Enzyme-Responsive Drug Carriers

Targeted Enzyme-Responsive Drug Carriers: Studies on the Delivery of a Combination of Drugs**

*Myung-Ryul Lee, Kyung-Hwa Baek, Hui Juan Jin, Yong-Gyu Jung, and Injae Shin**

A wide variety of new synthetic compounds have been screened for chemotherapeutic applications. However, many drug candidates exhibit poor membrane permeability and/or severe side effects caused by their lack of selectivity. These problems can often be overcome by using highly specific chemotherapeutic agents. On the other hand, considerable efforts have been devoted to the creation of delivery systems targeting infected cells (or malignant tumors); these systems improve membrane permeability, therapeutic efficacy, and selectivity.[1] In a recent effort aimed at designing novel systems that release free drugs in a stringently controlled fashion, we have synthesized and characterized carrier–drug conjugates connected by linkers that are only cleaved by an enzyme present in the infected cells. In addition, we have

[*] M.-R. Lee, K.-H. Baek, H. J. Jin, Y.-G. Jung, Prof. Dr. I. Shin
Department of Chemistry
Yonsei University
Seoul 120-749 (Korea)
Fax: (+82) 2-364-7050
E-mail: injae@yonsei.ac.kr

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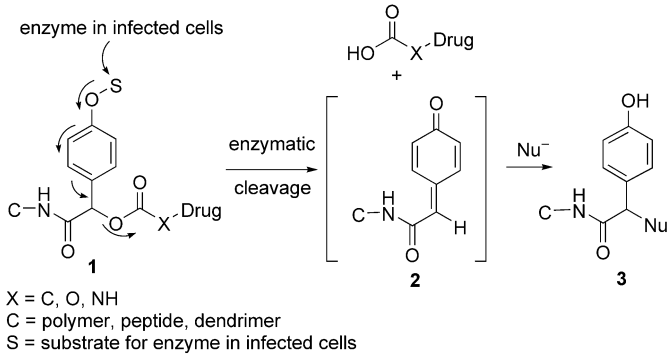
developed a new molecular system to deliver a combination of drugs to the target cells.

As shown in Scheme 1, 4-hydroxymandelic acid was used as the framework for constructing the enzyme-responsive

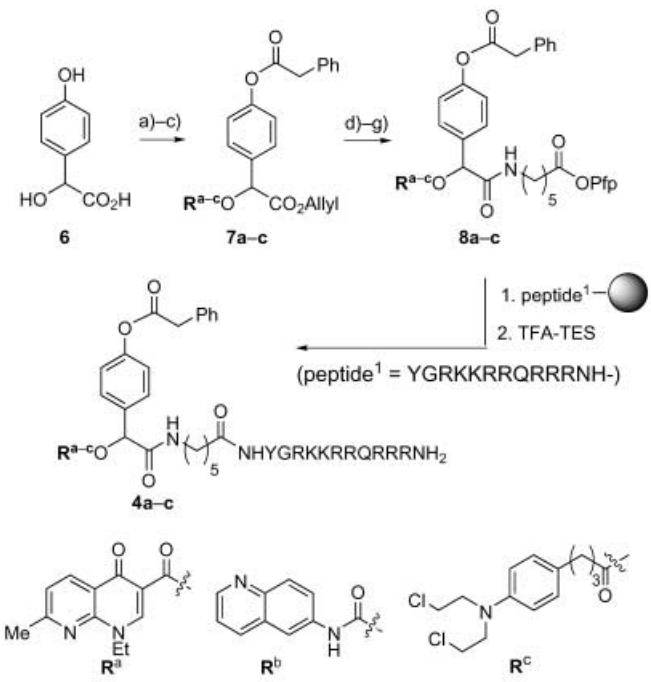


Scheme 1. General structure of carrier–drug conjugates linked by 4-hydroxymandelic acid. The pathway for enzyme-initiated release of free drugs is depicted.

linkers **1**.[2,3] Since the enzyme substrate portion in **1** is attached to the 4-hydroxy group of the mandelate core, the enzymatic cleavage processes should not be hindered by either the carrier or drug moieties attached at the *para* position. Enzymatic cleavage of the substrate portion in the infected cells will result in spontaneous release of the free drug by a 1,6-elimination process. The quinone methide intermediate **2** formed by this process will rapidly react with a nucleophile (for example, water) to generate **3**. Diverse drugs (or physical probes) containing OH, CO_2H, and NH_2 moieties can be coupled to the α-hydroxy group of the mandelate core through ether, ester, and carbamate linkages, respectively. Polymer, peptide, and dendrimer carriers can be coupled to the linker through amide bonds.[4]

