Synthesis and Application of Integrin Targeting Lipopeptides in Targeted Gene Delivery

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One of the main problems facing gene therapy is the ability to target the delivery of DNA to specific cells of choice. Recently, we developed a synthetic nonviral vector platform system known as LMD (liposome:mu:DNA) that was designed for further modular upgrading with tool-kits of chemical components. First-generation LMD systems were prepared from DC-Chol/DOPE cationic liposomes (DC-Chol = 3β-[N-(N′,N′-dimethylaminoethane)carbamoyl] cholesterol, DOPE = dioleoyl-L-α-phosphatidylethanolamine), μ peptide from the adenovirus core and plasmid DNA (pDNA). Here we report attempts to realise peptide-targeted gene delivery that build upon the LMD platform. Our strategy was to prepare novel lipopeptides with a lipid moiety designed to insert into the outer lipid bilayer of LMD particles whilst simultaneously presenting a peptide moiety for cell-surface receptor binding. One main functional peptide sequence was selected (PLAEIDGIELA; tenascin peptide sequence) known to target $\alpha_9\beta_1$-integrin proteins predominant on upper-airway epithelial cells. This sequence was investigated along with a corresponding control sequence. The syntheses of two classes (A and B) of lipopeptides are reported; the syntheses of class A lipopeptides requires a modification of Mitsunobu chemistry that could be of general utility to facilitate Mitsunobu reactions in other diverse systems. "Targeted" LMD and LD transfections with class A or B lipopeptides exhibit nonspecific peptide enhancements (up to one order of magnitude) over non-lipopeptide control transfections but few specific effects. Specific targeting effects can be seen if the overall LMD or LD particle cationic charge is lowered, but nonspecific effects are never eliminated. Whilst promising, these data now highlight the need for in vivo data and even a new modular, aqueous chemistry for the controlled adaptation of LMD particles in buffer in order for successful peptide-targeted, synthetic, nonviral gene delivery to be realised.

Introduction

Gene therapy can be defined as the delivery of nucleic acids to cells with a vector for some therapeutic purpose. The use of viral-based vectors still dominates gene therapy research and applications,[1,2] even though repeat dose administration is often severely compromised by viral immunogenicity and induced inflammation.[3] Synthetic nonviral vector systems should be ideal surrogates for viral vectors, particularly the cationic liposome/lipid-based systems.[4] Nonviral vectors have no size restrictions concerning the size of nucleic acid molecules that can be formulated (oligonucleotide up to artifical chromosome), they are less likely to elicit a substantial immune response, easier to handle in principle and easier to produce on a large scale. Furthermore, they possess substantially better pharmaceutical and regulatory requirements than viral vector systems. However, synthetic nonviral vector systems remain largely inefficient at nucleic acid delivery when compared with viral vector systems. This fundamental drawback must be corrected if synthetic nonviral vectors are to rival viral vector systems in clinical gene-therapy applications.

With this objective in mind, we constructed a robust and reproducible synthetic nonviral vector platform system that was designed for modular upgrading for in vivo use with tool-kits of purpose-designed chemical components. This platform system is known as liposome:mu:DNA (LMD).[5] LMD is a ternary vector based on the cationic adenoviral core peptide μ (mu) that precondenses plasmid DNA (pDNA) into mu:DNA (MD) nanoparticles (typically 100 ± 20 nm). These MD particles are typically introduced to a suspension of cationic liposomes under rapid vortex mixing conditions to give LMD particles (typically 120 ± 30 nm) that appear to comprise an MD core engulfed within a bilammellar cationic lipid outer coat. Cationic liposomes used to prepare first-generation LMD particles were DC-Chol/DOPE (6:4, m:m) liposomes prepared from cationic lipid (cytofectin) 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) and naturally available neutral lipid dioleoyl-L-α-phosphatidylethanolamine (DOPE). However, in the studies described here, we have also resorted to replacing DC-Chol/DOPE cationic liposomes with CDAN/DOPE (1:1, *m*/*m*) cationic liposomes in which DC-Chol is replaced by the more

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potent cytofectin N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN).[6]

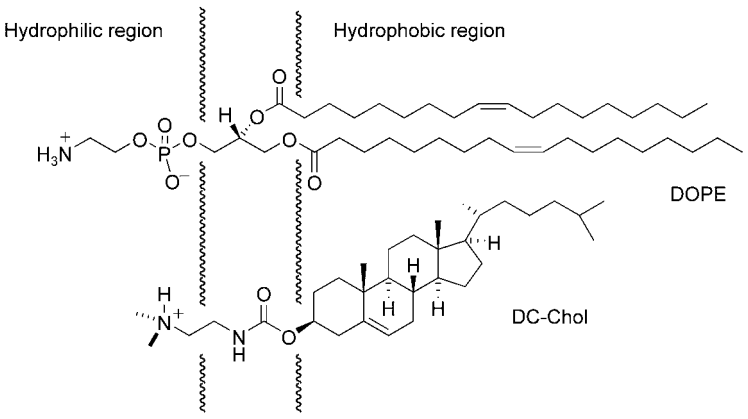
LMD particles have no targeting capacity and deliver pDNA to cells only by virtue of nonspecific electrostatic interactions between the cationic outer surface of particles and the anionic surface of cell membranes. These particles then enter cells within minutes shedding pDNA rapidly and allowing the mu peptide to enter cell nuclei in 15 min.[7] This behaviour inside cells needs to be improved to allow proper carriage of pDNA to the nucleus even when cells are quiescent, nevertheless LMD particles can be claimed to have some virus-like properties in this case.[7] Moreover, LMD particles have sufficient intrinsic stability,[8] in biological fluids to give credible transfection of mice lungs in vivo with an efficacy equivalent to the very best alternative synthetic nonviral vector systems.[5] Realistically, this level of transfection in vivo might need to be improved before more widespread therapeutic applications can be considered.

In an attempt to obtain this level of improvement, we elected to seek further inspiration from the adenovirus and introduce adenovirus-like targeting functionality into the first-generation LMD system. Adenoviridae enter cells by first binding to the coxsackie adenovirus receptor (CAR) followed by the cell-surface $\alpha_v\beta_3$ integrin protein, thereby triggering internalisation.[9,10] Integrins such as the $\alpha_v\beta_3$ integrin are heterodimeric transmembrane glycoproteins consisting of α and β subunits that are usually involved with cell–cell and cell–matrix interactions.[11,12] Several other pathogenic organisms including *Yersinia pseudotuberculosis* also make use of cell surface integrins for cellular entry.[9,13,14] However, although integrin-receptor-mediated entry into cells offers an appealing way to achieve efficient delivery of DNA to cells, care must be taken to select receptors relevant to the target cells of interest.

