Toward Targeted Oral Vaccine Delivery Systems: Selection of Lectin Mimetics from Combinatorial Libraries

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Purpose. Various lectins bind specifically to oligosaccharides on intestinal cells. Exploiting this specificity, *Ulex europaeus* agglutinin I (UEA1) has been used as a ligand for targeted oral vaccine delivery to M cells (antigen-presenting cells) in follicle-associated epithelium. In this study we characterized compounds identified from mixture-based positional scanning synthetic combinatorial libraries, which mimic UEA1 and, thus, may have properties applicable to targeted drug delivery.

Methods. Two UEA1 mimetics were synthesized and their activity was verified on live cells. The ability of the lead compound, a tetragalloyl D-Lysine amide construct (4-copy gallic acid construct), to deliver dye-loaded polystyrene particles to M cells was assessed in an *in situ* mouse gut loop model.

Results. The 4-copy gallic acid construct inhibited UEA1 binding to Caco-2 cell membranes with an IC_{50} of 3 μM, a 650- to 5000-fold increase over the natural UEA1 substrate α-L-fucose. The biotin-labeled derivative of this construct demonstrated comparable binding activity as verified on live cells by fluorescence-activated cell sorting. Preclinical studies confirmed its ability to mediate M cell-specific delivery of streptavidin-coated particles *in vivo*.

Conclusions. Polyphenolic compounds, D-Lysine scaffolds with multiple galloyl groups, can mimic functional activities of UEA1. Properties of such molecules, including low molecular weight, stability, ease of synthesis and low cost, highlight their potential for application in targeted vaccine delivery.

KEY WORDS: lectin; *Ulex europaeus* agglutinin I (UEA1); mixture-based synthetic combinatorial libraries (SCLs); gallic acid; polyphenols; M cells.

INTRODUCTION

Various procedures exist for encapsulation of pharmaceutically active ingredients in biodegradable particulate delivery systems, which allow the active substance to pass through the stomach and survive to reach the target region of the gastrointestinal tract (GIT). In practice, the bioavailability with these formulations is lower than with parenteral routes primarily because of poor particle uptake across epithelial cell barriers. Lectins, naturally occurring proteins with affinity for sugar residues, bind specifically to oligosaccharide moieties on the surface of intestinal cells and have been investigated as promoters of bioadhesion in the GIT (1,2). To this end lectins have been displayed on model particles, such as fluorescent dye-loaded polystyrene, as well as "real" delivery systems, such as polylactide particles and liposomes (2,3). *Ulex europaeus* agglutinin I (UEA1), an α-L-fucose-specific lectin, has been of particular interest because of its M cell specificity in the mouse model and so, its applicability for proof-of-concept studies of vaccine delivery to antigen-presenting cells (4). UEA1-coated polystyrene particles exhibited significantly greater M cell binding than lectin-free control particles in mice (2). Similarly 10.5% of UEA1-bearing liposomes were taken up from mouse GIT in contrast with 5.8% of wheat germ agglutinin bearing liposomes and 3.2% of lectin-free control liposomes (3). We and others have generated preclinical proof-of-concept data for vaccine delivery using UEA1 to enhance targeting to M cells (5,6).

The use of lectins as targeting ligands remains problematic, primarily because of their susceptibility to proteolytic degradation in the GIT and during typical procedures used for preparation of oral drug formulations. Potential immunogenic and cytotoxic effects also limit the use of lectins as targeting agents to deliver drugs and vaccines to and across the human GIT. One approach to overcome these limitations is to produce recombinant lectins with modified properties. Already, recombinant Mistletoe Lectin 1 (ML1), produced in *Escherichia coli*, has exhibited similar Chinese hamster ovary cell binding properties to the native lectin (7). Another approach is to select or design and synthesize peptides or organic molecules, which mimic the function of the lectins and thus, may have properties applicable to targeted drug delivery. Advantages of such mimetics include their size (typically less than 1500 da), stability (through non-natural composition), ease of synthesis and low cost, as well as their suitability for incorporation into delivery systems using routine chemical procedures. In this report we describe the characterization of small molecule UEA1 mimetics identified using mixture-based positional scanning synthetic combinatorial libraries (PS-SCLs) and competition assays on a human intestinal cell model. The potential for application of these mimetics in oral vaccine delivery is demonstrated using fluorescent dye-loaded polystyrene particles coated with a biotin-labeled construct in an *in situ* mouse gut loop model.

MATERIALS AND METHODS

Combinatorial Libraries and derivatives

Peptide, peptidomimetric, and organic molecule libraries were primarily in positional scanning format (8,9), and were used as a source of chemical diversity. Individual compounds were synthesized by the simultaneous multiple peptide synthesis method using the same scheme described recently (10). (See Table 1 of Reference 10 for chemical structures of the lead compounds [2a and 4a] used in this study). Purity and identity of each compound were characterized using an elec-

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trospray mass spectrometer interfaced with a liquid chromatography system.

Biotin-Labeled UEA1 Competition Assay

Caco-2 cell (ATCC, Rockville, ML, USA) membrane fractions were prepared from confluent cell monolayers grown in 75-cm^2 flasks for up to 1 week at 37°C and 5% CO_2 as previously described (11,12). The membrane fractions were coated onto 96-well microtiter plates at 10 μg mL^{-1} in 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C the plates were blocked with bovine serum albumin (Sigma, St. Louis, MO, USA; 1.5% bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline [DPBS]) for 1 to 4 h at room temperature. After extensive washing in distilled water, 50 μL of the test compounds were added in an equal volume of 1.5% BSA-DPBS. Biotin-labeled UEA1 (Vector Laboratories, Peterborough, UK) was added at a final concentration of 1 μg mL^{-1} in the same buffer and the plates were left to incubate overnight at 4°C. The plates were washed thoroughly and the biotin-labeled UEA1 was detected using streptavidin-horseradish peroxidase (CalBiochem, San Diego, CA, USA; 1:5000 dilution in 1.5% BSA-PBS; 1 h at room temperature) and an orthophenyl diamine substrate (Sigma; 1.6 mg mL^{-1}; 5–10 min at room temperature in the dark; reaction stopped by addition of 4 N sulfuric acid). Absorbance was measured at 490 nm using a conventional 96-well plate reading spectrophotometer.

