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Hypoxia-responsive amino acid

Development of Reduction-responsive Amino Acid that Induces Peptide Bond Cleavage in Hypoxic Cells

Akira Shigenaga,*[a] Keiji Ogura,[a] Hiroko Hirakawa,[a] Jun Yamamoto,[a] Koji Ebisuno,[a] Licht Miyamoto,[b] Keisuke Ishizawa,[b] Koichiro Tsuchiya,[b] and Akira Otaka*[a]

Bioreduction of a nitro group to an amine or a hydroxylamine is among the most attractive triggering reactions of antitumor prodrugs.[1,2] Prodrug **1**, possessing a nitroarylmethyl group, can be enzymatically reduced to corresponding nitro anion radical **2** in living cells (Figure 1). However, anion radical **2** is usually oxidized by ROS (reactive oxygen species) to regenerate parent prodrug **1** in aerobic non-malignant cells. On the contrary, the oxidation of nitro radical **2** is suppressed in hyopxic solid tumor, because concentration of molecular oxygen in hypoxic cells is lower than that in non-malignant cells. Therefore, radical anion **2** can be converted to cytotoxic drug **3** via further reduction followed by removal of the arylmethyl group specifically in tumor cells.[2] On the other hand, antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT) evoke a great clinical interest for cancer therapy, and they also utilize prodrugs with the nitroarylmethyl protective group.[2,3] For ADEPT or GDEPT, a bacterial nitroreductase is assembled on a tumor by conjugating to a tumor-specific antibody or is expressed in tumor cells by use of a tumor-specific gene vector to achieve a tumor-specific release of cytotoxin **3**, respectively. With these in mind, we decided to develop an NO_2 reduction-responsive amino acid that induces peptide bond cleavage after reduction of the nitro group, because it would potentially facilitate preparation of hypoxia-responsive peptidyl prodrugs and bioprobes.

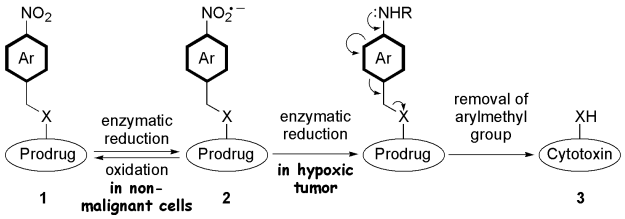
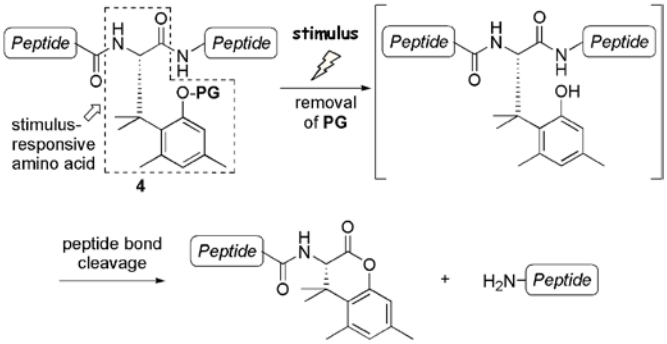


Figure 1. Reduction-responsive antitumor prodrug possessing nitroarylmethyl group. (Ar: aromatic ring; R = H or OH; X: heteroatom)



Scheme 1. Stimulus-responsive peptide bond cleavage. (**PG**: protective group removable by a corresponding stimulus.)

Previously, we reported a stimulus-responsive amino acid[4,5] and its application to controlling peptidyl function in living cells.[4] When peptide **4**, possessing the stimulus-responsive amino acid, was exposed to a corresponding stimulus, removal of **PG** (a protective group removable by the stimulus) followed by lactonization of trimethyl lock moiety[6] induced peptide bond cleavage (Scheme 1). In the present paper, development of the reduction-responsive amino acid and its application to a hypoxia-responsive peptide bond cleavage system are described.

Because *p*-nitrobenzyl phenyl ether is known to be split under hypoxic conditions via reduction of the nitro group,[7] it was used as the **PG** group. Fmoc protected reduction-responsive amino acid **8**, possessing the *p*-nitrobenzyl group, was synthesized as shown in Scheme 2. Phenol **5**[8] was treated with *p*-nitrobenzyl bromide in the presence of K_2CO_3 to afford ether **6**. The TBS group of **6** was then removed under acidic conditions. After two-step oxidation of generated alcohol **7**, the Boc group was replaced with an Fmoc group to give reduction-responsive amino acid derivative **8**. The amino acid derivative was then incorporated into model peptide **9** using Fmoc solid phase peptide synthesis (Fmoc SPPS) to demonstrate a capability to induce the reduction-responsive peptide bond cleavage.