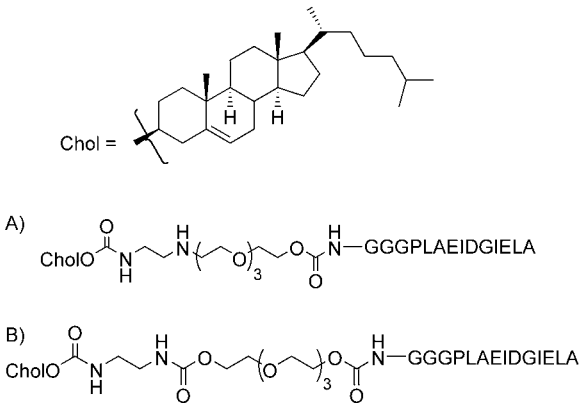
Previously, we developed the use of peptide mini-vectors for gene delivery to cells comprising a pDNA-binding peptide moiety (K_{16}) attached to a cyclic RGD-sequence-containing moiety whose arginine-glycine-aspartate sequence was known to target $\alpha_v\beta_3$ integrins.[15–19] However, whilst $\alpha_v\beta_3$ integrins dominate the lower airways in vivo, they are not prevalent in tracheal cells of the upper airways. This creates a problem, since many lung-associated diseases such as cystic fibrosis involve problems in the upper rather than lower airway. Fortunately, another class of integrin proteins ($\alpha_9\beta_1$) are found in the upper airway for which a targeting sequence (PLAEIDGIELA (**1**), tenascin peptide sequence) has recently been determined.[20,21] Therefore, we elected to try and utilise this sequence in order to obtain efficient targeting of LMD particles to tracheal cells, with potential concomitant improvements in transfection efficiency in vivo. This paper documents the syntheses of lipopeptides comprising the $\alpha_9\beta_1$ integrin-specific tenascin peptide sequence **1** and control sequences, followed by their application in "targeted"-LMD and LD transfection experiments.

Results and Discussion

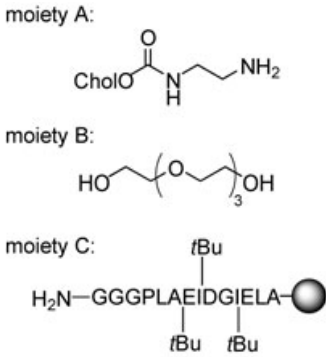
Lipopeptides were designed according to a model proposed by Cooper et al. showing how DOPE and DC-Chol might interact in the cationic liposome bilayer (Scheme 1).[17] Two main classes of lipopeptides were prepared and utilised in this study (Scheme 2). Class A lipopeptides were designed by direct analogy with the monobasic structure of DC-Chol cytofectin and hence possess a central amine-functional group linking cholesterol to the remainder of the molecule. Class B lipopeptides were designed with analogy to class A molecules by replacing the central amine with a neutral, polar amide-functional group. Consequently, we refer to class A lipopeptides as monobasic and class B lipopeptides as neutral. These lipopeptide classes A and B have three main features in common, a cholesteryl-amino amphiphilic moiety (A), a hydrophilic tetra(ethylene glycol) (TEG) moiety (B) and peptide moiety (C; Scheme 3). The TEG moiety (B) was introduced to enhance lipopeptide solubility in aqueous medium given the partial hydrophobic character



Scheme 1. Model of DOPE and DC-Chol interacting in the cationic liposome bilayer as proposed by Cooper et al.[17]



Scheme 2. Structures of the target lipopeptides. A) Class A, protonated at physiological pH; B) class B, neutral at physiological pH.



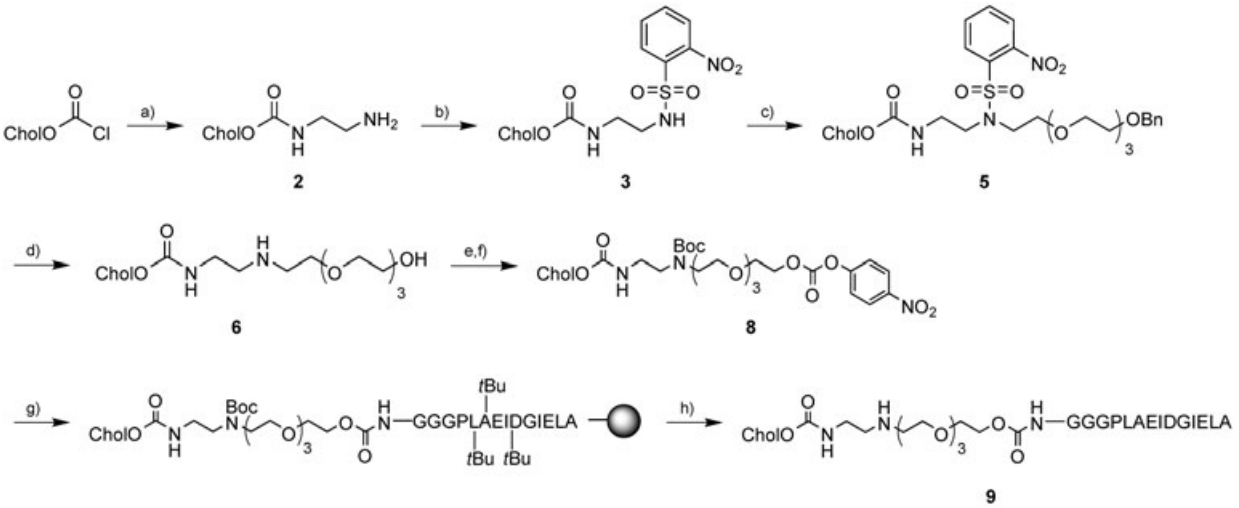
Scheme 3. Lipopeptide structural features.

of the peptide moiety. Moreover, the TEG group was expected to provide some degree of spacing for the peptide moiety from the lipid moiety suitable as a means to promote ligand presentation in the direction of $\alpha_9\beta_1$-integrin receptors. Peptide moieties such as **1** containing the $\alpha_9\beta_1$ integrin-specific sequence or alternate control sequences were all initially synthesised in fully protected form by using standard solid-phase peptide chemistry (Wang resin combined with α-amino Fmoc protecting-group strategy; Fmoc = 9-fluorenylmethyloxycarbonyl), and then coupled to combined cholesterol–TEG moieties whilst remaining on their respective solid supports. As a result, all lipopeptides were prepared by synthetic approaches completed by a similar solid-phase synthetic fragment coupling and final protecting-group removal.

Synthesis of class A lipopeptides

Class A lipopeptides are monobasic and were prepared by means of a highly convergent synthetic procedure involving a novel enhancement of the Mitsunobu reaction (Scheme 4).[22] Initially, ethylene diamine was coupled to cholesteryl chloroformate to generate cholesterylamine **2** in 65% yield, by using a 200-fold excess of ethylene diamine so as to maximise the formation of the monoacylation product. For the onward reaction of **2** with TEG, we reduced the pK_a of the primary amine functional group of **2** by functional-group modification with an arylsulfonyl group.[23–25] This was achieved by combining **2** with 2-nitrobenzylsulfonyl chloride with triethylamine, leading to the formation of an arylsulfonyl derivative, **3**, in good yield. TEG was monoprotected in the presence of silver(I) oxide and benzyl bromide giving ether **4**,[26] that was coupled to **3** to give sulfonamine **5** in the presence of diphenyl 2-pyridylphosphine (PPh_2py) and with the slow addition of di-*tert*-butyl azodicarboxylate (DTBAD) coupling reagent.[27] These Mitsunobu-coupling reaction conditions were developed after a considerable period for optimisation and now appear to represent an important enhancement in the Mitsunobu reaction procedure that might also have applications for increasing the yields of other Mitsunobu reactions in general.[27] Certainly, in our hands we saw the yield of sulfonamine **5** increase from 46% by using the best alternative conditions, to a much improved 71%. Reaction reliability was also markedly enhanced.

