200 MHz, CD_3OD): δ 5.03 (d, 2H, J = 7.8 Hz), 3.55–3.62 (m, 4H), 3.32–3.41 (m, 4H); ^{13}C NMR (50 MHz, CD_3OD): 145.4 (dm, J = 251.2 Hz), 140.4 (dm, J = 250.5 Hz), 121.0 (tt, J = 12.2 Hz, J = 3.1 Hz), 110.4 (td, J = 12.4 Hz, J = 8.4 Hz), 54.2, 49.1 (d, J = 5.0 Hz), 42.3; IR (film): 3241, 3113, 2964, 2159, 2124, 1654, 1496, 1238 cm^{-1}; MS (ESI$^+$): m/z (intensity), 424.0 ([M+H]$^+$, 100%), 426.0 ([M+H]$^+$ + 2, 65%), 428.0 ([M+H]$^+$ + 4, 10%), 465.1 ([M+H]$^+$ + CH_3CN, 20%), 467.1 ([M+H]$^+$ + 2 + CH_3CN, 13%), 469.1 ([M+H]$^+$ + 4 + CH_3CN, 2%).

5.7. Synthesis of TFA–L-glutamine (7)

To a solution of L-glutamine (14.6 g, 0.1 mol) in 1 N NaOH (100 mL, 0.1 mol) was added *S*-ethyl thioacetate (17 mL, 0.13 mol). The reaction was carried out at room temperature for 24 h. The reaction solution was acidified with 12 N HCl to pH ~2.0, saturated with NaCl, and then extracted with EtOAc. The combined EtOAc phase was washed with water, brine and dried over Na_2SO_4. After removal of Na_2SO_4 via filtration, the filtrate was concentrated to dryness to afford the desired product **7** (20.4 g) as white powder in a yield of 84%. ^{1}H NMR (200 MHz, CD_3-OD): δ 4.47 (dd, 1H, J = 9.0 Hz, J = 4.8 Hz), 2.35–2.42 (m, 4H), 2.21–2.32 (m, 1H), 1.96–2.18 (m, 1H); ^{13}C NMR (50 MHz, CD_3OD): 175.6, 171.4, 157.2 (q, J = 37.0 Hz), 115.5 (q, J = 285.0 Hz), 51.8, 30.5, 25.6; MS (ESI$^-$): m/z (intensity), 241.1 ([M−H]$^-$, 100%).

5.8. Synthesis of 4-(N^{α}-TFA–L-glutaminyl)aminobenzyl phosphoramide mustards 13a–e

To a solution of TFA-glutamine (2.0 mmol) and *N*-methylpiperidine (0.244 mL, 2.0 mmol) in THF (10 mL) was added a solution of isopropylchloroformate in toluene (2.0 mL, 2.0 mmol) at −15 °C. The resulting mixture was stirred for 30 min at −15 to −10 °C. Then, the obtained mixed anhydride solution was added into the freshly prepared solution of LiAlHSeH in THF via cannula over a period of 5 min. The reaction mixture was stirred for additional 30 min below 5 °C under nitrogen atmosphere. Then, a solution of azide **6a–e** (1.0 mmol) in THF (1 mL) was added into the above selenocarboxylate solution via syringe. The reaction was carried out at room temperature for 24 h. After evaporation of THF under reduced pressure, the residue was suspended in a saturated sodium bicarbonate aqueous solution (25 mL) followed by extraction with EtOAc (4 × 50 mL). The combined organic phase was washed with 1.0 M HCl (50 mL), water (50 mL) and saturated brine (50 mL), and dried over Na_2SO_4. After removal of Na_2SO_4 through filtration, the filtrate was concentrated to dryness under reduced pressure. The crude product was purified by flash column chromatography (FCC) (dichloromethane to 10% methanol/dichloromethane) on silica gel to provide desired product **13a–e**.