[a] *Prof. Dr. A. Shigenaga, K. Ogura, H. Hirakawa, J. Yamamoto, K. Ebisuno, Prof. Dr. A. Otaka*
Department of Bioorganic Synthetic Chemistry
Institute of Health Biosciences and
Graduate School of Pharmaceutical Sciences
The University of Tokushima
Shomachi, Tokushima 770-8505 (Japan)
E-mail: ashige@ph.tokushima-u.ac.jp (A. Shigenaga)
E-mail: aotaka@ph.tokushima-u.ac.jp (A. Otaka)
[b] *Dr. L. Miyamoto, Prof. Dr. K. Ishizawa, Prof. Dr. K. Tsuchiya*
Department of Medical Pharmacology
Institute of Health Biosciences and
Graduate School of Pharmaceutical Sciences
The University of Tokushima
Shomachi, Tokushima 770-8505 (Japan)

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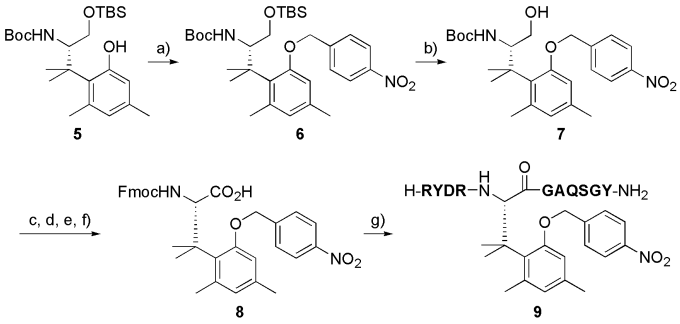
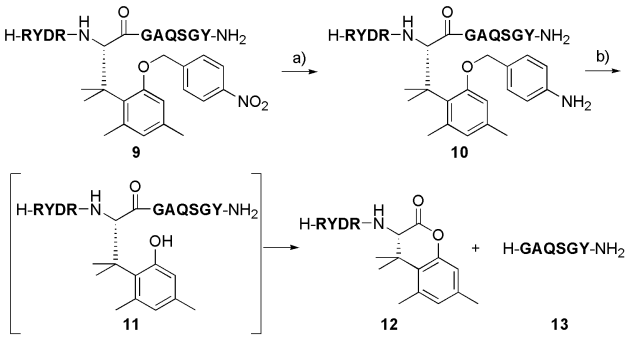


Scheme 2. Reagents and conditions. a) *p*-Nitrobenzyl bromide, K_2CO_3, DMF, 99%; b) AcOH aq., THF, quant.; c) PDC, DMF; d) $NaClO_2$, 2-methyl-2-butene, NaH_2PO_4, acetone, H_2O; e) HCl, AcOEt; f) FmocOSu, Na_2CO_3 aq., MeCN, 97% (4 steps); g) Fmoc SPPS. (**A**: alanine; **D**: aspartic acid; **G**: glycine; **Q**: glutamine; **R**: arginine; **S**: serine; **Y**: tyrosine).

To examine whether a reduction of the nitro group triggers the peptide bond cleavage, chemical reduction of peptide **9** was performed as shown in Scheme 3. To peptide **9** in aqueous solution of NH_4Cl was added a zinc powder under slightly acidic conditions, and the reaction mixture was incubated at 37 °C. During the reaction and HPLC purification under acidic conditions, removal of the *p*-aminobenzyl group of **10** was not observed. The obtained aniline **10** was incubated under physiological conditions (pH 7.4 of phosphate buffer, 37 °C). The reaction progress was monitored by HPLC, and the peptides were characterized by ESI-MS (Figure 2A). After 24 h of incubation, peptide **10** was completely converted to corresponding peptide fragments **12** and **13**. Intermediate **11** was not observed during the reaction; therefore, we concluded that the rate determining step was the removal of the *p*-aminobenzyl group. The disappearance of peptide **10** showed first-order dependence on the concentration of starting material **10**, and the half-life was determined as 3.1 h (Figure 2B). Because many tumor regions are known to be slightly acidic,[1] the half-life at pH 6.0 was also estimated, and was quite similar to that at pH 7.4 ($t_{1/2}$ = 3.7 h at pH 6.0).



Scheme 3. Reagents and conditions. a) Zn, NH_4Cl aq., 37 °C; b) Sodium phosphate buffer (pH 7.4 or 6.0, 20 mM), 37 °C.