Facile deprotection of **5** was achieved with sodium dissolved in dry THF in the presence of naphthalene.[28,29] Primary alcohol **6** was then isolated in excellent yield after reaction quenching with 2,6-di-*tert*-butyl-4-methyl phenol that is converted into non-nucleophilic phenolate anions as a result of the quenching process that do not interfere with product formed during the reaction, hence the excellent yields of **6**. Reprotection of the free secondary amino functional group of **6** was then accomplished with di-*tert*-butyl dicarbonate (Boc_2O) under standard conditions to give Boc-protected alcohol **7**. The use of the Boc protecting group was to prevent unwanted side reactions to-



Scheme 4. Synthesis of class A lipopeptide **9**. Reagents used: a) $H_2NCH_2CH_2NH_2$ (200 equiv), 2 days, 65%; b) 1) 2-NsCl (1.3 equiv), NEt_3 (1.5 equiv), DCM, 14 h, 87%; 2) BnBr (1.1 equiv), Ag_2O (1.5 equiv), 20 h, generating 55%; c) **3** (1.3 equiv), DTBAD (1.5 equiv) slow addition over 1 h in DCM, PPh_2py (1.5 equiv), DCM, 3 h, 71%; d) Na (10 equiv), naphthalene (10 equiv), −30 °C, 45 min, 74%; e) Boc_2O (1 equiv), NEt_3 (1.1 equiv), DCM, 10 h, **6** 84%; f) NEt_3 (2 equiv), DMAP (2 equiv), *p*-nitrophenyl chloroformate (3 equiv), DCM, 10 h, 92%; g) **1** (Fmoc deprotected on resin; 0.5 equiv), NEt_3 (2.5 equiv), DMF, 18 h; h) 95% TFA/H_2O, 90 min, 10%. DMAP = 4-dimethylaminopyridin; TFA = trifluoroacetic acid.

wards the end of the synthesis by use of a protecting group whose removal was also consistent with the acidic global-deprotection conditions available at the very end of the synthesis. Boc-protected alcohol **7** was then prepared for coupling to resin-bound protected peptide, by reaction with *p*-nitrophenyl chloroformate to give activated carbonate **8**, that was in turn used in solid-phase synthetic fragment-coupling reactions to resin-bound protected peptides followed by global protecting group removal. Two protected-peptide sequences were used in fragment coupling reactions to **8**, namely the resin-bound protected form of the $\alpha_9\beta_1$-integrin specific tenascin sequence **1** or the resin-bound protected form of a reordered control sequence **10**. Coupling of activated carbonate **8** with the protected form of **1** followed by global deprotection gave class A lipopeptide **9** in low (ca. 10%) but satisfactory yield post final purification by reversed-phase HPLC on a standard C_4 column (Figure 1). Fragment coupling of **8** with protected form of **10** similarly resulted in a low but satisfactory yield of class A lipopeptide **11** following deprotection and purification.

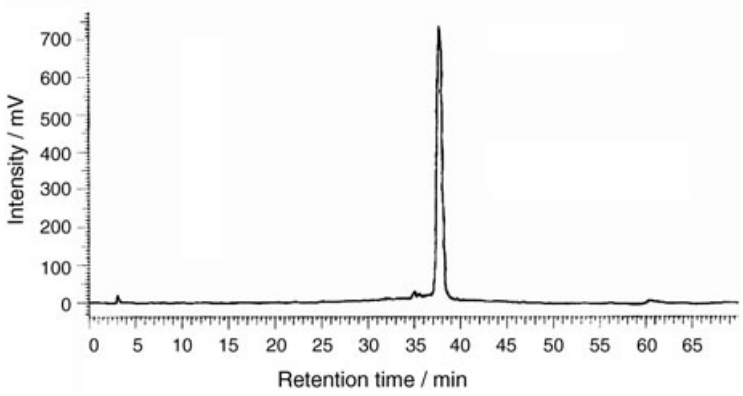


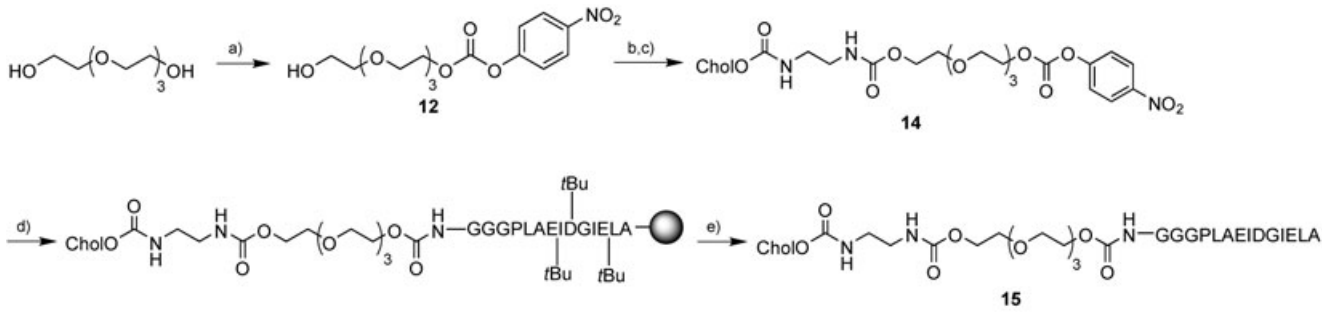
Figure 1. HPLC trace as detected by evaporative light-scattering, identifying the purified lipopeptide class A **9** (eluted with a 30–100% acetonitrile in water gradient over 40 min, 99.5% purity).

Synthesis of class B lipopeptides

As mentioned earlier, class B lipopeptides are neutral but for peptide-associated charges. The syntheses of class B lipopeptides were performed to determine the relative requirement for a monobasic amino functional group at the position shown in class A lipopeptides. Class B lipopeptides were prepared from cholesterylamine **2** that was coupled to TEG by means of the highly effective *p*-nitrophenyl chloroformate reagent (Scheme 5). In order to achieve this, TEG was combined with a mole equivalent of *p*-nitrophenyl chloroformate at 0 °C giving a mixture of mono- and diactivated TEG carbonates from which the desired monoactivated carbonate **12** was isolated in 45% yield. Carbonate **12** was then coupled to cholesterylamine **2** to give primary alcohol **13**. This was then prepared for coupling to resin-bound protected peptides by using *p*-nitrophenyl chloroformate once again in the presence of DMAP to give activated carbonate **14** in 66% yield. The yield fell to approximately 40% in the absence of DMAP. Coupling of **14** with the protected form of tenascin peptide **1** followed by global deprotection gave class B lipopeptide **15** in poor yield post final purification by reversed-phase HPLC on a C_4 column. Fragment coupling of **14** with the protected form of control peptide **10** similarly resulted in a poor but serviceable yield of class B lipopeptide **16** following deprotection and post purification.