Control wells contained purified UEA1 at concentrations in the range 0.04 μg mL^{-1} to 160 μg mL^{-1} to allow preparation of a standard curve. Results are illustrated as the percentage of inhibitory activity or inhibitory constant (IC_{50}) value, i.e., the concentration of a compound at which 50% inhibition of UEA1 binding occurred. A 'two-tier' assay, in which test compounds were allowed to incubate with the cell membranes prior to addition of the biotin-labeled UEA1, confirmed that the compounds were binding to surface receptors on the Caco-2 cell membranes and not to the co-incubated lectin. In this instance the plates were incubated overnight with the mixtures/compounds, washed thoroughly and the biotin-labeled UEA1 was added at a final concentration of 1 μg mL^{-1} in 1.5% BSA-PBS. After incubation for 2 h at room temperature the assay was completed according to routine procedures.

Direct Binding and Competition of Biotin-Labeled Compounds

Individual compounds were synthesized with biotin labels to allow confirmation of direct binding to Caco-2 cell membrane preparations. These biotinylated compounds were also competed against non-biotinylated UEA1 to further confirm competition. All assay conditions were similar to those described for detection of biotin-labeled UEA1 above.

Fluorescence-Activated Cell Sorting

Binding of biotin-labeled compounds and UEA1 to live Caco-2 cells was evaluated using a fluorescein-avidin D sandwich protocol. This protocol was developed based on a method to detect binding of peptides to major histocompatibility molecules in whole cells (13). Caco-2 cells were harvested in trypsin EDTA (Sigma, St Louis, MO, USA, cat. no. T4049), resuspended in culture medium (2×10^5 cells/vial; >90% viability) and incubated with individual compounds at 37°C for 3 h. Cells were washed twice in wash buffer (DPBS containing 1% BSA and 1% sodium azide) and bound biotin-labeled compounds were detected using fluorescein-avidin D (Vector Laboratories, Burlingame, CA, USA; 10 μg mL^{-1}; 30 min on ice) followed successively by biotin-labeled anti-avidin D (Vector Laboratories, Burlingame, CA, USA; 10 μg mL^{-1}; 30 min on ice), and fluorescein avidin (Vector Laboratories, Burlingame, CA, USA; 10 μg mL^{-1}; 30 min on ice) to enhance the signal. Samples were analyzed in duplicate on a FACScan instrument (Becton Dickinson, San Jose, CA, USA). Data were evaluated as percent gated cells, and mean × values. The gain values for UEA1 and the 4-copy construct were calculated as relative values by setting the background mean × value (FL1) equal to 100.

Polystyrene Particle Uptake in an *in Situ* Mouse Gut Loop Model

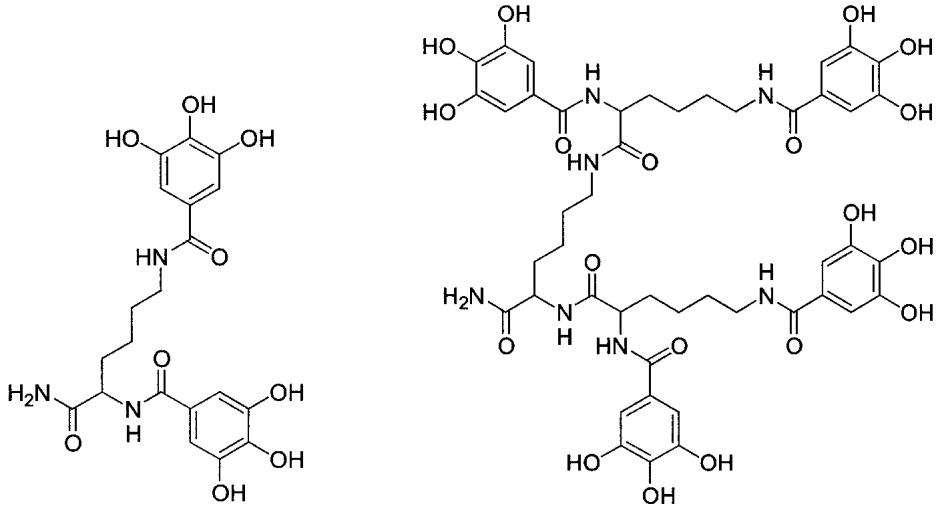
Biotin-labeled compounds were conjugated to yellow-green fluorescent streptavidin-coated polystyrene particles (Merck Eurolab, Cedex, France; 0.289 μm in diameter) according to manufacturer's instructions. The particles were resuspended in 0.3 mL of sterile PBS before administration. Control preparations included UEA1-coated (Vector Laboratories; positive control) and biocytin-coated (Molecular Probes, Leiden, The Netherlands; negative control) particle batches.

