

Endocytic Delivery of Vancomycin Mediated by a Synthetic Cell Surface Receptor: Rescue of Bacterially Infected Mammalian Cells and Tissue Targeting In Vivo

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Receptor-mediated drug delivery is a Trojan horse approach for the release of therapeutics into cells, tissues, or organs. By coupling drugs to ligands of cell surface receptors, ligand–drug conjugates can accumulate in specific tissues and can be internalized by cells through the mechanism of receptor-mediated endocytosis.¹ Receptors targeted in this way include the low-density lipoprotein (LDL) receptor,² transferrin receptors,³ and folate receptors.⁴ Receptors involved in the passage of nutrients across the blood–brain barrier through the related mechanism of receptor-mediated transcytosis have been similarly targeted to deliver drugs and imaging agents into the brain.⁵ This latter strategy is of significant interest because less than 2% of all drugs are capable of penetrating the blood–brain barrier.⁵

We report here a method for synthetic receptor-mediated drug delivery. This approach was applied to the glycopeptide antibiotic vancomycin (**1**), a drug of last resort against bacterial pathogens

(**3**) comprising the vancomycin-binding dipeptide D-Phe-D-Ala⁷ linked to an *N*-alkyl derivative of 3 β -cholesterylamine. When added to mammalian cells, this cholesterol-derived membrane anchor is known to access a membrane trafficking pathway involving rapid cycling between the plasma membrane and intracellular endosomes.⁸ Control compounds that do not bind vancomycin (L-Phe-L-Ala analogue **4**) or exhibit lower affinity for the plasma membrane (amide analogue **5**) were synthesized for comparison. Other vancomycin-binding motifs linked to lipids have been described previously.^{9–12}

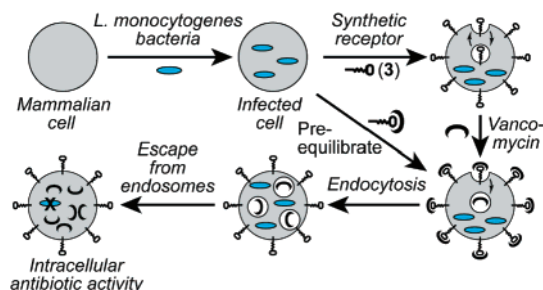
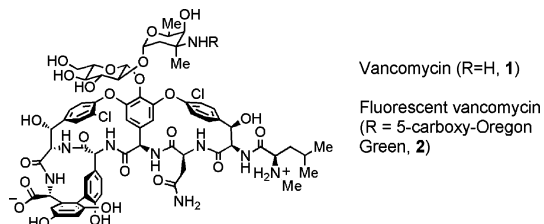
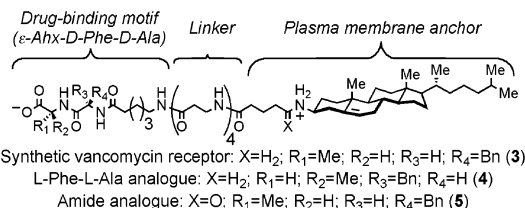


Figure 1. Delivery of vancomycin (**1**) into mammalian cells by synthetic receptor targeting. Delivery can be achieved by preloading the plasma membrane with synthetic receptor **3** followed by addition of vancomycin (**1**), or a pre-equilibrated complex of **1** and **3** can be added to living cells.

such as methicillin-resistant *Staphylococcus aureus*.⁶ By binding to peptidoglycan precursors that terminate in the peptide sequence D-Ala-D-Ala, vancomycin (**1**) blocks cross-linking of the bacterial cell wall, providing an effective mode of action against gram-positive bacteria. However, the high polarity and consequent low cell permeability of glycopeptide antibiotics render these drugs ineffective against bacteria capable of replicating either in the cytoplasm of mammalian host cells or in organs such as the brain that are protected by membrane barriers.