Competitive integrin-receptor binding studies

Prior to the performance of any formulation and transfection experiments, receptor-binding studies were performed to compare the relative $\alpha_9\beta_1$ receptor-binding efficiencies of naked tenascin lipopeptides **9** and **15** with those of control lipopeptides **11** and **16**. The natural ligand of $\alpha_9\beta_1$ integrin is known as the tenascin C protein. Both Yokosaki et al. and Schneider et al. published assays for the interaction between the binding domain of tenascin C and $\alpha_9\beta_1$ integrin.[20,21] In our case, we developed a competitive binding assay on the basis of the Schneider et al. procedure. Two cell lines were used both derived from a human colon carcinoma SW480 cell line (gift of Dean Sheppard), an $\alpha_9\beta_1$ integrin-expressing cell line (referred to as alpha 9 cells) and a nonintegrin expressing cell line (referred to as mock cells).[30]



Scheme 5. Synthesis of class B lipopeptide **15**. Reagents used: a) *p*-nitrophenyl chloroformate (1 equiv), py (1.25 equiv), DCM, 0 °C→RT, 20 h, 45%; b) **2** (1.25 equiv), NEt_3 (3.1 equiv), DCM, 12 h, 91%; c) *p*-nitrophenyl chloroformate (3 equiv), NEt_3 (2 equiv), DMAP (2 equiv), DCM, 20 h, 66%; d) **1** Fmoc-deprotected (0.5 equiv), NEt_3 (2.5 equiv), DMF, 18 h, e) 95% TFA/H_2O, 90 min, 4%. py = pyridine.

Competitive-binding studies were performed by preincubation of semiconfluent cell lines (either alpha 9 or mock cells) with various concentrations of free peptides or lipopeptides prior to the addition of individual combined mixtures to individual wells in assay plates (96-well plates) each coated with a fixed quantity of tenascin C. After a washing step, residual adherent cells remaining in each well post washing were determined by staining cells with crystal violet and then measuring absorbance from each well spectrophotometrically. Clearly in such competition binding studies in which alpha 9 cells are involved, the fewer the proportion of cells that remain adherent at the end of the experiment, the more effective must be the corresponding free peptide or lipopeptide in competing for cell-surface $\alpha_9\beta_1$ integrin-receptor binding. Furthermore, when mock cells are involved, these should exhibit only mild nonspecific interactions with the tenascin C coating in each well and therefore should barely adhere irrespective of the presence or otherwise of free peptides or lipopeptides.

Assay data are shown for free tenascin and control peptides (Figure 2). Free tenascin peptide **1** clearly acts to prevent the adherence of alpha 9 cells to tenascin C and therefore competes with tenascin C for interaction with the cell-surface $\alpha_9\beta_1$-integrin receptors of alpha 9 cells. In contrast, free control peptide **10** provides no competition for interactions between tenascin C and alpha 9 cells as expected. Finally mock cells appear to adhere only modestly to tenascin C in line with expectations since they do not express cell-surface $\alpha_9\beta_1$-integrin receptors. Compare this data set with data obtained by using class A lipopeptides **9** and **11** (Figure 3). Lipopeptide **9** (comprising a tenascin peptide moiety) appears to act similarly to free tenascin peptide **1**, if a little less efficiently whilst lipopeptide **11** (comprising a control peptide moiety) appears to act similarly to control peptide **10**. Similar results were obtained respectively with class B lipopeptides **15** and **16** (results not shown). These data clearly demonstrate that the biological efficacy of the tenascin peptide **1** and the precision of the control peptide **10** are retained post conjugation into lipopeptide structures.

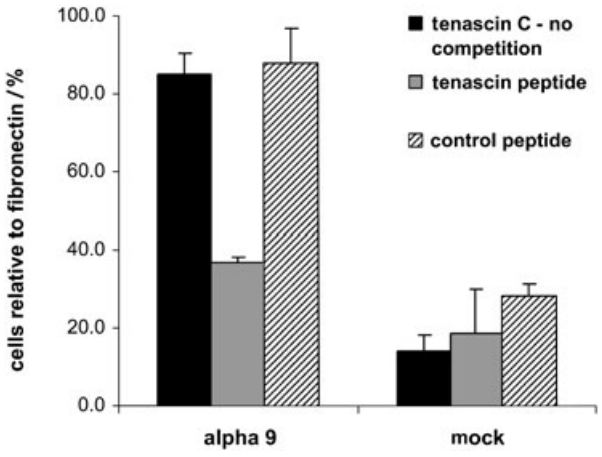


Figure 2. Competitive cell-binding assays with free tenascin peptide **1** and control peptide **10**. The graph illustrates the result of cell adherence when alpha 9 or mock cells are incubated without peptide, with free tenascin peptide **1** or with free control peptide **10** and then introduced into the wells of 96-well plates coated with tenascin C protein. In the absence of peptide or in the presence of control peptide, alpha 9 cells adhere to tenascin C easily, but not in the presence of tenascin peptide **1**. Mock cells do not adhere specifically to tenascin-coated wells under any circumstances.

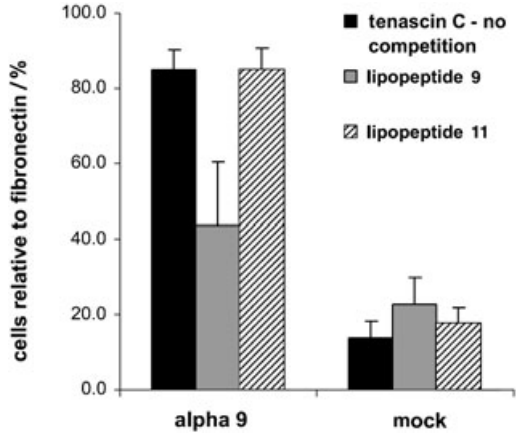


Figure 3. Competitive cell-binding inhibition assays with class A lipopeptides **9** and **11**. The graph illustrates the result of cell adherence when alpha 9 or mock cells are incubated without lipopeptide, with lipopeptide **9** (comprising tenascin peptide) or with lipopeptide **11** (comprising control peptide) and then introduced into the wells of 96-well plates coated with tenascin C protein. Lipopeptide **9** behaves like free tenascin peptide **1** (see Figure 2) and **11** (like free control peptide **10**). Hence **9** is an active ligand of the $\alpha_9\beta_1$ receptor.

LMD and LD transfections

LMD transfection experiments were then performed to determine the relative benefits of incorporating class A or B lipopeptides into the bilammellar liposomal outer coat of LMD particles. Comparisons were also made with corresponding cationic liposome–pDNA (LD; lipoplex) systems. In both cases, a premodification strategy was followed in which CDAN/DOPE cationic liposomes were prepared with class A or B lipopeptides (0.05–5 mol %) and then used to formulate LMD or corresponding LD systems from pDNA afterwards. The most striking aspect of our LMD transfection data (15 min transfection time) is the lack of pronounced "targeting effects", instead the presence of any lipopeptide (especially at low mol %) resulted in an impressive nonspecific boost to transfection efficiency of at least an order of magnitude irrespective of the cell type (either alpha 9 or mock cell line). Results are shown for "targeted" LMD transfections with class A lipopeptides **9** and **11** (Figure 4), LMD transfections with class B lipopeptides **15** and **16** gave similar results (data not shown). LD transfections (4 h transfection time) were also performed and showed the same nonspecific enhancement (data not shown); this suggested that we were observing a generic type of nonspecific peptide enhancement of cationic liposome-based transfection. Others have made similar observations working with "protein-targeted systems" in preference to the peptide-based systems described here. For instance, apotransferrin has been shown to confer

