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Published on Web 05/04/2002

Efficient Delivery of Streptavidin to Mammalian Cells: Clathrin-Mediated Endocytosis Regulated by a Synthetic Ligand

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Received February 8, 2002

Abstract: The efficient delivery of macromolecules to living cells presents a formidable challenge to the development of effective macromolecular therapeutics and cellular probes. We describe herein a novel synthetic ligand termed "Streptaphage" that enables efficient cellular uptake of the bacterial protein streptavidin by promoting noncovalent interactions with cholesterol and sphingolipid-rich lipid raft subdomains of cellular plasma membranes. The Streptaphage ligand comprises an *N*-alkyl derivative of 3β-cholesterylamine linked to the carboxylate of biotin through an 11-atom tether. Molecular recognition between streptavidin and this membrane-bound ligand promotes clathrin-mediated endocytosis, which renders streptavidin partially intracellular within 10 min and completely internalized within 4 h of protein addition. Analysis of protein uptake in Jurkat lymphocytes by epifluorescence microscopy and flow cytometry revealed intracellular fluorescence enhancements of over 300-fold (10 μM ligand) with >99% efficiency and low toxicity. Other mammalian cell lines including THP-1 macrophages, MCF-7 breast cancer cells, and CHO cells were similarly affected. Structurally related ligands bearing a shorter linker or substituting the protonated steroidal amine with an isosteric amide were ineffective molecular transporters. Confocal fluorescence microscopy revealed that Streptaphage-induced uptake of streptavidin functionally mimics the initial cellular penetration steps of Cholera toxin, which undergoes clathrin-mediated endocytosis upon binding to the lipid raft-associated natural product ganglioside GM1. The synthetic ligand described herein represents a designed cell surface receptor capable of targeting streptavidin conjugates into diverse mammalian cells by hijacking the molecular machinery used to organize cellular membranes. This technology has potential applications in DNA delivery, tumor therapy, and stimulation of immune responses.

Introduction

The effectiveness of therapeutics and cellular probes depends critically on the efficient delivery of molecules into living cells. Whereas many hydrophobic compounds of low molecular weight diffuse freely through low-polarity cell membranes, macromolecules such as proteins and DNA typically require active transport mechanisms to gain access to intracellular receptors.[1] To enhance the cellular uptake of poorly permeable molecules, covalent or noncovalent modification by cationic, hydrophobic, or amphipathic polymers[2−9] or hydrophobic lipids[10−12] can favorably modify the chemical properties of molecular cargo. Alternatively, cellular membranes can be chemically altered or permeabilized to facilitate macromolecular uptake.[13−15] However, the molecular mechanisms underlying many of these methods are not well understood. As a consequence, efficiencies of macromolecular delivery are often limited by variability, toxicity, and unpredictable cell-type specificity. Hence, novel methods that enhance uptake of poorly permeable molecules are needed in basic cell biology, tumor therapy, and genetic therapy.

Mammalian cells internalize specific small molecules, macromolecules, and particles through endocytosis, an active transport process dependent on time, temperature, pH, and energy.[16,17] This process targets extracellular materials to specific membrane-sealed compartments termed endosomes within the cytoplasm. The most common fate of molecules internalized in

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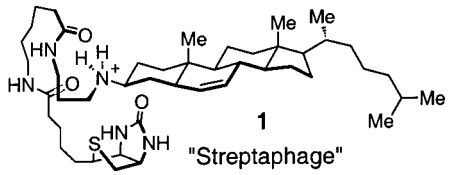
this fashion is degradation or recycling of material back to the cell surface, but internalized substances can also be released into the cytosol.[16,17] One of the most important and best-characterized endocytic mechanisms is receptor-mediated endocytosis via clathrin-coated pits. Clathrin is a protein that assembles at cellular plasma membranes into a basketlike concave framework of hexagons and pentagons that form pit structures.[18−20] Some endogenous proteins, such as the membrane-associated epidermal growth factor receptor, become clustered in coated pits upon binding to soluble ligands. This clustering results in invagination of the pit to form clathrin-coated vesicles that internalize the receptor−ligand complex and enable activation of signal transduction pathways.[16] Internalized clathrin-coated vesicles become intracellular endocytic compartments that are acidified to between pH 6.5 and 5.0 as a consequence of activation of proton pumps in endosomal membranes. This acidification activates proteolytic and other enzymes and promotes dissociation of ligands from bound receptors. Many membrane-associated receptors are subsequently recycled back to the cell surface, whereas ligands are typically degraded. This and other endocytic mechanisms are exploited by many viruses, toxins, and symbiotic microorganisms to gain entry into cells.[16]

Cholera toxin produced by *Vibrio cholerae* comprises a protein complex that penetrates cells by co-opting the molecular machinery controlling clathrin-mediated endocytosis.[21,22] This toxin consists of an A (activating) subunit that activates intracellular adenylyl cyclase activity and a pentameric B (binding) subunit that binds with high affinity to the plasma membrane-associated small molecule ligand, ganglioside GM1.[23] The GM1 ligand is a sphingolipid comprising a pentasaccharide linked to the lipid ceramide (*N*-acyl sphingosine). Each monomer of the toxin B subunit binds to the pentasaccharide moiety of GM1, which is localized to specific lipid raft domains in cellular plasma membranes. These lipid rafts form a liquid-ordered phase that is enriched in cholesterol and sphingolipids, and lipid rafts play key functional roles in segregation and concentration of membrane proteins.[24−26] Many proteins linked to cholesterol, glycosylphosphatidyl inositol (GPI), or saturated alkyl chains are localized to lipid rafts,[27] which are essential membrane features that control activation of numerous signal transduction pathways.[28] Binding of Cholera toxin to GM1 targets this protein to lipid rafts, promoting clathrin-mediated endocytosis through endogenous vesicular transport mechanisms.[21,22,29] In addition to Cholera toxin, other proteins that undergo clathrin-mediated endocytosis upon binding to lipid rafts include the epidermal growth factor receptor[30,31] and Shiga toxin.[32]

