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Phenylalanyl-aminocyclophosphamides as model prodrugs for proteolytic activation: Synthesis, stability, and stereochemical requirements for enzymatic cleavage

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Abstract—4-Aminocyclophosphamide (4-NH_2-CPA, **7**) was proposed as a prodrug moiety of phosphoramide mustard. Four diastereomers of phenylalanine-conjugates of 4-NH_2-CPA were synthesized and their stereochemistry was assigned based on chromatographic and spectroscopic data. All diastereomers were stable in phosphate buffer but only the *cis*-(4*R*)-isomer of **15** was efficiently cleaved by α-chymotrypsin with a half-life of 20 min, which is much shorter than the 8.9 h to >12 h half-lives found for the other diastereomers. LC–MS analysis of the proteolytic products of *cis*-(4*R*)-**15** indicated that 4-NH_2-CPA was released upon proteolysis and further disintegrated to phosphoramide mustard. These results suggest the feasibility of using peptide-conjugated *cis*-(4*R*)-4-NH_2-CPA as potential prodrugs for proteolytic activation in tumor tissues.

Most anticancer agents suffer severe side effects from their marginal selectivity against cancer cells over healthy ones. The side effects limit the maximum dose of these agents to below the amount that can kill all viable tumor cells and the survived tumor cells could be more drug resistant and malignant.[1,2] Among strategies explored to increase the tumor selectivity of anticancer agents, tumor-targeted prodrug therapy has attracted much attention.[3–5] In this strategy, an inert prodrug form of an anticancer agent is selectively activated through a biochemical mechanism associated with tumor cells, such as hypoxic reduction, enzymatic action, or receptor recognition. Recently, several proteases have been identified to be unique for tumor growth and metastasis, including plasmin,[6,7] plasminogen activator,[8] matrix metalloproteinases,[9,10] and prostate specific antigen (PSA).[11,12] The specificity of these enzymes to tumor cells has provided novel opportunities that are being explored for tumor-targeted prodrug therapy.

Cyclophosphamide is one of the often used anticancer drugs in the clinic. Because of its activity against both cycling and non-cycling cells, it is one of the few anticancer agents effective in the treatment of slow-growing solid tumors.[13] However, the clinical use of cyclophosphamide is associated with a life-threatening side effect, hemorrhagic cystitis, in addition to bone marrow and GI tract toxicities which are commonly seen for most other anticancer agents. The existing knowledge of cyclophosphamide has facilitated the development of a variety of phosphoramide mustard prodrugs for tumor-targeted activation.[14–17] In the last few years, we have focused on improving the therapeutic efficiency of cyclophosphamide through the strategy of tumor-targeted enzyme prodrug therapy. In one approach, we developed a series of nitrobenzyl phosphoramide mustard prodrugs for *Escherichia coli* nitroreductase activation in enzyme prodrug therapy.[18,19] In this communication, we describe our efforts toward the conversion of cyclophosphamide into peptide-conjugated 4-aminocyclophosphamide prodrugs in the form of **6** for tumor-targeted proteolytic activation. To our knowledge, this is the first time that 4-aminocyclophosphamide (4-NH_2-CPA, **7**) is proposed as a prodrug moiety of phosphoramide mustard.

Keywords: Anticancer prodrugs; Cyclophosphamide; 4-Aminocyclophosphamide; Site-specific activation; Proteolytic activation; *gem*-Diamines; Phosphoramide mustard.
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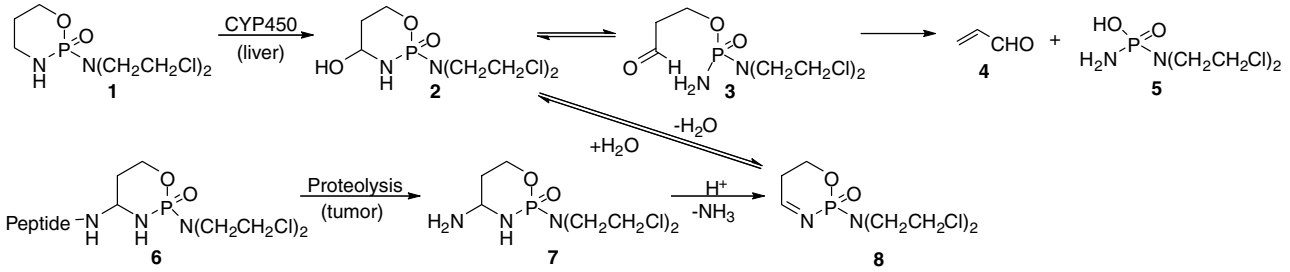
Cyclophosphamide (**1**) is a prodrug that requires activation by cytochrome P450 enzymes in the liver for its anticancer activity.[20,21] The enzymatic oxidation converts **1** to 4-hydroxycyclophosphamide (**2**) which generates acrolein (**4**) and phosphoramide mustard (**5**) after ring opening and β-elimination (Scheme 1).[20] Acrolein is responsible for the hemorrhagic cystitis associated with cyclophosphamide and phosphoramide mustard is the ultimate alkylating species that cross-links interstrand DNA.[22]