Figure 2, see page 3

Next, we designed FRET (Fluorescence Resonance Energy Transfer)-based peptide **14** to demonstrate the capability of the reduction-responsive amino acid to induce peptide bond cleavage in living hypoxic cells (Scheme 4). Peptide **14** possesses a dabsyl group as a quencher, a fluoresceinyl group as a fluorophore,[9] and an octaarginine as a cell-penetrating peptide sequence.[10] Once added to a cell, peptide **14** should penetrate into the cell, but it does not show fluorescence because of quenching by FRET. Upon the peptide bond cleavage, strong fluorescence of generated peptide fragment **15** can be observed. Peptide **14** and reference peptide **16**, possessing a tyrosine residue instead of the reduction-responsive amino acid, were synthesized using Fmoc SPPS (details are shown in Supporting Information). The peptide was incubated with Caco-2 cells under hypoxic (1% (v/v) O_2 and 5% (v/v) CO_2 in N_2) or aerobic (5% (v/v) CO_2 in air) conditions for 15 h, respectively. After exchanging medium followed by fluorescence microphotography, fluorescence intensity per cell area was calculated based on the fluorescence image. As shown in Figure 3, strong fluorescence was observed only when the cells were treated with peptide **14** under hypoxic conditions. Therefore, it was demonstrated that the reduction-responsive amino acid can induce the hypoxia-responsive peptide bond cleavage in living cells.

Scheme 4, see page 4

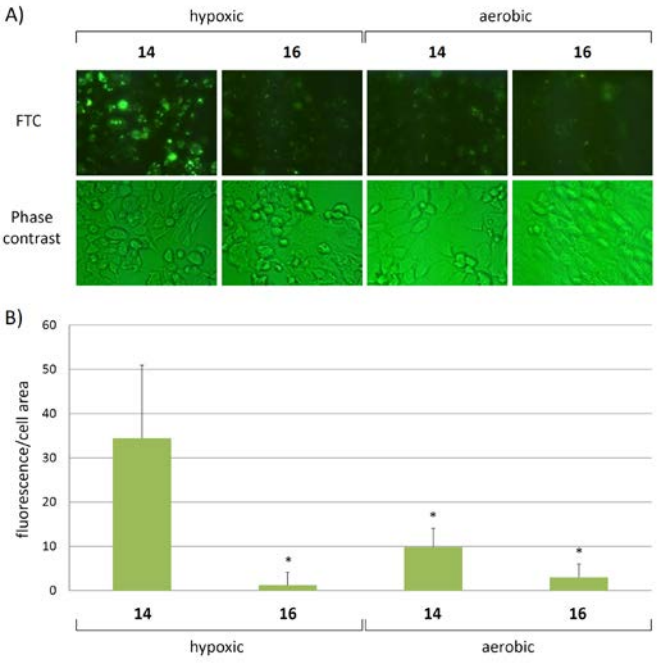


Figure 3. Visualization of peptide bond cleavage in living cells. Caco-2 cells were incubated with 300 nM peptide **14** or **16** under hypoxic (1% (v/v) O_2 and 5% (v/v) CO_2 in N_2) or aerobic (5% (v/v) CO_2 in air) conditions for 15 h. A) Microscopic images after exchanging medium. FTC: Fluorescence images at λ_{ex} = 460-490 nm and λ_{em} = 510 nm; B) Quantitative analysis of the fluorescence images. Fluorescence/cell area was average of 4 runs (10 cells/run), and statistical analyses were performed using Dunnett's test (*p<0.001). Error bars are + s.d.

In conclusion, we developed a reduction-responsive amino acid that induced peptide bond cleavage after reduction of a nitro group, and the cleavage products were obtained in high purity. Kinetic study of the peptide bond cleavage revealed that the half-life of peptide **10** at pH 6.0 was similar to that at pH 7.4. This suggests that the reduction-responsive amino acid could function even in acidic cancer cells. The capability of the reduction-responsive amino acid to induce hypoxia-responsive peptide

bond cleavage in living cells was then examined. Cell-based assay clarified that the peptide bond cleavage was efficiently induced only in the hypoxic cells. We believe that these results represent an indispensable step toward the development of hypoxia-responsive peptidyl prodrugs and bioprobes. Application of the reduction-responsive amino acid to a hypoxia-responsive antitumor prodrug is in progress.

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Keywords: hypoxia-responsive · peptide bond cleavage · reduction-responsive · stimulus-responsive · trimethyl lock