We report here the synthesis of a novel cholesterol-derived ligand (**1**) termed here "Streptaphage" (one that eats streptavidin). This ligand associates with lipid raft subdomains in plasma membranes of mammalian cells and efficiently promotes dose-dependent cellular uptake of the bacterial protein streptavidin (SA) through the mechanism of clathrin-mediated endocytosis. This compound is structurally related to a previously reported fluorescent cholesterylamine derivative that promotes endocytic cellular uptake of antifluorescein antibodies and associated Protein A from *Staphlococcus aureus*.[33]



Streptaphage (**1**) was designed to enable uptake of SA protein by promoting strong noncovalent interactions with lipid rafts of cellular plasma membranes. This compound comprises an *N*-alkyl derivative of 3β-cholesterylamine[34] linked to the carboxylate of D-biotin (vitamin H) through an 11-atom tether. The cholesterylamine moiety was chosen as a membrane anchor because cholesterol is a highly abundant membrane-associated steroid that is functionally linked to endocytosis[35−37] and is covalently attached to proteins involved in signal transduction.[28,38]

Streptaphage (**1**) differs from cholesterol by substituting a 3β-*N*-alkylamino headgroup for the 3β-cholesterol hydroxyl group to afford an amphiphilic molecule that is protonated at physiological pH (7.4). This amino group was incorporated because 3β-cholesterylamine binds much more tightly to model membranes than cholesterol as evidenced by ca. 30-fold slower off-rates of intervesicle transfer.[34] Structurally related cationic cholesterol derivatives that form nontoxic liposome complexes with DNA have been used as cellular transfection reagents.[39] Other synthetic cholesterylamine derivatives linked to the protein ligands estradiol[40] and mannose-6-phosphate[41] have been previously described, and lipid derivatives of biotin have also been reported.[42,43]

The biotin moiety of Streptaphage (**1**) provides a very high-affinity ligand ($K_d \sim 100$ fM)[44] for SA. The SA-biotin system is one of the strongest receptor−ligand interactions found in nature, and this complex has been extensively studied by thermodynamic, kinetic, and mutagenic methods in solution,[44]

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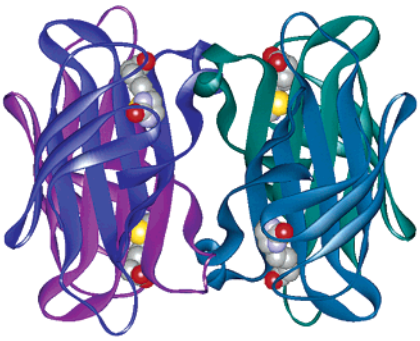
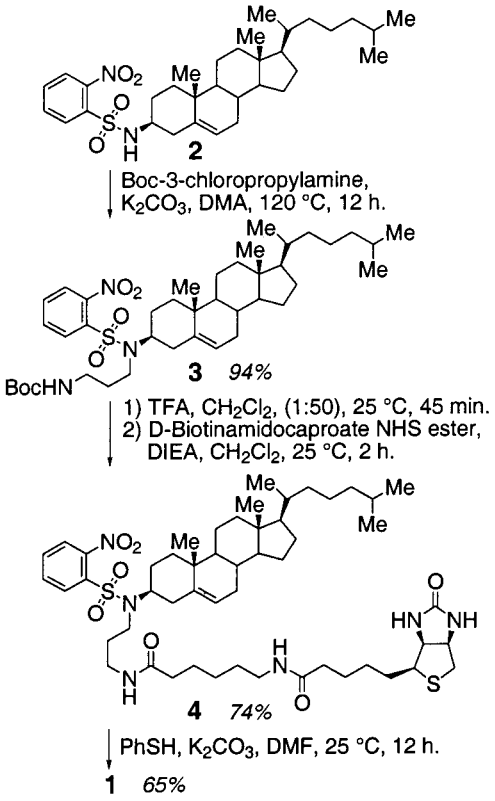


Figure 1. X-ray crystal structure (PDB 1SWR) of tetrameric streptavidin (ribbon diagrams) bound to biotin (CPK models).

in the solid state,[45−47] and at solid−liquid interfaces.[48−50] Structural studies have revealed that SA forms a tetramer that binds four biotin molecules positioned in pairs at opposite faces of the protein (Figure 1). The ease of functionalization of biomolecules with biotin or SA has made this system extremely useful for myriad biotechnological applications such as affinity separations,[51−54] diagnostic assays,[53−55] biomolecular imaging, and the delivery of therapeutics.[56−66] In addition, numerous conjugates of SA with fluorophores, enzymes, probes, and particles have been reported and many are commercially available.

Streptavidin is a particularly interesting target for enhanced cellular uptake because this protein has clinical applications in anticancer therapies employing monoclonal antibodies that bind tumor-associated antigens.[67−70] Pretargeting anticancer immunotherapeutics covalently link either SA or biotin to a monoclonal antibody that specifically recognizes tumor cells. Infusion of this antibody conjugate results in binding to specific tumor-associated antigens in vivo. Biotin or SA conjugates are subsequently administered to deliver toxins or imaging agents specifically to tumor cells. By uncoupling the kinetically slow step of antibody binding to tumor antigens from the fast kinetics of streptavidin−biotin recognition, this approach minimizes the duration of exposure to conjugated anticancer agents.[71] However, efficient endocytosis of these antibody conjugates by tumor cells is necessary to achieve optimal therapeutic effects, and many tumor antigens are not rapidly internalized. Hence, the identification of compounds that improve the delivery of SA-linked therapeutics into tumor cells may enhance the effectiveness of these agents.[72] We report here the synthesis of a nontoxic ligand that enables regulated dose-dependent delivery of SA conjugates into diverse mammalian cell lines through the well-characterized mechanism of clathrin-mediated endocytosis.