To demonstrate the potential of this targeted enzyme-responsive drug-delivery system we synthesized carrier–small-molecule conjugates **4a–c** and **5**, which contain a phenylacetic acid residue as the substrate for cleavage by bacterial penicillin G amidase (PGA)[5] and a TAT peptide as the peptide carrier. TAT peptides are one of a number of protein-transduction domains that have received considerable attention as efficient delivery systems recently.[4a] These carriers mediate intracellular uptake of small molecules and macromolecules in a receptor-independent manner.[4a] We selected the TAT peptide as the carrier because of its facile preparation by solid-phase synthesis and its biodegradability, which will be desirable following drug release.

The synthetic pathways for preparation of the conjugates containing one (**4a**, nalidixic acid; **4b**, 6-aminoquinoline; **4c**, chlorambucil) or two (**5**, nalidixic acid and 6-aminoquinoline) small-molecule agents are illustrated in Schemes 2 and 3. After protection of the carboxylic acid in 4-hydroxymandelic acid (**6**) with an allyl group, selective phenylacetylation of the phenolic hydroxy group and subsequent coupling of nalidixic acid, 6-aminoquinoline, or chlorambucil ($\mathbf{R^{a-c}}$ moieties, respectively) to the α-hydroxy group gave **7a–c** (Scheme 2).

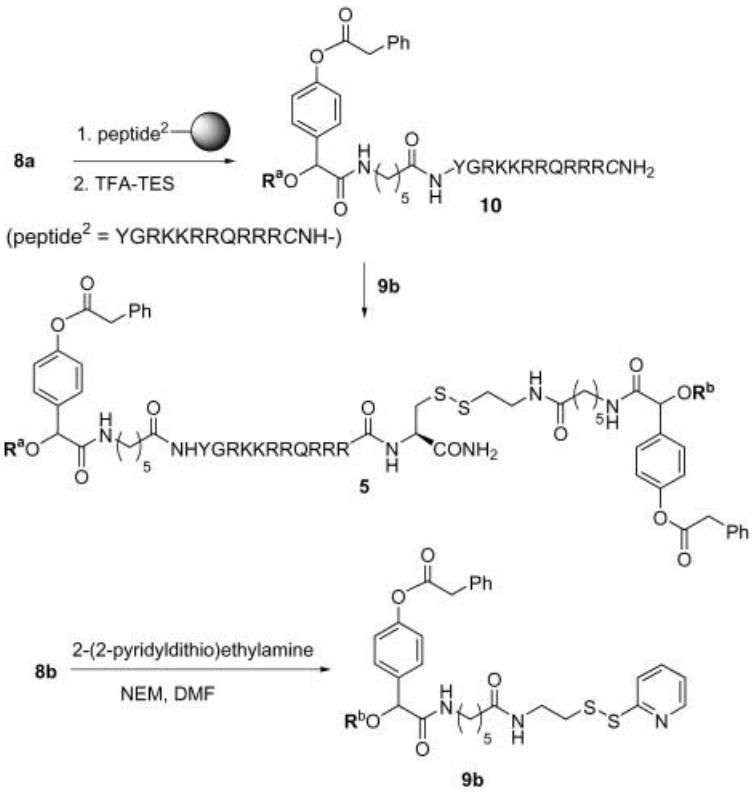


Scheme 2. Synthesis of conjugates **4**. Reagents: a) Cs_2CO_3, allyl bromide; b) phenylacetyl chloride, DIEA; c) **a**: nalidixic acid, EDC, DMAP; **b**: $COCl_2$, 6-aminoquinoline; **c**: chlorambucil, EDC, DMAP; d) $[Pd(PPh_3)_4]$, AcOH, NMA; e) *tert*-butyl-6-aminohexanoate, EDC, DMAP; f) TFA; g) pentafluorophenol, EDC. DIEA = diisopropylethylamine, DMAP = 4-dimethylaminopyridine, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, NMA = *N*-methylalanine, Pfp = pentafluorophenyl, TFA = trifluoroacetic acid, TES = triethylsilane.