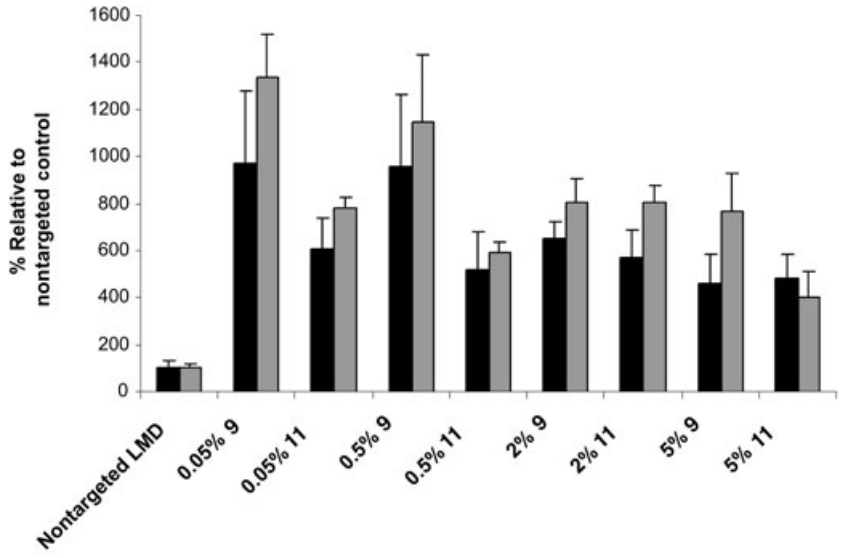


Figure 4. LMD transfection data (15 min transfection time) on alpha 9 (black) and mock (grey) cells obtained with LMD particles presenting class A lipopeptides (either lipopeptide **9** or control lipopeptide **11**) incorporated at the indicated mol%. All transfection data were determined in triplicate with the units of RLU mg^{-1} cellular protein and then normalised with respect to the transfection levels obtained with mock cells by using naked LMD particles without lipopeptide.

were always competitive with peptide-targeting effects even when near neutral systems were prepared.

The presented situation illustrates an important principle of balance between specific ligand-mediated processes of cellular uptake and nonspecific processes, suggesting that pDNA delivery systems comprised of condensed pDNA and lipid are unlikely to be amenable to pure receptor-specific targeting and uptake even with a biologically compatible ligand. A somewhat traditional view is that this balance problem between specific and nonspecific delivery can be corrected by coating the surface of an LD (or LMD) particle with a "stealth" polymer such as polyethylene glycol (PEG) to which a ligand might be attached. Such a surface coating is undoubtedly essentially for certain elements of biocompatibility such as resistance to aggregation in biological fluids and resistance to reticulo-endothelial system (RES)-mediated degradation,[33] owing to the formation of a dense network of fibril-like structures on particle surfaces.[34] Furthermore, such a surface coat-

similar nonspecific enhancements to LD transfections.[31] This effect was ascribed to a "nonreceptor"-mediated process that involves endocytosis, enhanced acidification of endosomes with enhanced endosomolysis, and potentially enhanced cytosolic transport of pDNA.

Recently Kono et al. have noted that transferrin also confers a nonspecific enhancement on LD transfection but that that effect becomes less nonspecific and more specific as LD positive charge is reduced.[32] Given this, additional LMD and LD transfection experiments were also conducted in which LMD and LD systems were prepared from DC-Chol/DOPE cationic liposomes with class A or B lipopeptides (0.05–5 mol%). In this instance, a modest but reproducible cell-specific effect could be observed overlaid on top of the nonspecific peptide enhancement effects seen above. Representative LD transfection data with class A lipopeptides are shown (Figure 5). The targeting effect (comparing alpha 9 to corresponding mock cell LD transfection data) is not more than two- to threefold. Similar effects were observed by using class B lipopeptides (results not shown), and for all LMD transfections with both classes of lipopeptide (data not shown).

The cytofectin DC-Chol has fewer available amino-functional groups than CDAN cytofectin and has a lower overall charge at neutral pH.[6] Therefore, LMD and LD systems formulated with DC-Chol rather than CDAN are of lower charge by default. Hence, the emergence of a modest targeting effect could be similarly correlated in our case with a reduction in the overall positive charge of LMD and LD particles as a result of the change in cytofectin. On this basis, further experiments were performed to study the effects of lowering the overall particle positive charge further. This proved unsuccessful since lipid-membrane interactions between liposomes and cell-surface

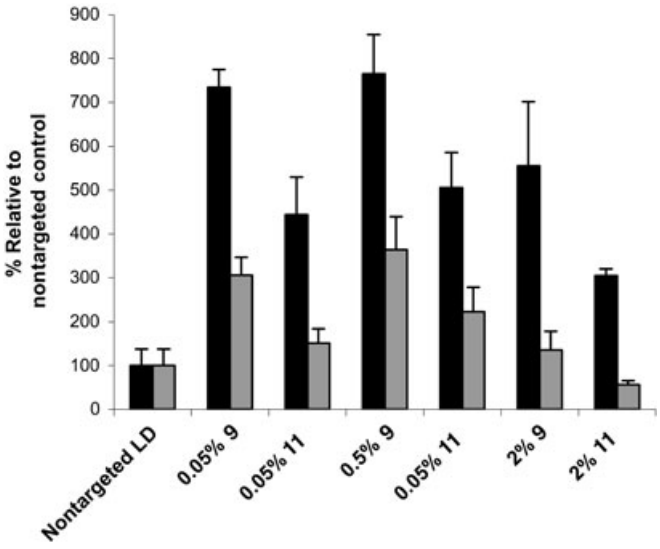


Figure 5. LD transfection data (4 h transfection time) obtained on alpha 9 (black) and mock (grey) cells with LD particles presenting class A lipopeptides (either lipopeptide **9** or control lipopeptide **11**) incorporated at the indicated mol%. All transfection data were determined in triplicate with the units of RLU mg^{-1} cellular protein and then normalised with respect to the transfection levels obtained in the mock cells by using naked LD particles without lipopeptide.

ing necessarily shields exterior positive charge and hence electrostatic interaction between a cationic LMD or LD particle and a cell-surface membrane. However, we have clearly shown that PEG^{3400}-coated LMD particles are more than competent to enter cells by nonreceptor-mediated endocytosis,[7] and in addition are unable to mediate further transfection owing to endosome-like vesicle entrapment.

Our conclusion to these data was that the PEG coating is very cell-surface interactive and does not prevent cellular uptake. Therefore, the PEG coating needs to be triggerable, that is stable and nonreactive in exterior biological fluids but unstable towards release within interior endosome compartments post cell entry, in order that naked LMD or LD particles might continue the transfection process unencumbered. Such triggerable systems are now under development in our laboratories. However, even when such systems become available, there remains one inescapable conclusion. Concerning the interactions between LD or LMD particles and cell-surface membranes, nonspecific interactions will always play an essential, dominant role in cellular uptake, irrespective of the transfection system involved and whether or not a stealth polymer coating is involved.