Scheme 1



Results and Discussion

Synthesis of Cholesterylamine Derivatives. Streptaphage (**1**) was prepared from the known[33] 2-nitrobenzenesulfonamide-protected cholesterylamine **2** as shown in Scheme 1. This approach employed the amine synthesis methodology of Fukuyama[73] to couple Boc-3-chloropropylamine[74] and install the protected primary amine of **3**. Removal of the Boc carbamate

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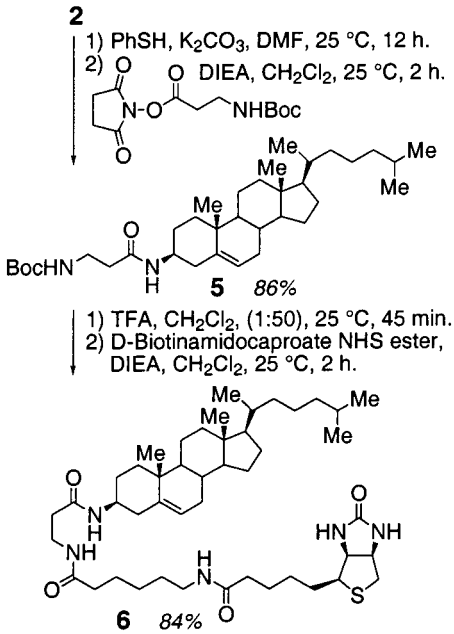
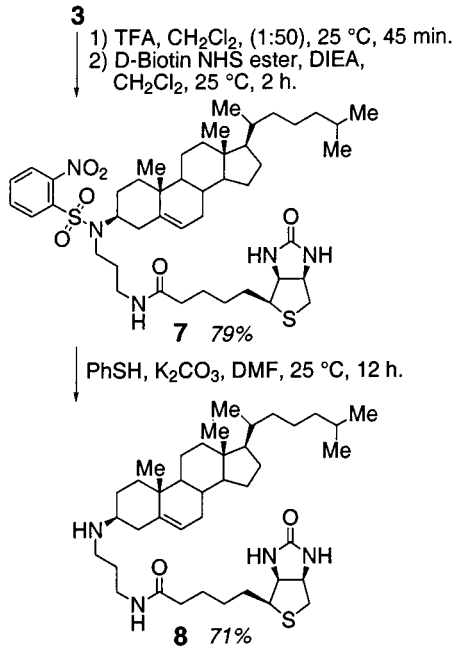
Scheme 2



Scheme 3



and acylation with commercially available D-biotinamidocaproate *N*-hydroxysuccinimidyl ester added the biotin side chain of **4**, which was further deprotected to afford **1**.

To probe the importance of the protonated cholesterylamine headgroup of Streptaphage (**1**), the isosteric amide **6** was prepared as shown in Scheme 2. In addition, the length of the linker between biotin and the steroid was investigated by synthesis of the shorter chain variant **8** as shown in Scheme 3. To compare the influence of amide and amine steroid headgroups on affinity for cellular plasma membranes, the related fluorescent probes **9** and **10** were synthesized as shown in Scheme 4.

Ligand-Regulated Uptake of SA by Mammalian Cells. To qualitatively evaluate whether combinations of these compounds and SA would affect mammalian cells, compound/SA-treated Jurkat T-lymphocytes were analyzed by epifluorescence

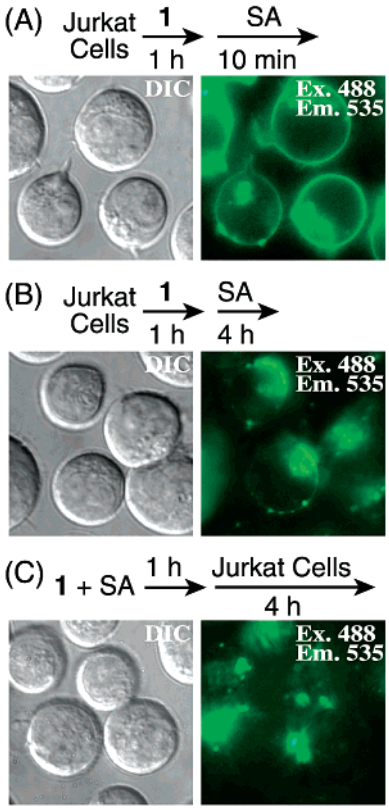
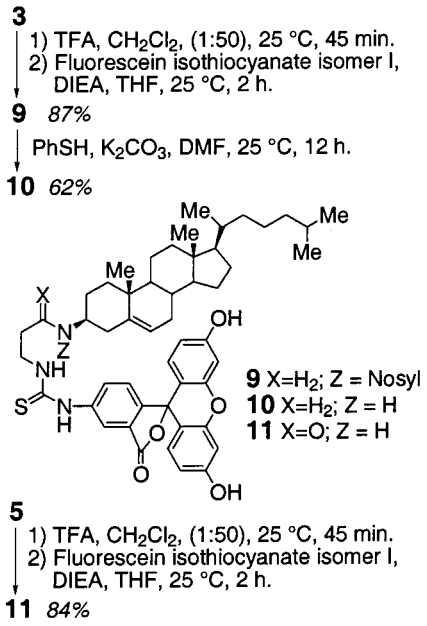


Figure 2. Differential interference contrast (DIC) and epifluorescence micrographs of Jurkat lymphocytes treated with Streptaphage (**1**, 10 μM) and green fluorescent SA Alexa Fluor 488 (20 μg/mL). Fluorescence excitation (Ex.) and emission (Em.) wavelengths (nm) are explicitly shown.