5.8.1. 4-(N^{α}-TFA–L-glutaminyl)amino-benzyl phosphoramide mustard (13a)

A yellow semi-solid (138 mg, 25%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.62 (d, 2H, J = 8.4 Hz), 7.39 (d, 2H, J = 8.4 Hz), 4.97 (d, 2H, J = 7.2 Hz), 4.56 (dd, 1H, J = 8.0 Hz, J = 5.4 Hz), 3.61–3.68 (m, 4H), 3.32–3.41 (m, 4H), 2.38–2.45 (m, 2H), 2.09–2.27 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 175.5, 168.7, 157.2 (q, J = 37.5 Hz), 137.5, 132.5 (d, J = 7.5 Hz), 127.6, 119.5, 115.3 (q, J = 285.0 Hz), 65.9 (d, J = 5.3 Hz), 53.5, 48.8 (d, J = 5.0 Hz), 41.2, 30.3, 26.4; IR (film): 3261, 3063, 1715, 1608, 1545, 1213, 1185, 1159, 980 cm^{-1}; MS (ESI$^+$): m/z (intensity), 572.1 ([M+Na]$^+$, 100%), 574.1 ([M+Na]$^+$ + 2, 65%), 576.1 ([M+Na]$^+$ + 4, 10%).

5.8.2. 4-(N^{α}-TFA–L-glutaminyl)amino-2-fluoro-benzyl phosphoramide mustard (13b)

A yellow semi-solid (312 mg, 55%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.84 (dd, 1H, J = 12.4 Hz, J = 1.8 Hz), 7.66 (t, 1H, J = 8.2 Hz), 7.54 (dd, 1H, J = 8.4 Hz, J = 2.2 Hz), 5.21 (d, 2H, J = 6.8 Hz), 4.75 (dd, 1H, J = 8.0 Hz, J = 5.2 Hz), 3.82–3.89 (m, 4H), 3.58–3.68 (m, 4H), 2.57–2.67 (m, 2H), 2.22–2.48 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 177.4, 170.8, 161.9 (d, J = 244.3 Hz), 159.0 (q, J = 36.8 Hz), 141.5 (d, J = 11.0 Hz), 131.7 (d, J = 5.3 Hz), 120.8 (dd, J = 15.0 Hz, J = 8.4 Hz), 117.3 (q, J = 284.9 Hz), 116.6 (d, J = 3.0 Hz), 108.1 (d, J = 26.6 Hz), 61.9, 53.4, 50.6 (d, J = 5.0 Hz), 43.1, 32.3, 28.2; IR (film): 3404, 2962, 1666, 1627, 1549, 1189, 1161, 998 cm^{-1}; MS (ESI$^+$): m/z (intensity), 348.2 ([M−OP(O)$NH_2N(CH_2CH_2Cl)_2$]$^+$, 25%), 366.2 ([M−OP(O)$NH_2N(CH_2CH_2Cl)_2$]$^+$ +H_2O, 100%), 407.3 ([M−OP(O)$NH_2N(CH_2CH_2Cl)_2$]$^+$ +H_2O + CH_3CN, 40%), 590.3 ([M+Na]$^+$, 20%), 592.3 ([M+Na]$^+$ + 2, 13%), 594.3 ([M+Na]$^+$ + 4, 2%).

5.8.3. 4-(N^{α}-TFA–L-glutaminyl)amino-3-fluoro-benzyl phosphoramide mustard (13c)

A yellow semi-solid (114 mg, 20%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.83 (t, 1H, J = 8.0 Hz), 7.15–7.26 (m, 2H), 4.93 (d, 2H, J = 7.2 Hz), 4.61 (dd, 1H, J = 8.6 Hz, J = 5.2 Hz), 3.59–3.66 (m, 4H), 3.33–3.45 (m, 4H), 2.37–2.44 (m, 2H), 2.09–2.27 (m, 2H); MS (ESI$^+$): m/z (intensity), 348.2 ([M−OP(O)$NH_2N(CH_2CH_2Cl)_2$]$^+$, 100%), 366.3 ([M−OP(O)$NH_2N(CH_2CH_2Cl)_2$]$^+$ +H_2O, 55%), 407.3 ([M−OP(O)$NH_2N(CH_2CH_2Cl)_2$]$^+$ +H_2O + CH_3CN, 15%), 568.3 ([M+H]$^+$, 40%), 570.3 ([M+H]$^+$ + 2, 26%), 572.3 ([M+Na]$^+$ + 4, 4%), 590.3 ([M+Na]$^+$, 30%), 592.3 ([M+Na]$^+$ + 2, 20%), 594.23 ([M+Na]$^+$ + 4, 3%).