Therefore, what is the role of specific ligand-mediated binding events given such a situation? In our view, specific ligand-mediated or receptor-specific binding events are primarily required for "residence-time" in vivo. In other words, specific binding events should exist to promote the accumulation of transfection competent particles within an organ of choice in association with target cells of interest, prior to internalisation by largely nonspecific effects. The power of receptor-mediated binding events to localise particles in vivo has been graphically demonstrated by Medina et al. in their recently described work concerning targeting to lymphocytes by highly selective integrin-receptor binding peptides.[35] Accordingly, we would argue that there are four main types of synthetic nonviral vector system that should be operable in various gene therapy scenarios:

I) Simple vector systems such as LMD that possess elements of stability and the ability to mediate transfection even in high serum conditions (ca. 100%), could have limited applications in which local delivery is possible or in which there is the possibility for rapid (minutes), passive accumulation in an organ of interest following systemic delivery.
II) Vector systems such as LMD that are also equipped with receptor-specific targeting ligands might have useful applications when the efficiency of rapid, passive organ accumulation needs to be enhanced.[36]
III) Vector systems such as triggerable LMD (trigLMD) that possess a biocompatible stealth polymer coating for enhanced stability in vivo, should be ideal for situations in which passive organ accumulation is possible but takes place only slowly (hours) following systemic delivery.[37]
IV) Vector systems such as trigLMD that are equipped with receptor-specific targeting ligands should be essential for those other applications in which there is a requirement to enhance the efficiency of slow, passive organ accumulation or elsewhere there is a requirement for active accumulation of particles into an alternative organ of choice.

Synthetic nonviral vector systems corresponding to types I and II are now available in our laboratories and await extensive evaluation in vivo in order to marry potential applications with vector properties. We anticipate that vector systems corresponding with types III and IV should also be available in our laboratories in the very near future. Clearly, from a pharmaceutical perspective, the simpler the system that mediates effective gene delivery is, the better, but appropriate in vivo performance is also paramount, and that might necessitate increasing the molecular complexity in ways that might otherwise be undesirable.

Conclusion

The aim of this study was to prepare novel classes of lipopeptides and investigate their capacity to target LMD and LD transfections. Data show that only partial targeting effects were obtained at best and that these were always competing with nonspecific background effects, even when the overall LMD or LD particle positive charge was reduced. However, we argue that the true value of specific receptor-mediated processes will be realised only in vivo. Therefore, LMD particles equipped with cell-surface receptor-specific targeting ligands should be validated in vivo at the earliest opportunity prior to the creation of more complex systems that involve the introduction of a triggerable stealth molecule surface coat.

Experimental Section

General chemistry: ^{1}H NMR spectra were recorded on either the Bruker DRX_{300}, Jeol GX-270Q or Bruker $Advance_{400}$ by using residual isotopic solvent ($CHCl_3$, $\delta_H = 7.26$ ppm) as an internal reference. ^{13}C spectra were also recorded on the same range of spectrometers employing $CDCl_3$ ($\delta_C = 77.0$ ppm) as an internal reference. FAB mass spectra were recorded on VG-7070B, Jeol SX-102 instruments, and ESI mass spectrometry was carried out by using a Bruker Daltronics ESI 6000 spectrometer. Infrared spectra were recorded on a Jasco 620 FTIR spectrometer. Where appropriate, a Pharmacia LKB–Ultrospec III (deuterium lamp at 300 nm) was used to read the UV absorbance. Chromatography refers to flash column chromatography on Merck-Kieselgel 60 (230–400 mesh). TLC refers to thin layer chromatography performed on precoated Merck Kieselgel 60 F_{254} aluminium-backed plates and visualised with ultraviolet light (254 nm) and acidic ammonium molybdate(IV), iodine, bromocresol green, Dragendorff's reagent, ninhydrin and chloranil. DCM was distilled from phosphorus pentoxide, other solvents were bought predried as required. All the reactions were performed under nitrogen with dry solvents unless otherwise stated. The FastMoc reagent *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluorium (HBTU) was obtained from Advanced Chemtech Europe (Cambridge, UK) and CN Bioscience (Nottingham, UK). DMF and acetonitrile were purchased from Rathburn (Walker–Burn, Scotland). All the reagents used in the syntheses were of the highest purity. The amino acids and resins were obtained from Nova Biochem. The numbering of cholesterol is in accordance with the literature.[38]

Synthesis of lipopeptides

*Tenascin peptide (**1**), deprotected*: This peptide was synthesised by using preloaded Fmoc-Ala-Wang resin (0.4 g, 0.2 mmol, 0.5 mmol g^{-1}). The amino acids were coupled to the resin by using standard solid-phase peptide chemistry techniques. Once the resin was swollen by using DMF the peptide sequence was built up by alternating coupling and Fmoc-deprotection steps. Fmoc deprotection was achieved by cleaving with a solution of 25% piperidine in DMF (2×5 min, 10 mL) followed by washing with DMF (5×2 min, 10 mL). After each Fmoc deprotection step, the collected cleavage and washing solutions were diluted and the absorbance was determined as for the initial loading calculations in order to check the completeness of the Fmoc cleavage. A Kaiser test[39] was performed; if it was negative the deprotection step was repeated. Once the Fmoc groups were removed a solution of Fmoc-protected amino acid (3 equiv), HBTU (3 equiv) and *N*,*N*-diisopropylethylamine (DIPEA, 5 equiv) in DMF (5–10 mL) was added to the resin and shaken for 45 mins. The resin was washed with DMF (5× 2 min, 10 mL) and another Kaiser test performed; again, if a negative result was not obtained the coupling was repeated. This process was repeated until the correct amino acid sequence was obtained. The resin was then washed with DCM (2×5 mL, 2 min) and MeOH (2×5 mL, 2 min). Batches (50 mg) were deprotected and cleaved for characterisation. The crude peptide was purified by reversed-phase HPLC (Vydac C4 column at a flow rate of 1 mL min^{-1}, 214 nm) with a gradient of 30–100% acetonitrile in water over 40 min. G_3PLAEIDGIELA (10 mg, 27% yield) eluting at R_t = 6.24 min, 99% purity. *m/z* (ESI) 1311 $[M+H]^+$, $C_{57}H_{95}N_{14}O_{21}$ requires $[M+H]^+$ 1311.