Scheme 4



microscopy. As shown in Figure 2 (Panel A), cells treated with Streptaphage (**1**) for 1 h followed by addition of SA conjugated to green fluorescent Alexa Fluor 488 revealed bright fluorescence at the cellular periphery of >99% of viable cells within 10 min of addition of the protein conjugate. This peripheral fluorescence was primarily localized at the cellular plasma membrane as determined by comparison with cells treated with previously described fluorescent plasma membrane probes.[33,75]

Remarkably, cells examined 4 h after protein addition revealed bright green fluorescence nearly exclusively in defined compartments in the interior of practically all viable cells, indicating that the complex between SA and **1** was internalized (Figure 2, Panel B). This effect was also observed when SA and **1** were preequilibrated prior to addition of the complex to cells (Figure 2, Panel C), even though partial precipitation of the preformed amphipathic protein−ligand complex was observed. Surprisingly, SA addition to cells treated with the isosteric amide analogue **6** or the short-chain analogue **8** did not appreciably affect cellular fluorescence (micrographs provided in the Supporting Information).

To quantify the compound dose-dependent uptake of SA observed by microscopy, treated cells were analyzed by flow cytometry. As shown in Figure 3 (Panel A), pretreatment of cells with **1** for 1 h followed by addition of green fluorescent SA for 4 h resulted in remarkable enhancements of cellular fluorescence with significant effects observed at concentrations of **1** as low as 100 nM. The intracellular fluorescence of cells pretreated with 10 μM **1** was increased by over 300-fold compared to cells treated with SA alone. Competition by addition of D-biotin (100 μM) completely blocked uptake promoted by **1** (1 μM), confirming that SA interacts specifically with **1** at plasma membranes. Preequilibration of SA and **1** also engendered dose-dependent uptake of SA, but the magnitude of the effect was diminished by 10-fold at 10 μM **1** (Figure 3, Panel B). This reduction was a consequence of aggregation and precipitation of the preformed SA-**1** complex as observed by microscopy. In contrast, compounds **6** and **8** did not substantially enhance uptake of SA by Jurkat lymphocytes. At compound concentrations of 10 μM, the uncharged amide **6** conferred only a 3-fold enhancement of cellular fluorescence and the shorter chain variant **8** enhanced cellular fluorescence by only 5-fold (Figure 3, Panels C and D). This result illustrated the importance of the protonated secondary amine of **1**, and revealed the dramatic influence of linker length on biological activity. Significant effects of the linker length on the affinity of other biotin derivatives for avidin and streptavidin proteins have been previously reported.[76,77]

Since cholesterol is a critical component of mammalian plasma membranes, the cholesterylamine moiety of Streptaphage (**1**) should enable delivery of SA into numerous mammalian cell lines. To test this hypothesis, uptake of SA by the suspension macrophage cell line THP-1, the adherent breast cancer cell line MCF-7, and adherent chinese hamster ovary (CHO) cells was analyzed by flow cytometry and epifluorescence microscopy. To release adherent cell lines from flask surfaces, cells were treated with the protease trypsin for analysis by flow cytometry.[78] As shown in Figure 3 (Panel E), flow cytometry revealed significant compound-mediated enhancements of cellular fluorescence in all cell lines examined. Compound-mediated cellular fluorescence was consistently observed in >99% of viable cells, illustrating the high efficiency of this delivery method. Examination of these cell lines by epifluorescence microscopy confirmed that the compound-induced fluorescence was intracellular and resulted from protein uptake (micrographs provided in the Supporting Information). These experiments confirmed that incorporation of Streptaphage (**1**) in cellular plasma membranes provides a highly efficient and general method for intracellular delivery of SA protein conjugates to mammalian cells.

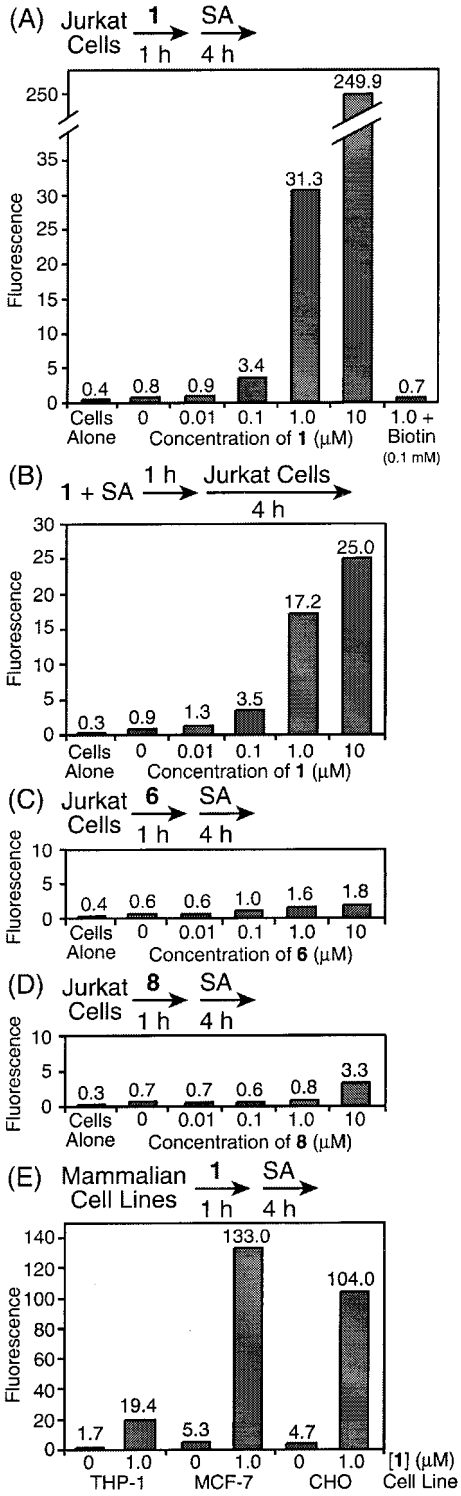


Figure 3. Analysis of ligand dose-dependent cellular uptake of SA Alexa Fluor 488 (20 μg/mL) by flow cytometry. Each bar represents the median fluorescence of 10 000 living cells.