5.8.4. 4-(N^{α}-TFA–L-glutaminyl)amino-2,6-difluoro-benzyl phosphoramide mustard (13d)

A yellow semi-solid (422 mg, 72%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.31 (d, 1H, J = 9.6 Hz), 5.00 (d, 2H, J = 7.0 Hz), 4.50 (dd, 1H, J = 8.6 Hz, J = 5.2 Hz), 3.53–3.64 (m, 4H), 3.33–3.44 (m, 4H), 2.30–2.41 (m, 2H), 2.04–2.25 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 177.3, 170.9, 163.0 (dd, J = 246.5 Hz, J = 9.9 Hz), 159.1 (q, J = 37.5 Hz), 142.4 (t, J = 14.0 Hz), 117.3 (q, J = 285.3 Hz), 109.2 (td, J = 19.8 Hz, J = 8.4 Hz), 103.7 (d, J = 29.6 Hz), 55.4, 50.6 (d, J = 4.9 Hz), 50.4, 42.9, 32.0, 28.0; IR (film): 3295, 3075, 2966, 1698, 1668, 1615, 1556, 1425, 1215, 1012 cm^{-1}; MS (ESI$^-$): m/z (intensity), 584.1 ([M−H]$^-$, 100%), 586.1 ([M−H]$^-$ + 2, 65%), 588.1 ([M−H]$^-$ + 4, 10%), 698.1 ([M−H]$^-$ + TFA, 40%), 700.1 ([M−H]$^-$ + 2 + TFA, 26%), 702.1 ([M−H]$^-$ + 4 + TFA, 4%).

5.8.5. 4-(N^{α}-TFA–L-glutaminyl)amino-2,3,5,6-tetrafluoro-benzyl phosphoramide mustard (13e)

A yellow semi-solid (541 mg, 87%); ^{1}H NMR (200 MHz, CD_3OD): δ 5.13 (d, 2H, J = 7.6 Hz), 4.66 (dd, 1H, J = 8.0 Hz, J = 5.2 Hz), 3.60–3.68 (m, 4H), 3.37–3.47 (m, 4H), 2.40–2.47 (m, 2H), 2.07–2.37 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 177.3, 171.3, 159.2 (q, J = 37.6 Hz), 147.6 (dm, J = 245.0 Hz), 143.8 (dm, J = 245.0 Hz), 118.5 (t, J = 14.8 Hz), 117.3 (q, J = 285.0 Hz), 114.9 (td, J = 17.7 Hz, J = 8.0 Hz), 55.5, 54.9, 50.6 (d, J = 4.9 Hz), 43.0, 32.0, 28.1; MS (ESI$^-$): m/z (intensity), 620.1 ([M−H]$^-$, 100%), 622.1 ([M−H]$^-$ + 2, 65%), 624.1 ([M−H]$^-$ + 2, 10%).

5.9. Synthesis of fluorinated and non-fluorinated 4-L-glutamylamino-benzyl phosphoramide mustards 8a–e

A solution of **13a–e** (1.0 mmol) and potassium carbonate (552 mg, 4.0 mmol) in 75% aqueous acetonitrile (20 mL) was stirred at room temperature for 48 h. The completion of reaction was confirmed by LC/MS and TLC. The aqueous phase was saturated with sodium chloride followed by extraction with acetonitrile (3 × 10 mL). The combined acetonitrile phase was dried over Na_2SO_4 followed by filtration off Na_2SO_4. The filtrate was concentrated to dryness. The crude was purified by flash column chromatography (FCC) (dichloromethane to 25% methanol/dichloromethane containing 1% concd NH_4OH) on silica gel to provide the desired product **8a–e**.

5.9.1. 4-L-Glutaminylamino-benzyl phosphoramide mustard (8a)

A yellow semi-solid (326 mg, 72%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.66 (d, 2H, J = 8.4 Hz), 7.40 (d, 2H, J = 8.4 Hz), 4.97 (d, 2H, J = 7.6 Hz), 3.61–3.69 (m, 4H), 3.38–3.48 (m, 4H), 2.36–2.45 (m, 2H), 2.09–2.27 (m, 2H); IR (film): 3202, 3064, 1671, 1203, 1134 cm^{-1}; MS (ESI$^+$): m/z (intensity), 476.1 ([M+Na]$^+$, 100%),

478.1 ([M+Na]$^+$ + 2, 65%), 480.1 ([M+Na]$^+$ + 4, 10%). HRMS (FAB$^+$) m/z calcd for $C_{16}H_{26}Cl_2N_5O_4P$, [M+H]$^+$, 454.1178, found 454.1178.