Our design of peptide-conjugates of 4-NH_2-CPA (**7**) as prodrugs for proteolytic activation takes advantage of the structural similarity of **7** to 4-hydroxycyclophosphamide (**2**) and the chemical instability of a *gem*-diamine. As illustrated in Scheme 1, proteolysis of conjugate **6** generates **7** and a peptide. Under physiological conditions, compound **7** is protonated and forms 3,4-dehydrocyclophosphamide (**8**) after elimination of ammonia. Compound **7** is known to be in equilibrium with **2** which is responsible for the generation of phosphoramide mustard in the activation mechanism of cyclophosphamide.[20] Chemically, **7** is a *gem*-diamine, which is known as a masked aldehyde.[23,24] *N*,*N*′-Diacylated or dicarbamylated *gem*-diamines are stable compounds and have been widely used in 'retro-inverso' peptide mimetics. Monoacyl or monocarbamyl *gem*-diamines are labile to acid- or base-catalyzed elimination reactions.

The phenylalanine-conjugated 4-NH_2-CPA (H-Phe-NH-CPA, **16**) and their benzyloxycarbonyl (Z)-protected analogs **15** were chosen as model prodrugs to examine the synthetic feasibility, chemical stability, and proteolytic activation of this class of compounds. Selection of the phenylalanine residue was based on the need of a chromophore to monitor reactions by UV and the known cleavage of amide bond after phenylalanine by α-chymotrypsin. Because of the presence of two chiral centers in the oxazaphosphorinane ring, compounds **15** and **16** exist as four configurational diastereomers. They are referred to as *cis*-(4*R*)-, *trans*-(4*R*)-, *cis*-(4*S*)-, and *trans*-(4*S*)- (Fig. 1) according to the oxazaphosphorinane C-4 configuration and the relative orientation of C-4 substituent to the oxygen atom of P═O bond in the oxazaphosphorinane ring (*cis* = *SR*/*RS*, *trans* = *RR*/*SS*).

The two diastereomers of (4*R*)-**16** were synthesized stereospecifically from L-homoserine ((*S*)-**9**) while the two diastereomers of (4*S*)-**16** were synthesized from D-homoserine ((*R*)-**9**) as shown in Scheme 2. Z-Phe-OH was preactivated using HOSu/DCC and the resulting activated ester reacted with **9** in a mixture of 1 M $KHCO_3$ and THF yielding the dipeptide **10**. Amidation of **10** was carried out using HOBt/EDC activation followed by treatment with saturated ammonium hydroxide,[25] and the hydroxyl group in **11** was protected with a TBDPS group. The bis(trifluoroacetoxy)iodobenzene (BTI)-mediated Hofmann rearrangement was employed to convert the amide **12** to the corresponding mono-acylated *gem*-diamine derivative **13**. This method was chosen over other methods such as the Curtius rearrangement because of its advantages of mild reaction conditions, high yield, and retention of stereochemistry.[26,27] The reaction was also conveniently monitored by the disappearance of the starting material on TLC. Protection of the hydroxyl group with TBDPS was necessary to avoid an intramolecular cyclization of the isocyanate intermediate during the Hofmann rearrangement to form 2-oxazolidinone.[28]

The TBDPS group in **13** was removed with TBAF at room temperature, which was accompanied by the formation of Z-Phe-NH_2 as a side product. Cyclization of 1,3-aminoalcohol **14** with bis(dichloroethyl)phospho-



Scheme 1. Proposed degradation mechanism of peptide-conjugated 4-aminocyclophosphamide as compared to the activation of cyclophosphamide.

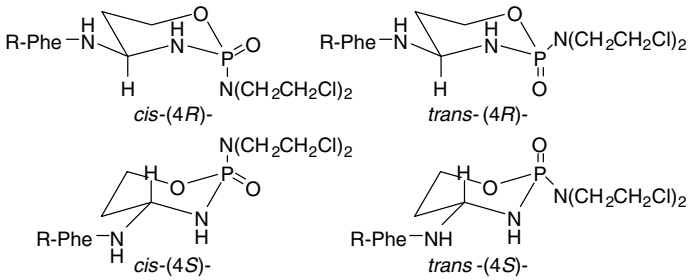


Figure 1. The four diastereomers of Z-protected and unprotected phenylalanine-4-aminocyclophosphamides **15** (R = Z) and **16** (R = H).