Molecular Modeling of SA−Ligand Complexes. The effect of linker length on the biological activity of Streptaphage (**1**)

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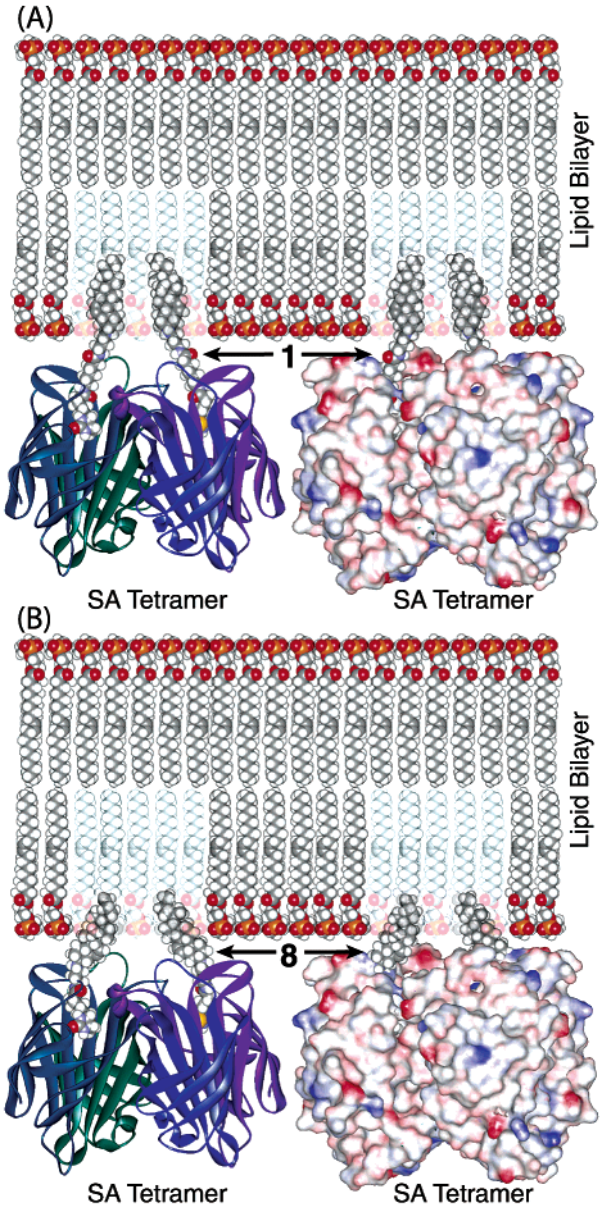

Figure 4. Ribbon and electrostatic solvent-exposed surface models of SA bound to Macromodel-minimized ligands (CPK models) inserted into a model membrane bilayer (CPK models). Panel A: SA bound to Streptaphage (**1**). Panel B: SA bound to the short-chain analogue **8**.

was examined by molecular modeling of SA−ligand complexes interacting with model membranes. Modeling employed substitution of two bound biotin ligands on one face of the tetrameric X-ray structure of SA with Macromodel (v. 6.5) Amber*-minimized[79] models of **1** and **8** in extended chain conformations. As shown in Figure 4, the area proximal to the ligand-binding site exhibits a concave topology as a consequence of nearby solvent-exposed loops on the protein exterior. This revealed that the binding site of SA allows complete penetration of the cholesterylamine moiety of **1** into a flat model membrane bilayer constructed from minimized phosphatidylcholine lipids (Figure 4, Panel A). In contrast, the shorter linker of **8** prohibits complete insertion of the steroid into the model membrane (Figure 4, Panel B). These results indicated that when bound

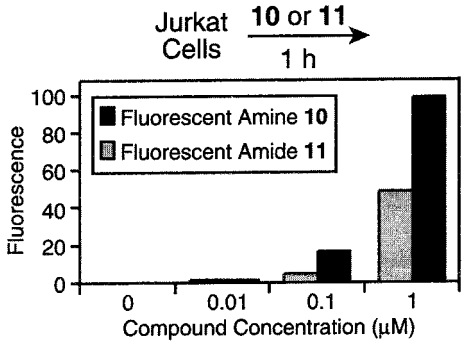

Figure 5. Analysis by flow cytometry of Jurkat lymphocytes treated with fluorescent derivatives of cholesterylamine. Cells were washed 3× with 1% BSA in PBS prior to analysis.

to SA, the greater linker length of **1** enables favorable interactions between the protonated amine of **1** and lipid headgroups. This protonated amino group is critical for uptake of SA as evidenced by the dramatically diminished ability of the uncharged amide **6** to affect the fluorescence of SA-treated cells (Figure 3, Panel C).