All animal experiments were performed according to previously described protocols (2) and adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Adult female Balb/c specific pathogen-free mice that had been fasted overnight and supplied with water *ad libitum* were anesthetized by intraperitoneal administration of 0.1 mL per 10 g midazolam/hypnorm (Janssen Pharmaceutical, Wantage, UK; Roche Products, Welwyn Garden City, UK). After laparotomy, 2- to 3-cm small intestinal loops containing one or more Peyer's patches were created by application of appropriate ligatures. Particle suspensions (0.3 mL containing 5.0×10^{10} particles mL^{-1} in PBS) were injected into the loops with 25-gauge needles, and the animals were maintained on heating pads for 30 min. The Peyer's patches were excised and pinned, mucosal surface uppermost, onto corkboards and washed in cold PBS. The tissues were fixed in methanol at –20°C for 60 min before cytochemical staining and confocal laser scanning microscopy. After fixation, the tissues were rinsed in PBS and villi were microdissected away from the domes to facilitate examination of the follicle-associated epithelium (FAE). M cells were located by immersion in 20 μg mL^{-1} of tetramethylrhodamine isothiocyanate labeled UEA1 (Vector Laboratories) for 60 min. Stained tissues were examined in a Bio-Rad MRC 600 CLSM equipped with an argon/krypton mixed gas laser.

Mann–Whitney analyses were performed to investigate whether there were significant differences at the 5% confidence limit between the number of microspheres bound per unit area of Peyer's patch FAE for each individual treatment.

RESULTS

A competitive assay was developed and optimized to allow for high-throughput screening of compounds for their



Structure I:
digalloyl D-Lysine amide construct
"2 copy gallic acid construct"

Structure II:
tetragalloyl D-Lysine amide construct
"4 copy gallic acid construct"

Fig. 1. Structure I, digalloyl D-Lysine amide construct (2-copy gallic acid construct) and Structure II, tetragalloyl D-Lysine amide construct (4-copy gallic acid construct).

ability to inhibit the binding of UEA1 to Caco-2 cell membrane preparations. A series of lead candidates were then identified as recently described (10). Based upon candidate IC_{50} data from the single tier competition binding assay, in which Caco-2 cell membrane preparations were incubated with test samples and biotin-labeled UEA1 simultaneously, as well as a two tier competition binding assay, in which Caco-2 cell membrane preparations were incubated with test samples first, washed, and then incubated with biotin-labeled UEA1, two lead compounds were identified: Structure I, a digalloyl D-Lysine amide construct ("2-copy gallic acid construct"), and Structure II, a tetragalloyl D-Lysine amide construct (4-copy gallic acid construct; Fig. 1). Lead compound IC_{50} data were compared with competition data for known inhibitors (Table I). In general, both the single and two tier assays yielded comparable results for the constructs. Both the 2- and the 4-copy gallic acid constructs were shown to compete better against biotin-labeled UEA1 for Caco-2 cell membrane preparations in single tier format than did α L-fucose, a known inhibitor of UEA1 binding. Because of the mechanism of L-fucose inhibition of UEA1, and to the nature of the second-tier competition assay, L-fucose does not compete with UEA1 biotin in second-tier format. This is expected if one considers that it is the UEA1 that binds to the L-fucose rather than L-fucose binding to the membrane preparations (23,2). The 2-copy construct exhibited an IC_{50} value of 96 μM, and the 4-copy construct 3 μM, when competed against biotin-labeled UEA1 in the single-tier competition assay, representing a significant enhancement over α L-fucose, which has been shown to have a range of inhibitory activity between 2000 and 15,000 μM (22,24,25). The 4-copy gallic acid construct consistently competes better than the 2-copy gallic acid construct in both the single and two tier competition assays.

Although the 4-copy gallic acid construct yielded better results in the initial competition studies against biotin UEA1 for binding to Caco-2 cell membrane fractions, both of the lead compounds, the 2-copy and the 4-copy gallic acid constructs were synthesized with biotin labels for additional binding studies. These biotin-labeled compounds were designed using two separate synthesis techniques (Fig. 2). Synthesis of both derivatives included an additional lysine as an attachment site for the label. For structure A, a seven-atom aminohexanoyl spacer was incorporated between the construct and biotin. In structure B, biotin was acylated directly to the lysine alpha amine. These two biotin-labeled derivatives of each of the two and four copy galloyl group structures were then tested via direct binding to Caco-2 cell membranes *in vitro* to determine whether steric hindrance of the label would affect the activity of the constructs. In addition, by using biotin-labeled compounds, binding of the compounds to UEA1 itself could be ruled out, further establishing the binding of the compounds to the Caco-2 cell membrane preparations.

The direct binding data for each of the two forms of the 2- and 4-copy biotin-labeled gallic acid compounds to Caco-2 cell membranes *in vitro* (Fig. 3) demonstrated that all samples bound to Caco-2 cell membrane preparations. In addition, the biotin-labeled constructs exhibited dose dependent binding to

Table I. Evaluation of Di- and Tetragalloyl D-Lysine Amide Compounds for Inhibition of Biotin-Labeled UEA1 Binding to Caco-2 Cell Membranes (IC_{50} Values in μM)

Competitors	First-tier assay	Second-tier assay
2-copy gallic acid construct[a] MW = 449.4	96 ± 71	20 ± 9
4-copy gallic acid construct[a] MW = 1008.9	3 ± 0.7	1 ± 0.05
UEA1 MW = 68,000	0.1 ± 0.04	0.2 ± 0.1
α L-Fucose MW = 164.2	2,000 to 15,000	>15,000

[a] See Reference 10 Table 1 for chemical structures of 2- and 4-copy gallic acid constructs (compounds 2a and 4a).

