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

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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.

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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

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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**.

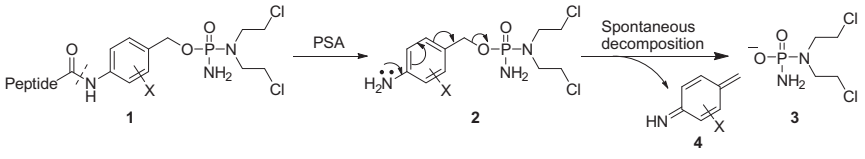
3.2. Synthesis of 4-azidobenzyl phosphoramide mustards 6a–e

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.

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