Nalidixic acid is an inhibitor of DNA gyrase,[6] and chlorambucil is an antitumor agent known to cross-link DNA strands.[7] 6-Aminoquinoline has an emission maximum at 370 nm in its conjugated form and at 550 nm in its free state. Thus, conjugates **4b** and **5** bearing 6-aminoquinoline were prepared to probe the enzymatic cleavage in cells.[8]

After removal of the allyl group in **7a–c** and condensation of the exposed acid with *tert*-butyl-6-aminohexanoate followed by deprotection of the *tert*-butyl group, the resulting acids were converted into the pentafluorophenyl esters (**8a–c**) for facile coupling with the TAT peptide in the next step. Finally, conjugates **4a–c** were obtained by the coupling of **8a–c** with peptide[1] (YGRKKRRQRRR), which was prepared on the solid support by the 9-fluorenylmethoxycarbonyl/*t*Bu strategy, and cleavage from the solid support.[9]

Conjugate **5** containing two small molecules (nalidixic acid and 6-aminoquinoline) was prepared for the study of the delivery of a combination of drugs into target cells (Scheme 3). Compound **8a** was coupled to the N-terminal amino group of peptide[2] (YGRKKRRQRRRC), which has cysteine at the C terminus, on a solid support. After cleavage of conjugate **10** from the solid support, the thiol group of the cysteine residue in **10** was chemoselectively coupled to **9b**, which was prepared from condensation of **8b** and 2-(2-pyridyldithio)ethylamine, to produce **5**.[9] This coupling method was highly efficient for the synthesis of the conjugate containing two different molecules.



Scheme 3. Synthesis of conjugate **5**. DMF = *N*,*N*-dimethylformamide, NEM = *N*-ethylmorpholine.

Initial examination of the enzyme-promoted release of drugs or physical probes from these conjugates was performed by analyzing mixtures of **4a–c** incubated with PGA from *Escherichia coli* in sodium phosphate buffer (pH 7.4) at 37 °C for 30 min. HPLC analysis of each mixture after incubation revealed the presence of nalidixic acid, 6-aminoquinoline, or chloroambucil along with phenylacetic acid and substances that were judged by mass spectrometry to be the products of water addition to the quinone methide intermediates and cyclized products resulting from intramolecular reactions of the quinone methide with lysine or arginine side chains present in the TAT peptide (Figure 1a). Importantly, these compounds were not produced when the conjugates were incubated in PGA-free solutions.

The antibacterial properties of **4a** were then investigated by determining the minimum inhibitory concentrations (MICs) of **4a** and nalidixic acid against an *E. coli* HB101 strain transformed with a PGA gene.[9,10] The conjugate **4a** exhibited an MIC value about 70 times lower (1.9 μM) than that of free nalidixic acid (138 μM). Many cationic peptides have been known to have antimicrobial activities.[11] Therefore, we tested the antibacterial activity of the TAT peptide and found that it did not exhibit antibacterial activity up to a concentration of 657 μM. We also examined the possible synergistic effect of the TAT peptide and nalidixic acid on antibacterial activity. It was found that the MIC value of nalidixic acid was not altered by the addition of this peptide at a concentration of 140 μM. Therefore, the better therapeutic efficacy of **4a** relative to free nalidixic acid resulted from its direct conjugation to the TAT peptide, not from a combined antibacterial effect. Importantly, **4a** did not display an antibacterial effect (up to a concentration of 15.2 μM) on *E. coli* without a PGA gene. This demonstrated that release of the free drug from the conjugate was controlled by intracellular enzymes.

The release of small molecules by intracellular enzymes was also examined by using the fluorescent probe-containing conjugate **4b**. For this purpose, fluorescence microscopy was employed to analyze the incubation mixtures of **4b** (7.6 μM) and *E. coli* HB101 with and without a PGA gene. It was revealed that PGA-expressing cells exhibited green fluorescence when incubated with **4b** for 20 min, whereas the characteristic green fluorescence of 6-aminoquinoline was not observed in *E. coli* cells without a PGA gene. To show that the release process is cell selective, **4b** was incubated with a mixture of *E. coli* cells with and without the PGA gene (Figure 2a and b). The observation that 6-aminoquinoline fluorescence was seen only in cells with the PGA gene clearly demonstrates that the developed delivery system was cell selective.

We then compared the rate of bacterial cell death with **4a** and nalidixic acid by counting colony-forming units (CFUs) after a certain time of exposure to the compounds (Figure 3).[12] Conjugate **4a** killed PGA-containing bacteria more rapidly than free nalidixic acid did at concentrations of 3.8 and 276 μM, respectively (2 multiples of the MICs of **4a** and nalidixic acid, respectively). However, *E. coli* cells that did not