5.9.2. 4-L-Glutaminylamino-2-fluoro-benzyl phosphoramide mustard (8b)

A yellow semi-solid (358 mg, 76%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.65 (dd, 1H, J = 12.4 Hz, J = 1.8 Hz), 7.46 (t, 1H, J = 8.4 Hz), 7.32 (dd, 1H, J = 8.4 Hz, J = 2.2 Hz), 5.00 (d, 2H, J = 7.4 Hz), 4.07 (t, 1H, J = 6.2 Hz), 3.59–3.66 (m, 4H), 3.36–3.46 (m, 4H), 2.46–2.53 (m, 2H), 2.09–2.25 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 176.9, 168.6, 162.1 (d, J = 244.7 Hz), 141.0 (d, J = 11.0 Hz), 132.0 (d, J = 5.0 Hz), 121.3 (dd, J = 13.7 Hz, J = 7.6 Hz), 116.5 (d, J = 3.4 Hz), 108.1 (d, J = 26.6 Hz), 61.8, 54.8, 50.7 (d, J = 5.0 Hz), 43.0, 31.7, 28.2; IR (film): 3200, 3064, 1670, 1623, 1555, 1203, 1135, 982 cm^{-1}; MS (ESI$^+$): m/z (intensity), 252.1 ([M−OP(O)NH$_2$N(CH$_2$CH$_2$Cl)$_2$]$^+$, 100%), 494.3 ([M+Na]$^+$, 80%), 496.3 ([M+Na]$^+$ + 2, 52%), 498.3 ([M+Na]$^+$ + 4, 8%). HRMS (FAB$^+$) m/z calcd for $C_{16}H_{25}Cl_2FN_5O_4P$, [M+H]$^+$, 472.1084, found 472.1034.

5.9.3. 4-L-Glutaminylamino-3-fluoro-benzyl phosphoramide mustard (8c)

A yellow semi-solid (348 mg, 74%); ^{1}H NMR (200 MHz, CD_3OD): δ 8.03 (t, 1H, J = 8.0 Hz), 7.18–7.29 (m, 2H), 4.96 (d, 2H, J = 7.6 Hz), 4.17 (t, 1H, J = 8.0 Hz), 3.60–3.68 (m, 4H), 3.39–3.55 (m, 4H), 2.38–2.47 (m, 2H), 1.90–2.21 (m, 2H); MS (ESI$^+$): m/z (intensity), 252.1 ([M−OP(O)NH$_2$N(CH$_2$CH$_2$Cl)$_2$]$^+$, 100%), 472.2 ([M+H]$^+$, 20%), 474.2 ([M+H]$^+$ + 2, 13%), 476.2 ([M+Na]$^+$ + 4, 2%), 494.2 ([M+Na]$^+$, 50%), 496.2 ([M+Na]$^+$ + 2, 33%), 498.2 ([M+Na]$^+$ + 4, 5%). HRMS (FAB$^+$) m/z calcd for $C_{16}H_{25}Cl_2FN_5O_4P$, [M+H]$^+$, 472.1084, found 472.1046.

5.9.4. 4-L-Glutaminylamino-2,6-difluoro-benzyl phosphoramide mustard (8d)