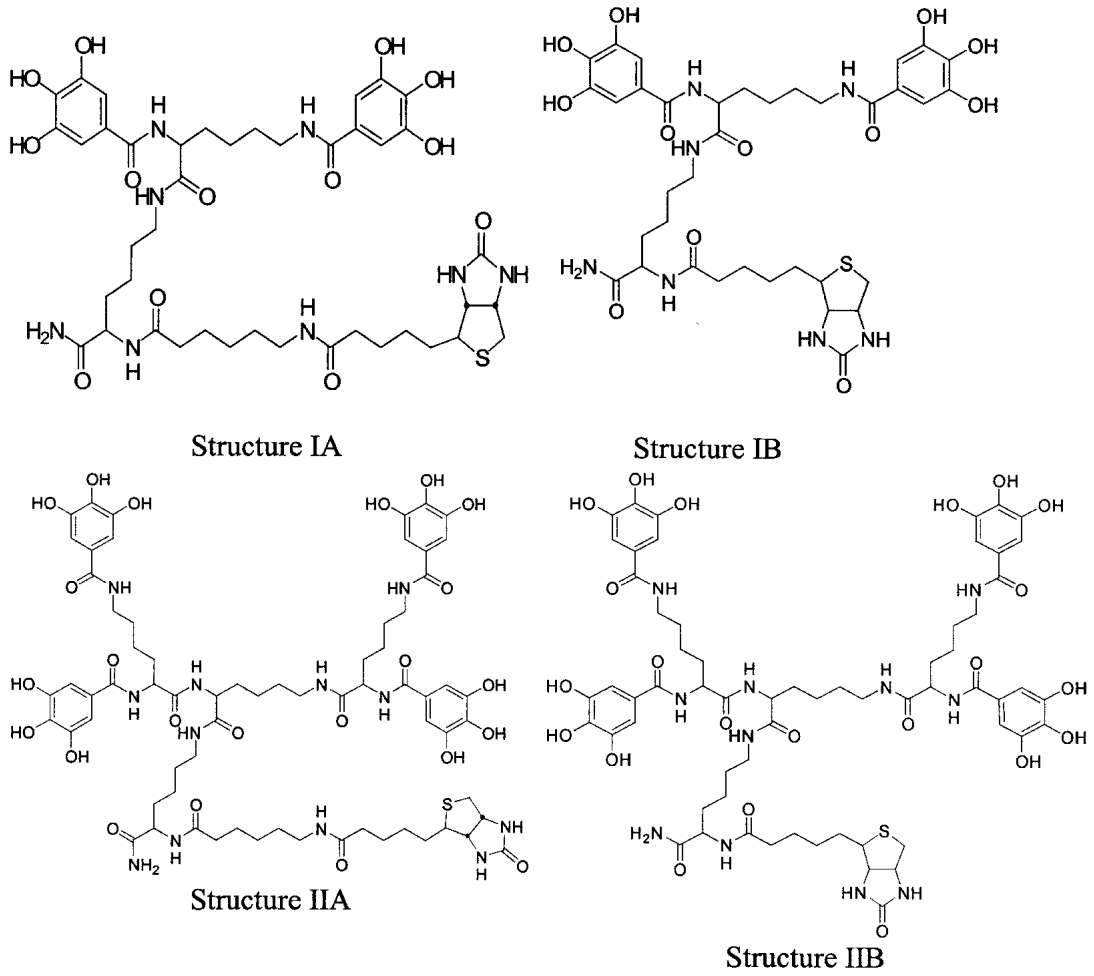


Fig. 2. Two forms of the biotin-labeled 2- and 4-copy gallic acid constructs. Synthesis of both derivatives included an additional lysine as an attachment site for the label. For structure A, a seven-atom aminohexanoyl spacer is incorporated between the construct and biotin. For structure B, biotin is acylated directly to the lysine alpha amine. IA, 2-copy gallic acid construct with biotin and spacer; IB, 2-copy gallic acid construct with biotin and no spacer; IIA, 4-copy gallic acid construct with biotin and spacer; IIB, 4-copy gallic acid construct with biotin and no spacer.

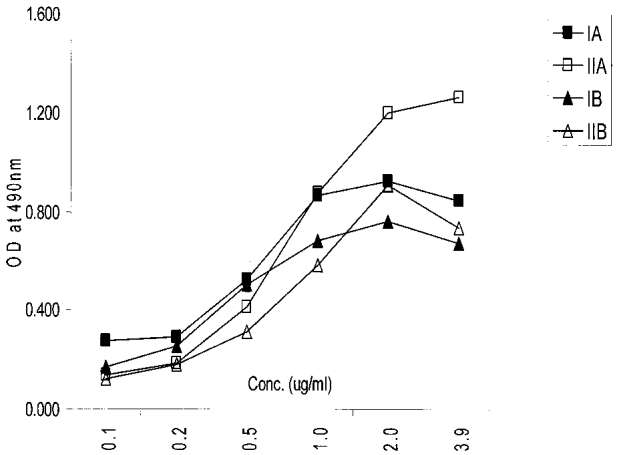


Fig. 3. Analysis of Caco-2 cell membrane fraction direct binding of biotin-labeled di and tetragalloyl D-Lysine amide constructs (optical density at 490 nm vs. ligand concentration in $\mu g\ mL^{-1}$). IA, 2-copy gallic acid construct with biotin and spacer; IB, 2-copy gallic acid construct with biotin and no spacer; IIA, tetragalloyl D-Lysine amide construct with biotin and spacer; IIB, tetragalloyl D-Lysine amide construct with biotin and no spacer.