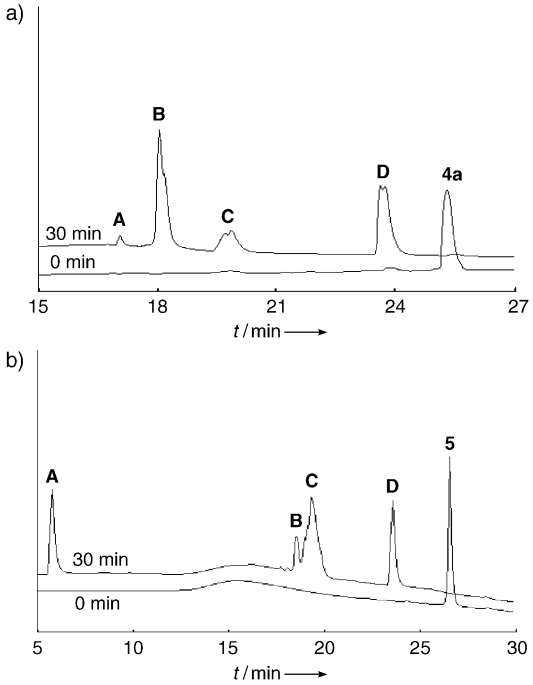


Figure 1. HPLC chromatogram of the incubation mixtures of a) **4a** or b) **5** with PGA. a) **A** = a water-quenched product ([*M*+1]: *m*/*z* = 1822); **B** = a cyclized product ([*M*+1]: *m*/*z* = 1804); **C** = phenylacetic acid; **D** = nalidixic acid. b) **A** = 6-aminoquinoline; **B** = a monocyclized product ([*M*+1]: *m*/*z* = 2245); **C** = a dicyclized product ([*M*+1]: *m*/*z* = 2227); **D** = nalidixic acid.

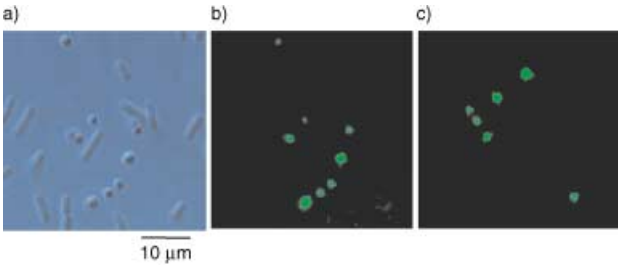


Figure 2. Images of the incubation mixture of **4b** and *E. coli* HB101 cells with and without the PGA gene: a) normal microsopic image, b) fluorescence image, c) fluorescence image of the incubation mixture of **5** and *E. coli* HB101 cells with the PGA gene.

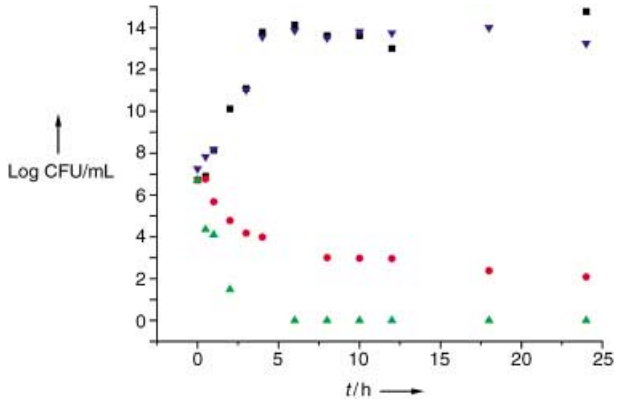


Figure 3. Rate of cell death caused by **4a** and nalidixic acid. ■: *E. coli* containing a PGA gene in the absence of active compounds; ▼: *E. coli* lacking a PGA gene in the presence of 3.8 μM **4a**; ●: *E. coli* containing a PGA gene in the presence of 276 μM nalidixic acid; ▲: *E. coli* containing a PGA gene in the presence of 3.8 μM **4a**.

contain a PGA gene grew at a similar rate in the presence of 3.8 μM **4a** as they did in the control experiment.

For over a decade, combination therapies have been exploited to improve therapeutic efficiency and/or to diminish the toxicity of drugs.[13] Although a variety of drug-delivery systems have been developed, these have rarely been designed to deliver drug combinations. We examined the enzyme-promoted release properties of conjugate **5**, which contained two different compounds. HPLC analysis of a mixture of **5** incubated with PGA for 30 min showed that nalidixic acid and 6-aminoquinoline were produced along with mono- and dicyclized products (Figure 1b). This observation indicates that the conjugate underwent efficient enzyme-responsive release of a combination of compounds.

To further investigate the possibility that two drugs could be delivered into cells, the same conjugate **5** (3.8 μM) was incubated with *E. coli* possessing the PGA gene. Fluorescence microscopy images of cells after incubation for 20 min showed that 6-aminoquinoline was released (Figure 2c). In addition, the bacterial cells were nearly completely killed by **5** after incubation for 8 h. These results clearly indicated that the designed systems were capable of delivering a combination of drugs to target cells.