A yellow semi-solid (416 mg, 85%); ^{1}H NMR (200 MHz, CD_3OD): δ 7.36 (d, 1H, J = 9.4 Hz), 5.02 (d, 2H, J = 6.8 Hz), 4.61 (t, 1H, J = 8.6 Hz), 3.52–3.66 (m, 4H), 3.35–3.45 (m, 4H), 2.35–2.43 (m, 2H), 1.91–2.16 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 177.6, 173.6, 163.1 (dd, J = 246.2 Hz, J = 10.3 Hz), 142.4 (t, J = 14.0 Hz), 109.4 (td, J = 19.8 Hz, J = 8.4 Hz), 103.7 (d, J = 30.4 Hz), 55.7, 55.5 (t, J = 3.4 Hz), 50.7 (d, J = 4.6 Hz), 43.0, 32.2, 30.6; IR (film): 3269, 3073, 1673, 1638, 1557, 1427, 1203, 1136, 1014 cm^{-1}; MS (ESI$^-$): m/z (intensity), 270.1 ([M−OP(O)NH$_2$N(CH$_2$CH$_2$Cl)$_2$]$^+$, 75%), 512.1 ([M+H]$^+$, 100%), 514.1 ([M+H]$^+$ + 2, 65%), 516.1 ([M+Na]$^+$ + 4, 10%). HRMS (FAB$^+$) m/z calcd for $C_{16}H_{24}Cl_2F_2N_5O_4P$, [M+H]$^+$, 490.0989, found 490.0998.

5.9.5. 4-L-Glutaminylamino-2,3,5,6-tetrafluoro-benzyl phosphoramide mustard (8e)

A yellow semi-solid (410 mg, 78%); ^{1}H NMR (200 MHz, CD_3OD): δ 5.14 (d, 2H, J = 8.2 Hz), 4.06 (dd, 1H, J = 7.0 Hz, J = 5.4 Hz), 3.60–3.68 (m, 4H), 3.35–3.47 (m, 4H), 2.47–2.54 (m, 2H), 1.90–2.28 (m, 2H); ^{13}C NMR (50 MHz, CD_3OD): 176.9, 169.6, 147.6 (dm, J = 245.1 Hz), 143.6 (dm, J = 247.4 Hz), 118.0 (t, J = 14.8 Hz), 115.3 (td, J = 17.7 Hz, J = 8.0 Hz), 55.5, 54.4, 50.6 (d, J = 5.0 Hz), 43.0, 31.5, 28.5; MS (ESI$^-$): m/z (intensity), 526.1 ([M+H]$^+$, 100%), 528.1 ([M+H]$^+$ + 2, 65%), 530.1 ([M+Na]$^+$ + 4, 10%), 548.1 ([M+Na]$^+$, 40%), 550.1 ([M+Na]$^+$ + 2, 26%), 552.1 ([M+Na]$^+$ + 4, 4%). HRMS (FAB$^+$) m/z calcd for $C_{16}H_{22}Cl_2F_4N_5O_4P$, [M+H]$^+$, 526.0801, found 526.0796.

5.10. Synthesis of fluorinated and non-fluorinated Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-benzyl phosphoramide mustards (1a–e)

To a solution of Fm-glutaryl-Hyp-Ala-Ser-Chg-OH (**9**) (0.1 mmol) and HBTU (0.11 mmol) in NMP (2 mL) was added DIPEA (0.11 mmol). The resulting mixture was stirred at room temperature for 30 min followed by addition of a solution of **8a–e** (0.1 mmol) in NMP (0.5 mL). The reaction was carried out at room temperature for additional 2 h and quenched by adding ice-cold 5% aqueous sodium bicarbonate (10 mL). The resulting mixture was stirred at 0–5 °C for 10 min and the resulting white precipitates were collected via centrifugation followed by successively washing with distilled water. The obtained solid was then dissolved in 50% MeOH/CH_3CN and treated with 10 equiv of DEA for 1 h. After removal of solvents under reduced pressure, the remaining solid was suspended in diethyl ether and collected via centrifugation. The crude products were purified by preparative-HPLC on reversed phase C18 column. A linear gradient was used from 10% solvent A to 90% solvent B with a flow rate of 12 mL/min, where solvent A was 0.1% TFA/H_2O and solvent B was 0.1% TFA/CH_3CN. The UV detection wavelength was set at 245 nm. Homogeneous fraction containing the desired products were pooled and lyophilized to afford the desired products as white powder. The purities and identities were confirmed by HPLC and LC/MS and the purity of the prodrug conjugates was >95%.

5.10.1. Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-benzyl phosphoramide mustard (1a)

White powder (30 mg, 30%); HRMS (FAB+) m/z calcd for $C_{40}H_{62}C_{l2}N_9NaO_{13}P$, [M+Na]$^+$, 1000.3479, found 1000.3477.