the Caco-2 cell membrane fractions. Structure A, the derivative incorporating the aminohexanoyl spacer on the 4-copy gallic acid construct, had a higher affinity for the Caco-2 cell membrane preparations than did the non-spacer or 2-copy gallic acid constructs. This biotin-labeled 4-copy gallic acid construct with linker was competed against unlabeled lectin UEA1, unlabeled 4-copy gallic acid construct (Table II), and also against α L-fucose. In the first two cases, competition was demonstrated for the construct in both single and second tier assays. Again, we saw no inhibition with L-fucose for the second tier assay, as expected, and as is the same for UEA1 biotin. Single-tier results indicated marginal inhibition of the biotin construct (between 10 and 15% inhibition at concentrations of greater than 4 mM α L-fucose). Competition of the unlabeled UEA1 with the labeled 4-copy gallic acid construct for Caco-2 cell membrane preparations in single and second tier assay format is indicative that this construct is in fact a UEA1 mimetic. Specificity of the mimetic for the UEA1 binding site was further confirmed by testing the 4-copy gallic acid construct in a Cholera Toxin B (CTB) subunit competition assay for ganglioside GM1 binding. The 4-copy gallic acid construct demonstrated no competition with CTB Biotin for binding to GM1 adsorbed on the plate, IC_{50} >500 μg/mL, or

Table II. Evaluation of 4-Copy Gallic Acid Construct and UEA1 for Inhibition of Biotin-Labeled 4-Copy Gallic Acid Construct Binding to Caco-2 Cell Membranes (IC_{50} Values in μM)

Competitors	First-tier assay	Second-tier assay
UEA1	3 ± 3	3 ± 3
4-copy gallic acid construct	4 ± 4	40 ± 3

>495 μM, and the CTB competed with CTB Biotin at an IC_{50} of approximately 1μg/ml or 0.1μM. Based on this initial characterization data, the 4-copy gallic acid construct was chosen for further *in vitro* and *in vivo* studies.

Binding to the surface of live intact Caco-2 cells was verified by fluorescence-activated cell sorting (FACS) analysis. The biotin-labeled 4-copy gallic acid construct, which exhibited dose-dependent binding to Caco-2 cell membrane fractions in the direct binding assays, also demonstrated a dose response for whole cells in solution (Fig. 4, III and IV; Table III), with binding equivalent to that observed using biotin-labeled UEA1 (Fig. 4, II). Binding at such concentrations, i.e., in the low micromolar range that had previously been established as adequate to achieve site-specific particle delivery prompted us to evaluate the construct *in vivo*. Adherence of fluorescent streptavidin-polystyrene particles coated with the biotin-labeled 4-copy gallic acid construct to