We hypothesized that a synthetic receptor capable of promoting the endocytosis of vancomycin (**1**) might allow this cell-impermeable antibiotic to access the cytoplasm of cells infected with pathogenic bacteria and cross tissue barriers that restrict the delivery of this drug. To test these hypotheses, we synthesized a fluorescent derivative of vancomycin (**2**) and an artificial cell surface receptor

We investigated the effects of **3** and **1** on mammalian cells infected with the pathogen *Listeria monocytogenes*. This facultative gram-positive intracellular bacterium can replicate in the cytoplasm of mammalian host cells and can cause fatal food poisoning in humans.¹³ Insertion of **3** into the plasma membrane of mammalian cells, binding of the D-Phe-D-Ala motif of **3** to **1** (or **2**), and cycling of **3** between the cell surface and endosomes were proposed to shuttle glycopeptides into endosomes. In mammalian cells infected with *L. monocytogenes*, we hypothesized that endosomes would be disrupted,¹⁴ allowing the antibiotic to enter the cytoplasm. This delivery strategy, termed synthetic receptor targeting,⁸ is illustrated in Figure 1. Previous studies of vancomycin in liposomes¹⁵ and other strategies for chemical modification of cell surfaces¹⁶ have been reported.



Confocal laser scanning microscopy and flow cytometry were used to examine the delivery of fluorescent vancomycin (**2**) into J-774 mouse macrophage cells and human HeLa cells. Treatment with **2** alone did not appreciably enhance cellular fluorescence, but cells treated with **3** and **2** internalized this fluorescent probe (Figures 2 and 3). In non-infected cells, this intracellular fluorescence was localized in endosomes and lysosomes as evidenced by colocalization with red fluorescent low-density lipoprotein (DiI-LDL)¹⁷ (images provided in the Supporting Information). However, in living

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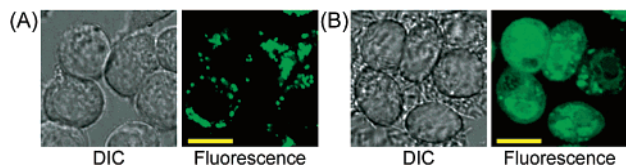


Figure 2. Confocal laser scanning and differential interference contrast (DIC) microscopy of living J-774 macrophages alone (panel A) and infected with *L. monocytogenes* (panel B). Prior to microscopy, receptor **3** ($10\ \mu\text{M}$) was added to cells for 1 h at $37\ ^\circ\text{C}$, cells were washed, and **2** ($3.6\ \mu\text{M}$) was added for 2 h at $37\ ^\circ\text{C}$. Scale bar: $10\ \mu\text{m}$.