5.10.2. Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-2-fluorobenzyl phosphoramide mustard (1b)

White powder (40 mg, 38%); HRMS (FAB$^+$) m/z calcd for $C_{40}H_{61}Cl_2FN_9NaO_{13}P$, [M+Na]$^+$, 1018.3385, found 1018.3357.

5.10.3. Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-3-fluorobenzyl phosphoramide mustard (1c)

White powder (28 mg, 28%); HRMS (FAB$^+$) m/z calcd for $C_{40}H_{61}Cl_2FN_9NaO_{13}P$, [M+Na]$^+$, 1018.3385, found 1018.3363.

5.10.4. Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-2,6-difluorobenzyl phosphoramide mustard (1d)

White powder (41 mg, 40%); HRMS (FAB$^+$) m/z calcd for $C_{40}H_{60}Cl_2F_2N_9NaO_{13}P$, [M+Na]$^+$, 1036.3291, found 1036.3181.

5.10.5. Glutaryl-Hyp-Ala-Ser-Chg-Gln-NH-2,3,5,6-tetrafluorobenzyl phosphoramide mustard (1e)

White powder (32 mg, 30%); HRMS (FAB$^+$) m/z calcd for $C_{40}H_{58}Cl_2F_4N_9NaO_{13}P$, [M+Na]$^+$, 1072.3103, found 1072.2985.

5.11. Stability study of 1a–e in buffers

The peptide conjugate **1a–e** (1 mg) was dissolved in 0.5 mL of 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4) containing 2% DMSO or 0.5 mL of 50 mM Tris–HCl/10 mM $CaCl_2$/0.1% Tween-20® buffer (pH 8.0) containing 2% DMSO, respectively. At different time intervals, aliquots (25 μL) were withdrawn and frozen prior to HPLC analysis (Waters Symmetry C_{18} column −3.5 μm, 4.6 × 150 mm, gradient elution from 10% to 80% acetonitrile containing 0.1% TFA at a flow rate at 1 mL/min, detection wavelength at 220 nm and 245 nm). The half-life was determined based on the disappearance of the peptide conjugate **1a–e**.

5.12. Prostate-specific antigen (PSA) assay of 1a–e

PSA was purchased from CALIBIOCHEM. The stock solution of peptide conjugate **1a–e** (5 μL, 10 mM in DMSO) was added to a PSA buffer (295 μL, 50 mM Tris–HCl/10 mM $CaCl_2$/0.1% Tween-20® buffer, pH 8.0), respectively. The prepared solution was warmed up to 37 °C and then 245 μL of solution was transferred to an eppendorf vial containing PSA (5 μL, 2.45 mg/mL), respec-

tively. At different time intervals, aliquots (20 μL) were withdrawn, quenched with acetonitrile (5 μL) and frozen prior to HPLC analysis (Waters Symmetry C_{18} column —3.5 μm, 4.6 × 150 mm, gradient elution from 10% to 80% acetonitrile containing 0.1% TFA at a flow rate at 1 mL/min, detection wavelength at 220 nm and 245 nm). The half-life was determined based on the disappearance of peptide conjugate **1a–e**.

5.13. Cell culture and antiproliferative assays of 1a–e

LNCaP (PSA positive, ATCC) and DU145 (PSA negative, ATCC) human prostate carcinoma cells were grown as monolayer cultures in the culture medium (RPMI 1640 with L-glutamine and phenol red, supplemented with 10% FBS, 100 units/mL penicillin G and 100 units/mL streptomycin sulfate). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Media were routinely changed every 72 h. Cells were split at 80% confluence followed by trypsinization and subcultured at 1:4. Cells were plated in 96-well plates (7500 LNCaP cells/well and 500 DU145 cells/well) and grown for 48 h in the culture medium. The medium was then replaced by the serum-free medium (RPMI 1640 with L-glutamine and without phenol red, supplemented with 2% TCM, 100 units/mL penicillin G and 100 units/mL streptomycin sulfate). Subsequently, cells were incubated with various peptide-conjugate concentrations (100–0.01 μM) for 72 h where medium alone was used as a negative control. An MTT dye solution (5 mg/mL) was then added to the wells. The cells were then incubated at 37 °C for 4 h followed by addition of a solubilization solution (sodium laurate) and incubation at room temperature overnight in dark. The plates were read at a wavelength of 570 nm by a Dynatech MR5000 microtiter plate reader. Results were expressed at a percentage of control growth. IC_{50} values and the concentration required to reduce the cell number to 50% of the control were obtained by interpolation.