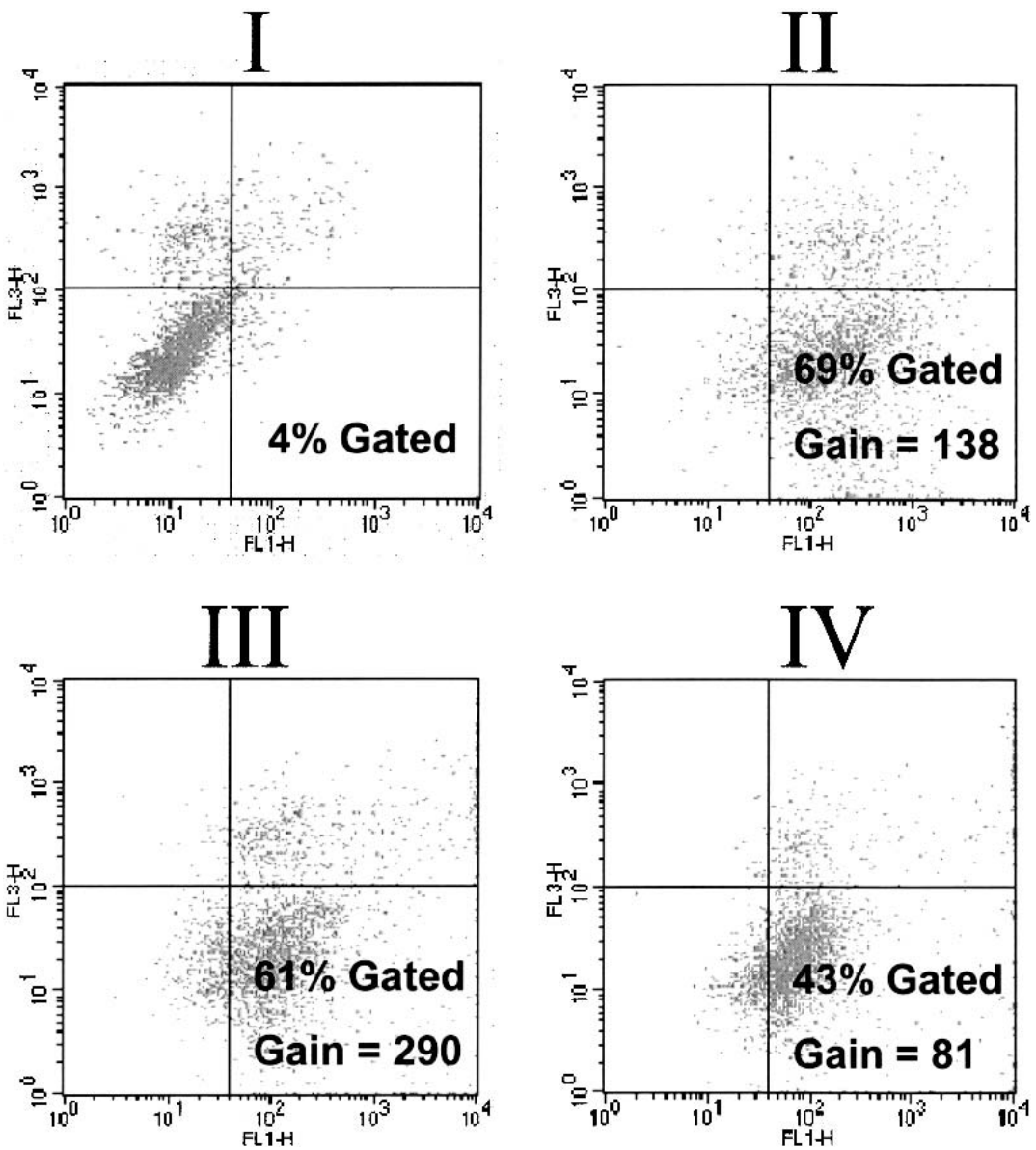


Fig. 4. Fluorescence-activated cell sorting sandwich analysis of Caco-2 cell binding by the biotin-labeled 4-copy gallic acid construct and UEA1. Values were generated in a single representative FACS assay, the values for which were included in the averages reported in Table III. Live Caco-2 cells in solution by FACS analysis (FL1 [FITC] at 50 μM and at 5 μM, vs. FL3 [7AAD, seven-amino-actinomycin D, Becton Dickinson, San Diego, CA, USA] 0.25 μg per tube, to identify dead cells). I, negative control (no ligand), mean x = 161; II, biotin-labeled UEA1 (concentration: 0.1 μM), mean x = 222; III, tetragalloyl D-Lysine amide construct with biotin and spacer (concentration: 50 μM), mean x = 468; IV, tetragalloyl D-Lysine amide construct with biotin and spacer (concentration: 5 μM), mean x = 130. The gain values for UEA1 and the 4-copy construct were calculated as relative values by setting the background mean x value (FL1) equal to 100.

Table III. FACS Sandwich Analysis of Caco-2 Cell Binding by the Biotin Labeled 4 Copy Gallic Acid Construct and UEA1

Ligand	% gated cells[a]	× Mean[a]	Gain
Negative control	3.6 ± 1.8	105.6 ± 35.6	NA
UEA1, 0.1 μM	68.2 ± 10.9	409.0 ± 106.6	387.3
4-copy construct, 50 μM	55.0 ± 12.2	541.6 ± 316.5	513.0
4-copy construct, 5 μM	64.6 ± 17.0	297.4 ± 187.8	281.7

[a] Values reported reflect the net value after subtraction of negative control values.

mouse M cells in FAE was demonstrated *in situ* (Fig. 5). Adherence was restricted to M cells (Fig. 5, II) and was significantly increased relative to biocytin-coated controls (Fig. 5, I). Binding and uptake of particles could be clearly differentiated by vertical cross section (Fig. 5, III) and z series sequence (Fig. 5, IV; 0.0–14.0 μm) respectively. The number of particles that adhered to the FAE was comparable to that observed using UEA1 (Fig. 6) thus providing proof-of-concept data relevant to targeting vaccine delivery to antigen-presenting cells.

DISCUSSION

We have used PS-SCLs to identify, design and generate organic molecules, which can mimic the functional activity of the lectin UEA1 and thus, may have properties applicable to targeted drug delivery. Although combinatorial chemistry is an integral part of the drug discovery process, there have been few reports of its application in the oral drug delivery field. This is understandable in that early combinatorial strategies focused on peptides, primarily because of the existence of phage display libraries featuring extracellular presentation of millions of candidate ligands, and peptides were associated with biologic instability and poor membrane permeability in the GIT. It should be noted that peptide ligands that facilitate transport of bacteriophage across intestinal epithelial barriers