Molecular Recognition of Cellular Plasma Membranes by Structurally Related Fluorescent Probes. To further investigate the importance of the protonated cholesterylamine headgroup for molecular recognition of cellular plasma membranes, Jurkat lymphocytes were treated with fluorescent probes **10** and **11**. Epifluorescence microscopy revealed that cells treated with **10** or **11** exhibited green fluorescence localized persistently and nearly exclusively at cellular plasma membranes (micrographs provided in the Supporting Information). However, compound **10** bearing a free amino group exhibited significantly higher affinity for cellular plasma membranes compared with the analogous amide as evidenced by qualitative differences in fluorescence intensity by microscopy. Furthermore, quantitative differences of 2-fold (1 μM) to 4-fold (0.1 μM) were measured by flow cytometry (Figure 5). The enhanced affinity of the protonated amine of **10** for plasma membranes may derive from favorable electrostatic interactions with anionic phospholipid headgroups.[80] However, the cholesterol hydroxyl group is thought to form favorable H-bonding interactions with amide and alcohol functionality of sphingolipids in cholesterol-rich lipid rafts,[27] and these H-bonding interactions should be strengthened by the presence of a protonated amino substituent. Comparison of fluorescent probes **10** and **11** with SA-bound **1** and **6** suggests that the enhanced affinity for plasma membranes engendered by a free amino group contributes significantly to the stability of membrane-bound complexes between SA and **1**. However, it is remarkable that amide **6** does not appreciably promote association between SA and cellular plasma membranes (Figure 3, Panel C). This result may relate to the much longer linker between the biotin derivatives and cholesterylamine compared with the fluorescent probes. The short hydrophilic thiourea linker between fluorescein and cholesterylamine presumably interacts favorably with the hydrophilic lipid headgroups of the plasma membrane. In contrast, the long relatively hydrophobic linker in the biotin derivatives may magnify differences in membrane affinity between the amide and amine by positioning the hydrophilic biotin moiety distal to hydrophilic headgroups of membrane lipids.

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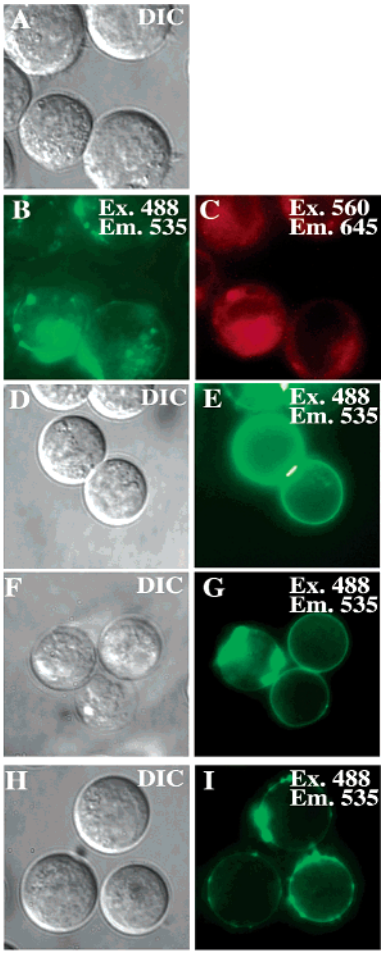

Figure 6. DIC and epifluorescence micrographs of Jurkat lymphocytes treated with Streptophage (**1**, 10 μM) for 1 h followed by green fluorescent SA Alexa Fluor 488 (20 μg/mL) for 4 h. Panels A−C: Cells colabeled with red fluorescent Lysotracker Red dye. D, E: Cells pretreated with chlorpromazine (100 μM). F, G: Cells pretreated with hypertonic sucrose (400 mM). H, I: Cells pretreated with methyl-β-cyclodextrin (10 mM).

Streptaphage (1) Enables SA to Associate with Lipid Rafts and Access a Clathrin-Mediated Endocytosis Pathway. Streptaphage (**1**) was found to target SA conjugates to defined intracellular compartments. These compartments were identified as acidic endosomes by addition of the acid-sensitive red fluorescent endosome probe Lysotracker Red (Figure 6, Panels A−C), indicating that the protein is internalized by an endocytic mechanism. To examine whether uptake of SA involved clathrin-mediated endocytosis, specific inhibitors of this pathway were investigated. Cationic amphipathic drugs such as chlorpromazine specifically block clathrin-mediated endocytosis by disrupting the assembly/disassembly of clathrin associated with coated pits and endosomes.[20] In addition, cells treated with hypertonic sucrose exhibit defects in clathrin-coated lattices that block clathrin-mediated endocytosis.[81] Uptake of SA promoted by **1** was inhibited by chlorpromazine (Figure 6, Panels D and E) and hypertonic sucrose (Figure 6, Panes F and G). In addition, cellular cholesterol was also critical for ligand-regulated uptake of SA. Cells pretreated with methyl-β-cyclodextrin to deplete plasma membrane cholesterol levels trapped SA at the plasma membrane. Similar effects of this cholesterol-binding compound on lipid rafts and clathrin-mediated endocytosis have been previously described.[35−37,82] Treatment with these compounds predominantly trapped SA at the cellular plasma membrane, which indicated that uptake of SA was controlled by a clathrin-dependent mechanism.

Cholera toxin invades cells by binding to the lipid raft-associated ganglioside GM1 in cellular plasma membranes.[23,29] Hence, fluorescent derivatives of the Cholera toxin B subunit have been employed as markers of lipid rafts.[21] To investigate whether green fluorescent cholesterylamine **10** or the SA-**1** complex might similarly colocalize with lipid rafts, confocal fluorescence microscopy was employed to compare localization in cellular plasma membranes with the membrane distribution of red fluorescent Cholera toxin B subunit. As shown in Figure 7, compound **10** and the SA-**1** complex exhibited a heterogeneous distribution in plasma membranes of Jurkat lymphocytes. This distribution substantially colocalized with Cholera toxin, consistent with the cholesterylamine moiety of **10** and **1** associating with lipid rafts. These results indicate that both **10** and complexes between SA and **1** preferentially bind to the sphingolipid and cholesterol-rich liquid-ordered phase of lipid rafts in cellular plasma membranes.