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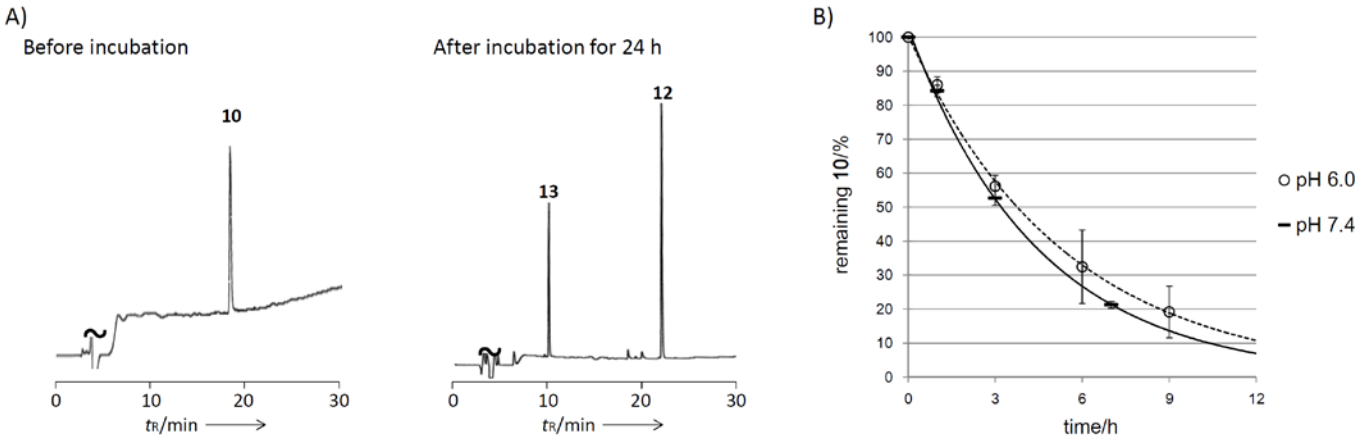
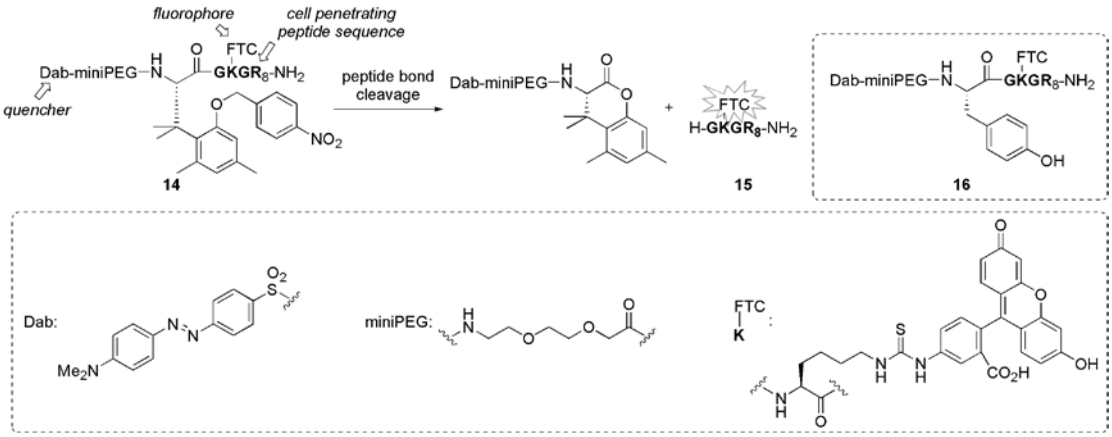


Figure 2. Reaction monitoring of the peptide bond cleavage of aniline **10** as shown in Scheme 3; A) HPLC profile of the peptide bond cleavage reaction. Analytical HPLC conditions: linear gradient of 0.1% (v/v) TFA/MeCN in 0.1% (v/v) TFA aq., 1-50% over 30 min; B) First order kinetic treatment of the data. Percentage of remaining substrate **10** was estimated based on HPLC peak area. Error bars are ± s.d.



Scheme 4. Design of FRET-based hypoxia-responsive peptide **14** and reference peptide **16**. (**G**: glycine; **K**: lysine; **R**: arginine)

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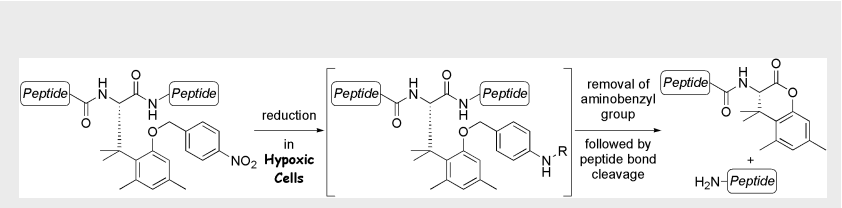

Utilization of a hypoxia-responsive amino acid is indispensable in the preparation of hypoxic tumor-specific peptidyl prodrugs. Bioreduction of a nitro group is among the most attractive triggering reactions in the hypoxia-responsive prodrugs. In this paper, design and synthesis of a reduction-responsive amino acid that induces peptide bond cleavage after reduction of the nitro group are described. Application to hypoxia-responsive peptide bond cleavage system is also reported.

A. Shigenaga, K. Ogura, H. Hirakawa, J. Yamamoto, K. Ebisuno, L. Miyamoto, K. Ishizawa, K. Tsuchiya, A. Otaka**

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