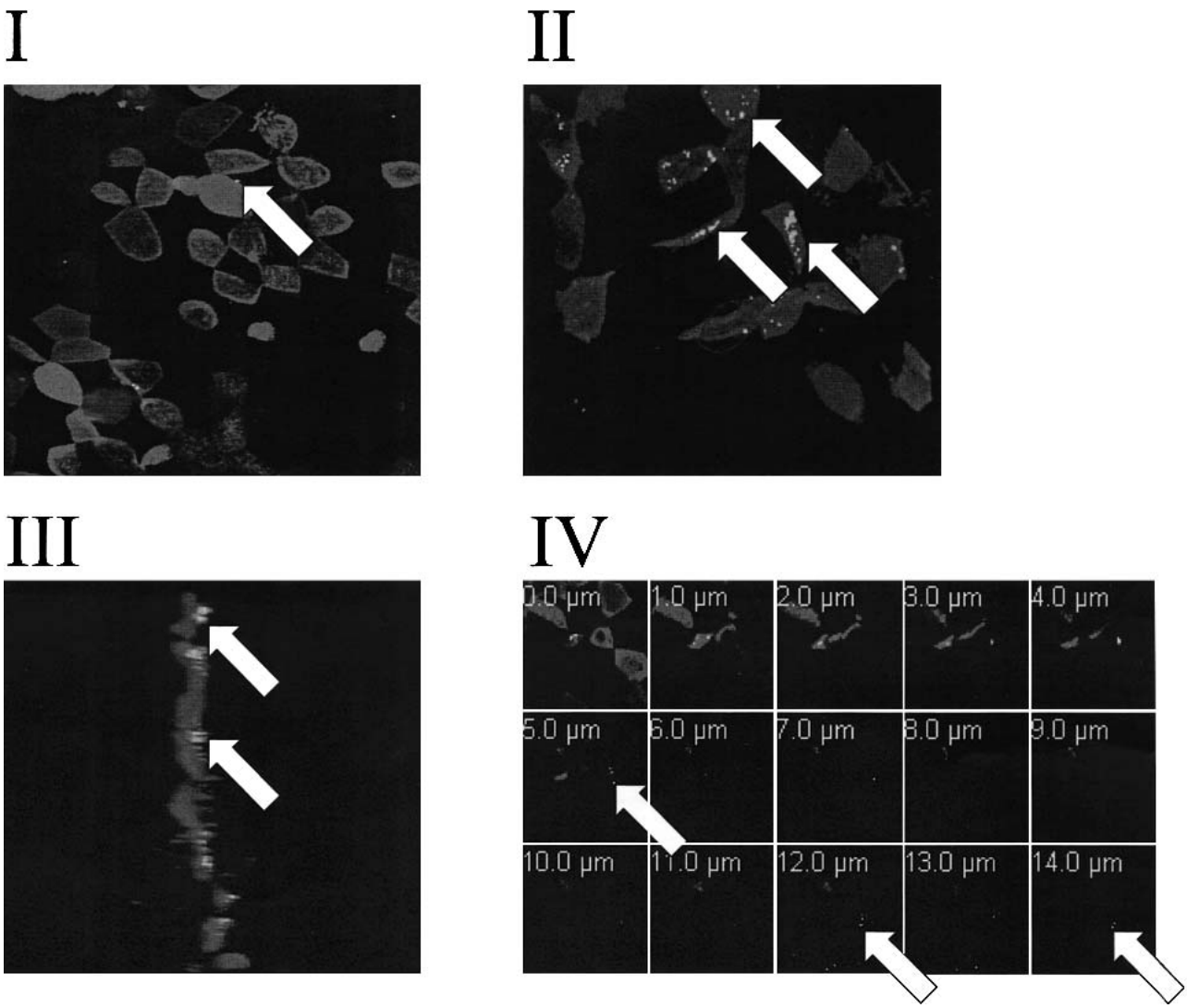


Fig. 5 Confocal laser scanning microscopy images illustrating the association of biotin-labeled 4-copy gallic acid coated polystyrene particles with mouse Peyer's patch FAE after 30 min-incubation in gut loops (projected z series of images). I, biocytin-coated control particles (arrow); II, biotin-labeled 4-copy gallic acid coated particles (arrows) adhered to TRITC-UEA1 stained M-cells (z series); III, biotin-labeled 4-copy gallic acid coated particles (arrows) adhered to TRITC-UEA1 stained M-cells (vertical cross section); IV, biotin-labeled 4-copy gallic acid coated particles (arrows) adhered to TRITC-UEA1 stained M-cells (z series sequence 0.0-14.0 μm). Objective = ×100.

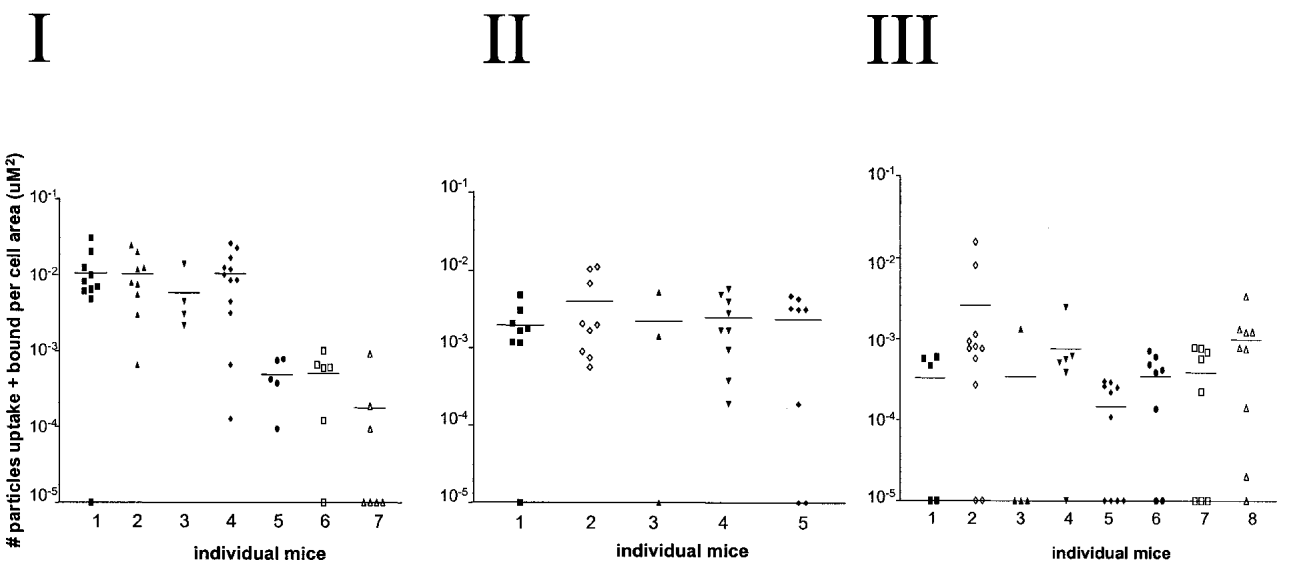


Fig. 6. The number of biotin-labeled 4-copy gallic acid, UEA1, and biocytin-coated polystyrene particles bound/taken up per unit area (linear scale) of mouse Peyer's patch FAE. I, biotin-labeled 4-copy gallic acid; II, UEA1; III, biocytin. Significantly more ($p < 0.05$) 4-copy gallic acid-coated particles adhered to M-cell surface compared with biocytin-coated particles.

into the systemic circulation have been isolated using random phage display libraries in screenings *in vivo* (14). It is postulated that the nature of the screenings in an environment as complex as the intestinal lumen, intracellular space and systemic circulation may intrinsically select for peptide stability in addition to membrane permeability. Several synthetic libraries have also been screened to identify moieties, which are transported across intestinal epithelial cell monolayers (15,16). For example, Glaxo Wellcome disclosed the use of human Caco-2 cell monolayers to screen a synthetic mixture-based tripeptide library for information relating to the permeability of small peptides (15). Little or no transport was observed for most of the tripeptides although some permeable structures were identified by liquid chromatography combined with mass spectrometry, and it was proposed that they would aid design of pharmaceutically bio-available drugs. It is generally accepted, however, that peptides must be reengineered to develop viable leads for targeting of drug-loaded delivery systems to enterocytes.