Streptaphage (1) Exhibits Minimal Toxicity to Cells in Culture. Streptavidin is sufficiently nontoxic for clinical applications such as pretargeting immunotherapeutics.[67−71] Additionally, structurally related cationic cholesterol derivatives exhibit low toxicity and have been used as transfection reagents.[39] To evaluate the toxicity of Streptaphage (**1**) alone and in combination with SA, compound-treated Jurkat lymphocytes were analyzed by flow cytometry. As shown in Figure 8, Streptaphage (**1**) does not adversely affect the viability of lymphocytes even at concentrations as high as 100 μM. Similarly, SA protein alone at 0.2 mg/mL in cell culture was nontoxic. Under conditions that promote uptake of SA by over 300-fold (10 μM **1**, 0.02 mg/mL SA), only a slight reduction in viability was observed. However, cellular viability was dramatically affected by coadministration of **1** and SA at 10-fold higher concentrations, which may result from rupture of cellular membranes due to overwhelmingly excessive protein delivery.

Summary and Conclusions

We report the synthesis of a novel small-molecule ligand termed Streptaphage (**1**) that enables efficient uptake of SA by mammalian cells by hijacking the molecular machinery used by cells to organize cellular membranes. This ligand comprises biotin linked to cholesterylamine, which promotes clathrin-mediated endocytosis by anchoring SA in lipid raft subdomains of cellular plasma membranes. Streptaphage (**1**) associates with lipid rafts by functionally mimicking cholesterol, which packs with sphingolipids through hydrophobic and hydrogen bonding interactions to form the liquid ordered phase that characterizes lipid rafts.[24−27,83]

The Cholera toxin B subunit gains access to the cellular interior by similarly binding a lipid raft-associated small molecule. This pentameric protein penetrates cellular membranes

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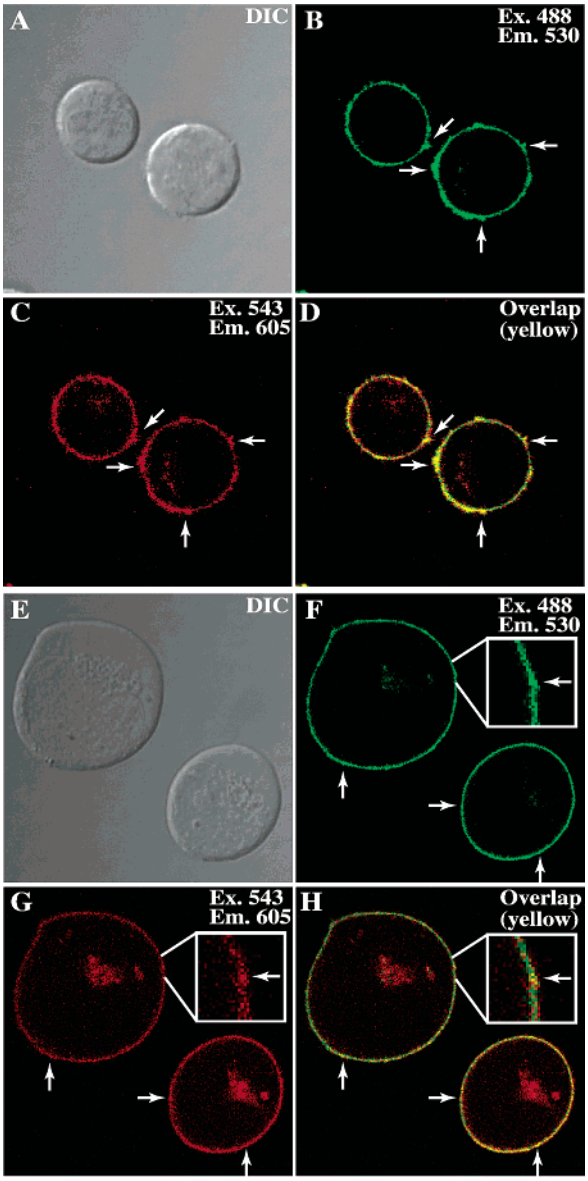

Figure 7. Confocal fluorescence microscopy of Jurkat lymphocytes treated with red fluorescent Alexa Fluor 594−conjugated Cholera toxin B subunit (8 μg/mL) for 5 min. Panels A−D: Cells pretreated with green fluorescent cholesterylamine **10** (10 μM) for 1 h. Panels E−H: Cells pretreated with **1** (10 μM) for 1 h, followed by addition of green fluoresent SA Alexa Fluor 488 (20 μg/mL) for 5 min. White arrows illustrate points of colocalization at the plasma membrane. Overlap images (D and H) display colocalization as yellow pixels.

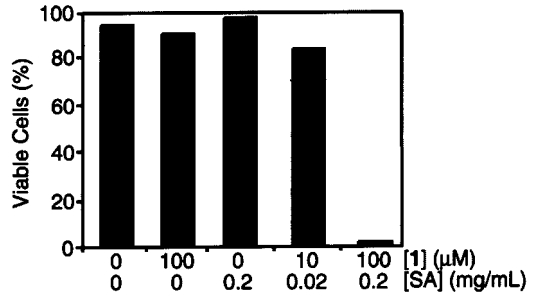

Figure 8. Analysis of toxicity of **1** and/or SA by flow cytometry. Jurkat lymphocytes were treated with compounds for 24 h and viability quantified by forward and side-scatter dot plots. Dead cells were counterstained with propidium iodide.