Acknowledgements

We gratefully acknowledge the financial support of grant SNJ-CCR 700-009 from the State of New Jersey Commission on Cancer Research, a pilot grant from the Gallo Prostate Cancer Center of the Cancer Institute of New Jersey, and grant RSG-03-004-01-CDD from the American Cancer Society.

References and notes

1. Siegel, R. L.; Miller, K. D.; Jemal, A. *CA Cancer J. Clin.* **2016**, *66*, 7.
2. Arlen, P. M.; Gulley, J. L. *Future Oncol.* **2005**, *1*, 19.
3. Bono, J. S. d.; Oudard, S.; Ozguroglu, M.; Hansen, S.; Machiels, J. P.; Kocak, I.; Gravis, G.; Bodrogi, I.; Mackenzie, M. J.; Shen, L.; Roessner, M.; Gupta, S.; Sartor, A. O. *Lancet* **2010**, *376*, 1147.
4. Kantoff, P. W.; Higano, C. S.; Shore, N. D.; Berger, E. R.; Small, E. J.; Penson, D. F.; Redfern, C. H.; Ferrari, A. C.; Dreicer, R.; Sims, R. B.; Xu, Y.; Frohlich, M. W.; Schellhammer, P. F. *N. Eng. J. Med.* **2010**, *363*, 411.
5. Ryan, C. J.; Smith, M. R.; Bono, J. S. d.; Molina, A.; Logothetis, C. J.; Souza, P. d.; Fizazi, K.; Mainwaring, P.; Piulats, J.; Ng, S.; Carles, J.; Mulders, P. F. A.; Basch, E.; Small, E. J.; Saad, F.; Schrijvers, D.; Poppel, H. V.; Mukherjee, S. D.; Suttmann, H.; Gerritsen, W. R.; Flaig, T. W.; George, D. J.; Yu, E. Y.; Efstathiou, E.; Pantuck, A.; Winquist, E.; Higano, C. S.; Park, Y.; Kheoh, T.; Griffin, T.; Scher, H. I.; Rathkopf, D. E. *N. Eng. J. Med.* **2013**, *368*, 138.
6. Scher, H. I.; Fizazi, K.; Saad, F.; Taplin, M. E.; Sternberg, C. N.; Miller, K.; Wit, R. d.; Mulders, P.; Chi, K. N.; Shore, N. D.; Armstrong, A. J.; Flaig, T. W.; Fléchon, A.; Mainwaring, P.; Fleming, M.; Hainsworth, J. D.; Hirmand, M.; Selby, B.; Seely, L.; Bono, J. S. d. *N. Eng. J. Med.* **2012**, *367*, 1187.
7. Parker, C.; Nilsson, S.; Heinrich, D.; Helle, S. I.; O'Sullivan, J. M.; Fosså, S. D.; Chodacki, A.; Wiechno, P.; Logue, J.; Seke, M.; Widmark, A.; Johannessen, D. C.; Hoskin, P.; Bottomley, D.; James, N. D.; Solberg, A.; Syndikus, I.; Kliment, J.; Wedel, S.; Boehmer, S.; Dall'Oglio, M.; Franzén, L.; Coleman, R.; Vogelzang, N. J.; O'Bryan-Tear, C. G.; Staudacher, K.; Garcia-Vargas, J.; Shan, M.; Bruland, Ø. S.; Sartor, O. *N. Eng. J. Med.* **2013**, *369*, 213.
8. Aloysius, H.; Hu, L. *Med. Res. Rev.* **2015**, *35*, 554.
9. Christensson, A.; Laurell, C.-B.; Lilja, H. *Eur. J. Biochem.* **1990**, *194*, 755.
10. Lilja, H.; Chrlstensson, A.; Dahlén, U.; Matlkainen, M.-T.; Nilsson, O.; Pettersson, K.; Lövgren, T. *Clin. Chem.* **1991**, *37*, 1618.
11. Otto, A. B. J.; Birkenmeier, G. *J. Urol.* **1998**, *159*, 297.
12. Sinha, A.; Quast, B.; Reddy, P.; Elson, M.; Wilson, M. *Anticancer Res.* **1999**, *19*, 893.