Peptidomimetics and small organic molecules possess many features required for the final orally available drug or targeting ligand, including stability, reduced cost, and ease of synthesis. These factors prompted us to use mixture-based libraries of stabilized peptides, peptidomimetics, and organic molecules in high-throughput screenings relevant to identification of novel targeting ligands for oral drug delivery. The design and application of mixture-based synthetic combinatorial libraries has been reviewed extensively elsewhere (8,17). The power of this technology lies in the ability to screen millions of chemical compounds in a format suited to cell-based assays in a cost-effective manner. The compounds making up each mixture are present in approximately equimolar amounts, they are not support-bound (which allows each compound to interact freely in solution with relevant receptors) and each compound is present at a concentration expected to yield a detectable signal in high affinity *in vitro* bioassays (9). The assay used in this instance took advantage of the UEA1 binding capacity of human intestinal Caco-2 cells, which had been deemed sufficient for use in drug delivery applications (1). The identification of individual *N*-acylamine compounds derived from the library mixtures verified that the competition assay was suitable for the identification of UEA1 mimetics. Furthermore, the predominance of active *N*-acylamines containing hydroxy- and methoxybenzoyl substitutents led to the design of lead candidates with polyphenolic moieties. Evaluation of these individual compounds allowed us to hone in on the specific features required to mimic UEA1 and to identify a polygalloyl construct as a lead candidate ligand. Structure-activity studies subsequently confirmed that activity was related to the specific galloyl structure rather than being a general feature of carboxylic acids and that activity increased with the increase in number of galloyl groups displayed on the scaffold, i.e., increasing valency has a direct impact on binding affinity. The ligands exhibited significantly higher activity than the known UEA1 binding inhibitor α-L-fucose, illustrating the power of the screening methodology to select clinically relevant leads.

Multivalency is evident in nature as demonstrated in a number of biologic systems. Protein–carbohydrate interactions are notable examples where enhanced affinity is achieved through polyvalent binding (18). Structurally, lectins are large proteins that contain multiple carbohydrate binding sites and thus potentially bind to cell surfaces in a polyvalent fashion. For example, UEA1 is a homodimeric metalloglycoprotein (68 kd) whose quaternary structure consists of a 12-stranded anti-parallel β-sheet scaffold with carbohydrate binding sites present at both ends (19). UEA1 binds the H-type 2 human blood group determinant [α-L-Fucα(1→2)-β-D-Galβ(1→4)-β-D-GlcNAcα-] and recognition is coordinated by an invariant triad of residues, a common feature of legume lectins (19). Therefore, by presenting the polyphenolic moiety in a multivalent format, the multiple interactions emulated UEA1 binding and thus, a stronger receptor-ligand binding affinity was achieved. In addition, the display of the active moieties on an amide-linked backbone or scaffold may have played an important role in imitating a polypeptide en-

vironment. These factors could account for the ability of compounds with molecular masses of less than 1.5 kd to mimic such complex protein–carbohydrate interactions. Interestingly, clustered derivatives of quinic and shikimic acid attached to a lysinyl core were recently reported as efficient carbohydrate mimics (20). The acid derivatives were selected on the basis of X-ray crystallography studies of the related rat mannose-binding lectin and were proposed for use in targeting of drugs to the mannose receptor on dendritic cells (20). Two- and four-copy constructs derived from quinic and shikimic acid were evaluated in our direct binding assay studies and did not demonstrate significant activity compared with the galloyl constructs (data not shown).

A two-tier competitive assay, in which individual compounds and biotin-labeled UEA1 were incubated separately with Caco-2 cell membranes, provided indirect evidence that the compounds and UEA1 were binding to the same component on the cells. Specificity for the UEA1 binding site was further supported when the 4-copy compound showed no competition for GM1 binding in a CTB/GM1 competition assay. This was subsequently verified using the two lead compounds, biotin-labeled 2- and 4-copy gallic acid constructs, in direct binding and competition assays on cell membranes, and more importantly, binding of the biotin-labeled 4-copy gallic acid construct on whole cells. Detection of binding to whole cells by fluorescence-activated cell sorting required signal amplification as might be expected given that the organic molecule contained a single biotin while UEA1 typically contains biotin at a 4:1 molar ratio (biotin:protein). It was very encouraging to achieve binding with the 4-copy gallic acid construct at concentrations in the low micromolar range with a ligand of much smaller molecular weight than that of UEA1, e.g., between 1 and 2 orders of magnitude lesser in size than UEA1, and this prompted us to evaluate the lead compound *in vivo*. It has been established that the surface expression of oligosaccharides differs between M cells and adjacent enterocytes and that UEA1 labels the surface of mouse Peyer's patch M cells in a highly specific manner (4,21). Using this specificity UEA1 coated latex microspheres have previously been targeted to M cells and indeed, both internalization and transcytosis have been demonstrated (2). The biotin-labeled 4-copy gallic acid construct also mediated M cell-specific adherence of fluorescent streptavidin-polystyrene particles thus providing proof-of-concept data relevant to targeting vaccine delivery to antigen presenting cells. Particle uptake was equivalent to that observed using UEA1.

In summary a tetragalloyl D-lysine amide construct, which mimics the functionality of UEA1 and exhibits substantially higher inhibitory activity than natural UEA1 competitors such as L-fucose, has been characterized. The potential for use of polygalloyl lectin mimetics in oral targeted drug delivery applications has been demonstrated using a model particulate system. The M cell-specific nature of these mimetics in the mouse intestinal loop model is of particular interest in the context of vaccine delivery to antigen presenting cells. Structure activity analysis of the mimetics is ongoing to identify further derivatives with enhanced activity as well as functionality determinants.

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