N^1-*Cholesteryloxycarbonyl-1,2-diaminoethane (**2**)*: A round-bottomed flask was charged with cholesteryl chloroformate (8 g, 17.8 mmol), ethylenediamine (240 mL, 3.56 mol) was slowly added with stirring. The reaction mixture was then stirred for two days until the reaction went to completion. The base was neutralised with an icy slurry, then washed with water (2×20 mL), extracted with DCM (2× 50 mL), dried (Na_2SO_4), and the solvent removed by reduced pressure. The resultant residue was redissolved in DCM and loaded onto a silica flash column (DCM/MeOH/NH_3 92:7:1) yielding amine **2** (5.5 g, 65% yield) as a white solid. R_f = 0.15 (DCM/MeOH/NH_3 92:7:1); ^{1}H NMR (270 MHz, $CDCl_3$): δ = 0.64 (s, 3H; H-18′), 0.83 (d, *J* = 6.5 Hz, 6H; H-26′, H-27′), 0.85 (d, *J* = 6.5 Hz, 3H; H-21′), 0.92 (s, 3H; H-19′), 0.99–1.62 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.64–2.04 (m, 5H; H-2′, H-7′, H-8′), 2.09–2.39 (m, 2H; H-24′), 2.76 (t, *J* = 6 Hz, 2H; H-2), 3.16 (q, *J* = 5.5 Hz, 2H; H-1), 4.44 (m, 1H; H-3′), 5.33 (br m, 2H; H-6′, CholOCON*H*); ^{13}C NMR (270 MHz, $CDCl_3$): δ = 11.8 (C-18′), 18.6 (C-21′), 19.2 (C-19′), 20.9 (C-11′), 22.5 (C-26′), 22.7 (C-27′), 23.7 (C-23′), 24.2 (C-15′), 27.9 (C-16′), 28.1 (C-2′, C-25′), 31.7 (C-7′, C-8′), 35.7 (C-20′), 36.1 (C-22′), 36.4 (C-10′), 36.9 (C-1′), 38.3 (C-24′), 39.4 (C-4′), 39.6 (C-12′), 41.7 (C-13′), 42.2 (C-2), 43.5 (C-1), 49.9 (C-9′), 56.0 (C-17′), 56.6 (C-14′), 73.6 (C-3′), 122.4 (C-6′), 139.7 (C-5′), 156.5 (NCOO); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 2940, 2867, 1697, 1467 cm^{-1}; FABMS: *m/z*: 473 $[M+H]^+$, 369 $[Chol]^+$; HRMS: calcd for $C_{30}H_{52}N_2O_2$ $[M+H]^+$: 473.4110, found: $[M+H]^+$ 473.4125.

N^1-*Cholesteryloxycarbonyl-1-amino-2″-nitrobenzenesulfonamidoethane (**3**)*: 2-Nitrobenzylsulfonyl chloride (2.6 g, 13.8 mmol) and NEt_3 (2.2 mL, 15.9 mmol) were added to a solution of amine **2** (5.0 g, 10.6 mmol) in dry DCM (50 mL) and stirred for 14 h. The reaction mixture was washed with water (2×20 mL), extracted with DCM (2×20 mL), dried (Na_2SO_4), concentrated under vacuum, and purified by column chromatography producing nosyl **3** (6 g, 87%) as a white solid. R_f = 0.2 (DCM/MeOH/NH_3 99:0.87:0.13); ^{1}H NMR (270 MHz, $CDCl_3$): δ = 0.65 (s, 3H; H-18′), 0.82 (d, *J* = 6.5 Hz, 6H; H-26′, H-27′), 0.85 (d, *J* = 6.5 Hz, 3H; H-21′), 0.94 (s, 3H; H-19′), 0.99–1.66 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.68–2.05 (m, 5H; H-2′, H-7′, H-8′), 2.09–2.33 (m, 2H; H-24′), 3.08–3.34 (m, 4H; H-1, H-2), 4.38 (m, 1H; H-3′), 5.30 (m, 1H; H-6′), 5.41 (br m, 1H; CholOCON*H*), 6.11 (br m, 1H; N*H*SO_2), 7.70 (m, 2H; H-4″, H-5″), 7.76 (m, 1H; H-6″), 8.07 (m, 1H; H-3″); ^{13}C NMR (270 MHz, $CDCl_3$): δ = 11.7 (C-18′), 18.6 (C-21′), 19.2 (C-19′), 20.9 (C-11′), 22.4 (C-26′), 22.7 (C-27′), 23.7 (C-23′), 24.1 (C-15′), 27.8 (C-16′), 27.9 (C-2′), 28.1 (C-25′), 31.7 (C-7′, C-8′), 35.6 (C-20′), 36.0 (C-22′), 36.3 (C-10′), 36.8 (C-1′), 38.3 (C-24′), 39.3 (C-4′), 39.6 (C-12′), 40.4 (C-2), 42.1 (C-13′), 43.5 (C-1), 49.7 (C-9′), 56.0 (C-17′), 56.5 (C-14′), 74.5 (C-3′), 122.3 (C-6′), 125.1 (C-3″), 130.8 (C-6″), 132.7 (C-4″), 133.2 (C-1″), 133.5 (C-5″), 139.5 (C-5′), 147.7 (C-2″), 156.5 (NCOO); IR (Nujol): $\tilde{\nu}_{max}$ = 3337, 2923, 1697, 1540, 1461, 1376, 1162 cm^{-1}; FABMS: *m/z*: 680 $[M+Na]^+$, 658 $[M+H]^+$, 369 $[Chol]^+$; HRMS: calcd for $C_{36}H_{55}N_3O_6S$ $[M+H]^+$: 658.3890, found: 658.3896.

*3,6,9-Trioxa-11-benzyloxyundecan-1-ol (**4**)*:[26] A dry flask was charged with a solution of TEG (predried with Na_2SO_4; 1 g, 5.15 mmol) in dry DCM (100 mL), silver(I) oxide (1.8 g, 7.73 mmol) was added, and the mixture was placed under nitrogen. It was left stirring for 15 min before benzyl bromide (0.67 mL, 5.57 mmol) was added. The reaction was monitored by TLC and went to completion after 20 h. The solvent was concentrated and loaded directly onto a silica flash column (ethyl acetate/hexane/dioxane (72:24:4); R_f 0.2) producing ether **4** as a colourless oil (0.8 g, 55% yield). ^{1}H NMR (270 MHz, $CDCl_3$): δ = 2.98 (br m, 1H; OH), 3.52–3.71 (m, 16H; H-1, H-2, H-4, H-5, H-7, H-8, H-10, H-11), 4.52 (s, 2H; H-13), 7.18–7.35 (m, 5H; H-1′-5′); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 61.3 (C-1), 69.2 (C-11), 70.0 (C-10), 70.3 (C-4, C-5, C-7, C-8), 72.4 (C-2), 73.0 (C-13), 127.4 (C-4′), 127.5 (C-2′, C-6′), 128.1 (C-3′, C-5′), 138.0 (C-1′); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3462, 3060, 2871, 1456, 1098 cm^{-1}; FABMS: *m/z*: 285 $[M+H]^+$, 91 (C_7H_7); HRMS: calcd for $C_{15}H_{24}O_5$ $[M+H]^+$: 285.1702, found: 285.1693.