13. Mhaka, A.; Denmeade, S. R.; Yao, W.; Isaacs, J. T.; Khan, S. R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2459.
14. Tang, X.; Xian, M.; Trikha, M.; Honn, K. V.; Wang, P. G. *Tetrahedron Lett.* **2001**, *42*, 2625.
15. Jones, G. B.; Mitchell, M. O.; Weinberg, J. S.; D'Amico, A. V.; Bubley, G. J. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1987.
16. Denmeade, S. R.; Jakobsen, C. M.; Janssen, S.; Khan, S. R.; Garrett, E. S.; Lilja, H.; Christensson, S. B.; Isaacs, J. T. *J. Natl. Cancer Inst.* **2003**, *95*, 990.
17. Jakobsen, C. M.; Denmeade, S. R.; Isaacs, J. T.; Grady, A.; Olsen, C. E.; Christensen, S. B. *J. Med. Chem.* **2001**, *44*, 4696.
18. Brady, S. F.; Pawluczyk, J. M.; Lumma, P.; Wai, J.; Jones, R. E.; DeFeo-Jones, D.; Wong, B. K.; Miller-Stein, C.; Lin, J. H.; Oliff, A. *J. Med. Chem.* **2002**, *45*, 4706.
19. Kumar, S. K.; Williams, S. A.; Isaacs, J. T.; Denmeade, S. R.; Khan, S. R. *Bioorg. Med. Chem.* **2007**, *15*, 4973.
20. Williams, S. A.; Merchant, R. F.; Garrett-Mayer, E.; Isaacs, J. T.; Buckley, J. T.; Denmeade, S. R. *J. Natl. Cancer Inst.* **2007**, *99*, 376.
21. Yang, Y.; Aloysius, H.; Inoyama, D.; Chen, Y.; Hu, L. *Acta Pharm. Sin. B* **2011**, *1*, 143.
22. Lee, J.; Huang, W.; Broering, J. M.; Barron, A. E.; Seo, J. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 2849.
23. Graeser, R.; Chung, D.-E.; Esser, N.; Moor, S.; Schachtele, C.; Unger, C.; Kratz, F. *Int. J. Cancer* **2008**, *122*, 1145.
24. Elsadek, B.; Graeser, R.; Esser, N.; Schäfer-Obodozie, C.; Ajaj, K. A.; Unger, C.; Warnecke, A.; Saleem, T.; El-Melegy, N.; Madkor, H.; Kratz, F. *Eur. J. Cancer* **2010**, *46*, 3434.
25. Tai, W.; Shukla, R. S.; Qin, B.; Li, B.; Cheng, K. *Mol. Pharm.* **2011**, *8*, 901.
26. Garsky, V. M.; Lumma, P.; Feng, D.-M.; Wai, J.; Ramjit, H. G.; Sardana, M. K.; Oliff, A.; Jones, R. E.; DeFeo-Jones, D.; Freidinger, R. *J. Med. Chem.* **2001**, *44*, 4216.
27. Jiang, Y.; Hu, L. *Bioorg. Med. Chem.* **2013**, *21*, 7507.
28. Wu, X.; Hu, L. *Tetrahedron Lett.* **2005**, *46*, 8401.
29. Surabhi, P.; Wu, X.; Hu, L. *Tetrahedron Lett.* **2006**, *47*, 4609.
30. Wu, X.; Chen, Y.; Aloysius, H.; Hu, L. *Beilstein J. Org. Chem.* **2011**, *7*, 1030.
31. Wu, X.; Hu, L. *J. Org. Chem.* **2007**, *72*, 765.
32. *Peptide Synthesis Protocols*; Humana Press: Totowa, New Jersey, 1995.
33. Hu, L. Q.; Yu, C. Z.; Jiang, Y. Y.; Han, J. Y.; Li, Z. R.; Browne, P.; Race, P. R.; Knox, R. J.; Searle, P. F.; Hyde, E. I. *J. Med. Chem.* **2003**, *46*, 4818.
34. Jiang, Y.; Han, J.; Yu, C.; Vass, S. O.; Searle, P. F.; Browne, P.; Knox, R. J.; Hu, L. *J. Med. Chem.* **2006**, *49*, 4333.