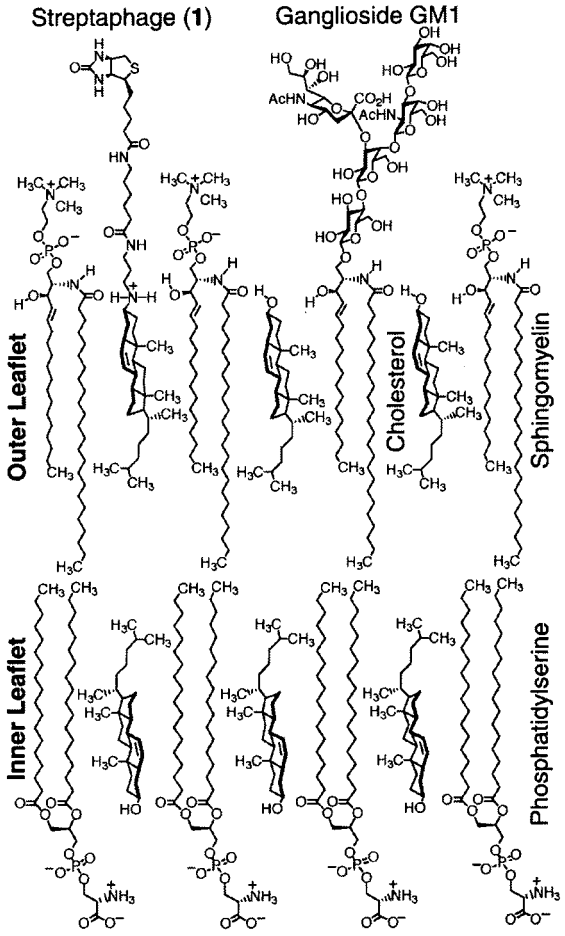

Figure 9. Model of a plasma membrane lipid raft segment containing Streptaphage (**1**) and ganglioside GM1. The cholesterylamine moiety of **1** partitions preferentially into the liquid-ordered phase of the exoplasmic (outer) leaflet, which primarily comprises cholesterol, sphingomyelin, and glycosphingolipids. The inner leaflet predominantly contains cholesterol and saturated glycerolipids such as phosphatidylserine.

by binding the raft-associated natural product ganglioside GM1. This natural product is a sphingolipid that comprises ceramide bearing a pentasaccharide headgroup (Figure 9). Binding of Cholera toxin to GM1 enables this protein to localize in lipid rafts and undergo clathrin-mediated endocytosis by accessing endogenous mechanisms of vesicular transport.[21,22,29] Because lipid rafts contain cholesterol packed with sphingolipids, synthetic analogues of GM1 replacing the ceramide lipid moiety with a cholesterol ester have yielded non-natural GM1 mimics more potent than the natural product in mediating actions of Cholera toxin.[84] This previously reported result and the results described herein illustrate the functional equivalence of sphingolipid and cholesterol derivatives in mediating protein uptake through this mechanism.

Analysis by confocal fluorescence microscopy revealed that the SA-**1** complex and Cholera toxin-GM1 colocalize in lipid rafts. Furthermore, both proteins are internalized by clathrin-mediated endocytosis. Thus, uptake of the SA-**1** complex functionally mimics the initial steps of cellular penetration employed by the Cholera toxin-GM1 complex. A proposed model of Streptaphage (**1**) associated with a portion of a lipid raft[27] is shown in Figure 9. In this model, **1** is shown in the

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exoplasmic leaflet of the bilayer because 3β-cholesterylamine is unequally distributed in model liposomal membranes and favors the outer leaflet.[34]

Streptaphage (**1**) was found to exhibit remarkable low toxicity to cells in culture, and SA protein is sufficiently nontoxic for clinical applications such as pretargeting immunotherapeutics. These results suggest that ligand-regulated uptake of SA conjugates and fusion proteins may have applications in tumor therapy. Many proteins such as serum albumin tend to accumulate in solid tumors due to the enhanced microvasculature of tumors and the lack of functional lymphatic drainage systems in tumor tissue.[85,86] Hence, endosome-activated antitumor agents conjugated to serum albumin can exhibit some selectivity for solid tumors and promote tumor regression in nude mice.[85,87] SA-linked cytotoxins that become activated upon protolysis or acidification in endosomes may similarly accumulate in tumor tissue. Subsequent administration of synthetic ligands that promote endocytosis may trigger tumor regression. Streptaphage (**1**)-regulated cytotoxicity of SA proteins linked to endosome-activated toxins may be particularly effective because the fate of internalized SA protein appears to be irreversible entrapment in endosomal compartments due to the high affinity of bound cholesterylamine for membranes. This approach of labeling lipid rafts of cell membranes with small molecules that internalize SA toxins has the potential to be useful for targeting multidrug resistant (MDR) cancers, which exhibit distinct plasma membrane compositions as a consequence of dramatic upregulation of production of lipid raft components.[88,89] Lipid raft-rich plasma membranes of MDR cancers may permit selective cellular labeling by Streptaphage (**1**) or related compounds for targeted delivery of antitumor agents.

The synthesis of Streptaphage (**1**) analogues bearing acid-labile or other reactive linkers may facilitate escape of SA proteins from acidic endosomes into the cytosol or nucleus. This approach may be useful for stimulation of immune responses by regulating uptake and processing of SA-linked protein antigens in antigen presenting cells such as macrophages, B-cells, and dendridic cells. Streptaphage (**1**) may also enable regulated gene delivery since avidin conjugated to the DNA-binding polymer polyethyleneimine has previously been employed for cellular transfection.[15] This general strategy of coupling protein ligands to molecules that associate with lipid rafts has potential for regulated delivery of diverse ligand-binding proteins.

Acknowledgment. We thank the NIH (CA83831) for financial support. S.L.H. thanks the DOD for a predoctoral fellowship.

Supporting Information Available: Experimental procedures, compound characterization data, and additional micrographs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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