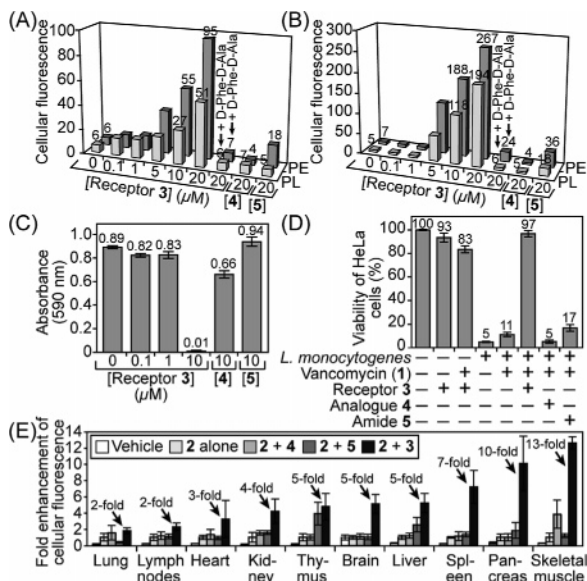


Figure 3. Panels A and B: Flow cytometric analysis of uptake of **2** by J-774 cells (A) and HeLa cells (B) promoted by **3**–**5**. PL = Preequilibration conditions: **3**, **4**, or **5** was added to cells for 1 h at $37\ ^\circ\text{C}$, cells were washed, and **2** ($3.6\ \mu\text{M}$) was added for 4 h at $37\ ^\circ\text{C}$. PE = Preequilibration conditions: **3**, **4**, or **5** was combined with **2** ($3.6\ \mu\text{M}$) at $23\ ^\circ\text{C}$ for 1 h followed by addition to cells for 4 h at $37\ ^\circ\text{C}$. [D-Phe-D-Ala] = $2\ \text{mM}$. Panels C and D: Effects of preequilibrated **3** and **1** on HeLa cells infected with *L. monocytogenes*. Infected cells were treated with **1** ($50\ \mu\text{M}$) and **3**, **4**, or **5** ($10\ \mu\text{M}$) for 6 h at $37\ ^\circ\text{C}$. Cells were subsequently washed with media lacking antibiotics. Panel C: Viability of *L. monocytogenes* cultured from infected HeLa cells. Bacterial growth after 18 h at $30\ ^\circ\text{C}$ was quantified by absorbance. Panel D: Viability of HeLa cells after 40 h at $37\ ^\circ\text{C}$ quantified by flow cytometry. Panel E: Delivery of **2** into tissues of Balb/c mice in vivo. Compounds were injected ip ($50\ \text{mg}/\text{kg}$), mice were sacrificed after 8 h, and fluorescence of cells from tissues was analyzed by flow cytometry. Error bars reflect the standard deviation ($n = 3$).

J-774 cells infected with *L. monocytogenes*, the fluorescence of **2** could be observed distributed throughout the cytoplasm and nucleus (Figure 2, panel B), presumably due to disruption of endosomes/phagosomes by the pathogen.

The dose-dependent effectiveness of delivery of **2** into mammalian cells mediated by receptor **3** was examined quantitatively by flow cytometry. As shown in Figure 3 (panels A and B), preequilibration of **3** with **2** prior to addition to cells was ca. 2-fold more effective for delivery compared with preloading of cellular plasma membranes with **3**, washing of cells to remove unincorporated receptor, and subsequent addition of **2**. However, both conditions substantially enhanced ligand uptake. Addition of the soluble competitor AcNH-D-Phe-D-Ala- CO_2H blocked these effects, and treatment with the L-Phe-L-Ala-analogue **4** did not enhance cellular fluorescence, demonstrating a requirement for specific recognition, and further confirming that **3** does not enhance cellular fluorescence by disruption of plasma membranes. Consistent with previous studies of amide derivatives of 3β -cholesterylamine,⁸ analogue **5** was much less active.

Treatment of HeLa cells with receptor **3** at $20\ \mu\text{M}$ for 1 h, followed by washing of cells to remove unincorporated receptors, installed an average of ~ 19 million receptor molecules per cell surface. This value was determined by flow cytometry at $4\ ^\circ\text{C}$ to block endocytosis⁸ (images and methods provided in the Supporting Information). Under the same conditions, J-774 cells incorporated an average of ~ 1.5 million receptors per cell surface.

The ability of **3** to deliver vancomycin (**1**) into HeLa cells infected by *L. monocytogenes* was examined. As shown in Figure 3 (panel C), **3** ($10\ \mu\text{M}$) uniquely enabled **1** to eliminate this intracellular microorganism. Control compound **4** or **5** did not confer antibiotic activity under the same conditions. Moreover, the combination of **3** and **1** rescued HeLa cells from the lethal effects of this pathogen (Figure 3, panel D), whereas neither **4** nor **5** combined with **1** blocked the toxicity of *L. monocytogenes*.

We next examined the potential of synthetic receptor targeting in vivo by injecting fluorescent vancomycin (**2**) into mice. Balb/c mice were injected intraperitoneally (ip) with **2** or premixed solutions of **2** combined with **3** or control compounds (**4**, **5**), and fluorescence of cells isolated from specific tissues was quantified by flow cytometry. As shown in Figure 3, **3** uniquely enabled **2** to accumulate in the brain and other tissues with no apparent toxicity. This delivery was highly dependent on molecular structure; altering the stereochemistry of the vancomycin-binding motif (**4**) or acylation of 3β -cholesterylamine (**5**) substantially reduced tissue targeting in vivo.

Our results demonstrate that a simple synthetic cell surface receptor (**3**) derived from *N*-alkyl- 3β -cholesterylamine can enhance the uptake of a glycopeptide antibiotic by mammalian cells, enabling efficacy against intracellular bacteria in vitro. Importantly, this receptor (**3**) can define new pathways across biological membrane barriers both in vitro and in vivo, including the blood–brain barrier. Small synthetic mimics of cell surface receptors hold significant promise as new tools for drug delivery.

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Supporting Information Available: Supporting figures (S1–S2), synthetic methods, compound characterization data and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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