In conclusion, we have developed an efficient enzyme-responsive drug-delivery system. In the studies described above, we have shown that the newly designed drug–carrier conjugates effectively and selectively kill only those *E. coli* cells containing a specific enzyme (for example, PGA). We also demonstrated that the carrier lowered the effective concentration of drug needed to kill the bacteria. Furthermore, we observed that two different compounds (a drug and a fluorescent probe) were released from a bisconjugate by an intracellular enzyme. This approach could be used for targeted combination therapy. The general method developed in this work may be useful for targeting virus-infected cells by using carrier–antivirus-drug conjugates connected by linkers that are viral-enzyme labile; malignant tumors with genes encoding specific enzymes could be induced to release free anticancer drugs from conjugates.[14]

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[1] a) M. Yokoyama, T. Okano, *Adv. Drug Delivery Rev.* **1996**, *21*, 77; b) K. Kataoka in *Controlled Drug Delivery: Challenges and Strategies* (Ed.: K. Park), American Chemical Society, Washington, **1997**, pp. 49–71.
[2] Enzyme-labile safety-catch linkers have been developed for solid-phase synthesis: a) R. Reents, D. A. Jeyaraj, H. Waldmann, *Adv. Synth. Catal.* **2001**, *343*, 501; b) U. Grether, H. Waldmann, *Angew. Chem.* **2000**, *112*, 1688; *Angew. Chem. Int. Ed.* **2000**, *39*, 1629; c) B. Sauerbrei, V. Jungmann, H. Waldmann, *Angew. Chem.* **1998**, *110*, 1187; *Angew. Chem. Int. Ed.* **1998**, *37*, 1143.
[3] During the preparation of this manuscript, a similar approach for the development of a tumor-targeting delivery system was reported: A. Gopin, N. Pessah, M. Shamis, C. Rader, D. Shabat, *Angew. Chem.* **2003**, *115*, 341; *Angew. Chem. Int. Ed.* **2003**, *42*, 327.
[4] a) S. R. Schwarze, S. F. Dowdy, *Trends Pharm. Sci.* **2000**, *21*, 45; b) A. T. Florence, N. Hussain, *Adv. Drug Delivery Rev.* **2001**, *50*, S69.
[5] J. Y. Lu, D. A. Lowe, M. D. Kennedy, P. S. Low, *J. Drug Targeting* **1999**, *7*, 43; b) V. M. Vrudhula, P. D. Senter, K. J. Fischer, P. M. Wallace, *J. Med. Chem.* **1993**, *36*, 919.
[6] W. A. Goss, W. H. Deitz, T. M. Cook, *J. Bacteriol.* **1965**, *89*, 1068.
[7] S. R. Rajski, R. M. Williams, *Chem. Rev.* **1998**, *98*, 2723.
[8] R. P. Haugland, *Handbook of Fluorescenct Probes and Research Chemicals*, 6th ed., Molecular Probes, Eugene, **1996**, chap. 10.
[9] See the Supporting Information.
[10] MIC values were determined with an inoculum of 10^7 CFU mL^{-1} of *E. coli*; "Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically": Approved Standards M7-A4, 4th ed., National Committee for Clinical Laboratory Standards, Wayne, PA, **1997**.
[11] R. E. Hancock, R. Lehrer, *Trends Biotechnol.* **1998**, *16*, 82.
[12] S. Navon-Venezia, R. Feder, L. Gaidukov, Y. Carmeli, A. Mor, *Antimicrob. Agents Chemother.* **2002**, *46*, 689.
[13] a) E. Gradelski, B. Kolek, D. P. Bonner, L. Valera, B. Minassian, J. Fung-Tomc, *Int. J. Antimicrob. Agents* **2001**, *17*, 103; b) H. C. Neu, *Rev. Infect. Dis.* **1989**, *11*(Suppl. 5), S1025.
[14] a) K. W. Culver, Z. Ram, S. Wallbridge, H. Ishii, E. H. Oldfield, R. M. Blaese, *Science* **1992**, *256*, 1550; b) B. E. Huber, C. A. Richards, E. A. Austin, *Ann. N.Y. Acad. Sci.* **1994**, *716*, 104; c) T. A. Connors, *Gene Ther.* **1995**, *2*, 235; d) G. Cavallaro, G. Pitarresi, M. Licciardi, G. Giammona, *Bioconjugate Chem.* **2001**, *12*, 143.