N^1-*Cholesteryloxycarbonyl-3-aza*-N^3-*2″-nitrobenzenesulfonyl-,6,9,12-trioxa-15-benzyloxy-1-amino-tetradecane (**5**)*: Ether **4** (1 g, 3.5 mmol) was dissolved in dry DCM (100 mL) and charged to a dry round-bottomed flask under nitrogen. Nosyl **3** (2.3 g, 3.5 mmol) and diphenyl 2-pyridylphosphine (1.4 g, 5.3 mmol) were added to the flask and stirred for 10 min. A solution of DTBAD (1.2 g, 5.3 mmol) in dry DCM (10 mL) was transferred at 10 mL h^{-1} to the reaction vessel. The solution was stirred vigorously during the slow addition and for additional 2 h. The solvent was removed under reduced pressure, and the crude product was purified as described above for **4** to produce the Mitsunobu product **5** (2.3 g, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.68 (s, 3H; H-18′), 0.85 (d, *J* = 6.5 Hz, 6H; H-26′, H-27′), 0.90 (d, *J* = 6.5 Hz, 3H; H-21′), 0.99 (s, 3H; H-19′), 1.00–1.62 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.78–2.09 (m, 5H; H-2′, H-7′, H-8′), 2.15–2.38 (m, 2H; H-24′), 3.38 (m, 2H; H-1), 3.45 (m, 2H; H-4), 3.52 (t, *J* = 4.8 Hz, 2H; H-2), 3.58 (m, 2H; H-5), 3.59 (m, 12H; H-7, H-8, H-10, H-11, H-13, H-14), 4.47 (m, 1H; H-3′), 4.58 (s, 2H; H-16), 5.38 (m, 1H; H-6′), 5.55 (br m, 1H; CholOCON*H*), 7.27–7.37 (m, 5H; H-1‴-5‴), 7.62 (m, 1H; H-4″), 7.68 (m, 2H; H-5″, H-6″), 8.05 (m, 1H; H-3″); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.7 (C-18′), 18.6 (C-21′), 19.1 (C-19′), 20.9 (C-11′), 22.4 (C-26′), 22.7 (C-27′), 23.7 (C-23′), 24.1 (C-15′), 27.9 (C-16′), 28.0 (C-2′), 28.1 (C-25′), 31.7 (C-7′, C-8′), 35.6 (C-20′), 36.0 (C-22′), 36.4 (C-10′), 36.8 (C-1′), 38.4 (C-24′), 39.1 (C-1), 39.6 (C-4′), 39.6 (C-12′), 41.1 (C-13′), 48.3 (C-4), 48.7 (C-2), 49.2 (C-9′), 56.0 (C-17′), 57.5 (C-14′), 69.3 (C-5), 69.8 (C-14), 70.1 (C-7), 70.2 (C-13), 70.3 (C-8), 70.4 (C-10), 70.5 (C-11), 73.1 (C-16), 74.1 (C-3′), 122.2 (C-6′), 124.0 (C-3″), 127.5 (C-4‴), 127.6 (C-2‴, C-6‴), 128.2 (C-3‴, C-

5‴), 130.7 (C-6″), 131.7 (C-4″), 132.8 (C-1″), 133.4 (C-5″), 138 (C-1‴), 139.7 (C-5′), 148.0 (C-2″), 156.2 (NCOO); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3345, 3045, 2945, 2873, 1711, 1455, 1123, 1010 cm^{-1}; FABMS: *m/z*: 946 $[M+Na]^+$, 924 $[M+H]^+$, 369 [Chol]$^+$; HRMS: calcd for $C_{51}H_{77}N_3O_{10}S$ $[M+H]^+$: 924.5408, found: 924.5376.

N^1-*Cholesteryloxycarbonyl-3-aza-6,9,12-trioxa-1-amino-15-hyrdoxytetradecane* (**6**):[28,29] A dry round-bottomed flask was charged with naphthalene (4.5 g, 34.7 mmol) and sodium metal (0.8 g, 34.7 mmol) under nitrogen. Dry THF (100 mL) was added to the flask, and the mixture was vigorously stirred for 1 h. The solution was cooled to −30 °C with cardice and acetone. A solution containing the Mitsunobu product **5** (1.6 g, 1.7 mmol) in dry THF (10 mL) was added to the flask over 5 min, and the reaction mixture was stirred for 45 min at −30 °C, at which point it had had gone to completion. A solution of 2,6-di-*tert*-butyl-4-methyl phenol (7.48 g, 34.7 mmol) in THF (2 mL) was slowly added, and the reaction mixture was allowed to warm to room temperature. The solvent was removed under reduced pressure, and the resultant residue was dry loaded onto a silica flash column (DCM/MeOH/NH_3 (97:2.5:0.5)) to give the deprotected product **6** as a viscous white solid (0.83 g, 74 %). R_f = 0.25 (DCM/MeOH/NH_3 92:7:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.67 (s, 3H; H-18′), 0.85 (d, J = 6.5 Hz, 6H; H-26′, H-27′), 0.90 (d, J = 6.5 Hz, 3H; H-21′), 0.99 (s, 3H; H-19′), 1.01–1.65 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.76–2.08 (m, 5H; H-2′, H-7′, H-8′), 2.17–2.41 (m, 2H; H-24′), 2.74 (m, 4H; H-2, H-4), 2.98 (br, 1H; OH), 3.30 (m, 2H; H-1), 3.58–3.79 (m, 14H; H-5, H-7, H-8, H-10, H-11, H-13, H-14), 4.50 (m, 1H; H-3′), 5.36 (m, 1H; H-6′), 5.72 (br m, 1H; CholOCON*H*); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.8 (C-18′), 18.7 (C-21′), 19.3 (C-19′), 21.0 (C-11′), 22.5 (C-26′), 22.8 (C-27′), 23.8 (C-23′), 24.2 (C-15′), 28.0 (C-16′), 28.2 (C-2′), 29.6 (C-25′), 31.8 (C-7′, C-8′), 35.7 (C-20′), 36.0 (C-22′), 36.5 (C-10′), 36.9 (C-1′), 38.6 (C-24′), 39.5 (C-4′), 39.7 (C-12′), 40.3 (C-13′), 42.2 (C-1), 48.4 (C-4), 48.7 (C-2), 49.9 (C-9′), 56.1 (C-17′), 56.6 (C-14′), 61.3 (C-14), 69.7 (C-7), 70.1 (C-8), 70.3 (C-10, C-11), 70.5 (C-5), 73.0 (C-13), 74.1 (C-3′), 122.4 (C-6′), 139.9 (C-5′), 156.3 (NCOO); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3610, 3345, 2936, 2863, 1698, 1456, 1260 cm^{-1}; FABMS: *m/z*: 649 $[M+H]^+$, 369 [Chol]$^+$; HRMS: calcd for $C_{38}H_{68}N_2O_6$ $[M+H]^+$: 649.5156, found: 649.5144.

N^1-*Cholesteryloxycarbonyl-3-aza*-N^3-tert-*butoxycarbonyl-6,9,12-trioxa-1-amino-15-hydroxytetradecane* (**7**): Di-*tert*-butyldicarbonate (10 mg, 0.046 mmol) and NEt_3 (4.1 μL, 0.05 mmol) were added under nitrogen to a solution of alcohol **6** (30 mg, 0.046 mmol) in dry DCM (1 mL). The reaction was stirred for 10 h until it had gone to completion. The solvent was removed in vacuo, and the mixture was dry loaded onto a silica flash column (ethyl acetate/hexane (1:1)) to produce the Boc-protected alcohol **7** as a white viscous solid (28.5 mg, 84 %). R_f = 0.3 (DCM/MeOH/NH_3 92:7:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.69 (s, 3H; H-18′), 0.88 (d, J = 6.5 Hz, 6H; H-26′, H-27′), 0.93 (d, J = 6.5 Hz, 3H; H-21′), 1.02 (s, 3H; H-19′), 1.03–1.72 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.47 (s, 9H; Me of *t*Bu), 1.76–2.10 (m, 5H; H-2′, H-7′, H-8′), 2.22–2.48 (m, 2H; H-24′), 3.14 (br, 1H; OH), 3.26–3.48 (m, 6H; H-1, H-2, H-4), 3.52–3.85 (m, 14H; H-5, H-7, H-8, H-10, H-11, H-13, H-14), 4.49 (m, 1H; H-3′), 5.35 (m, 1H; H-6′), 5.65 (br m, 1H; CholOCON*H*); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.5 (C-18′), 18.4 (C-21′), 19.0 (C-19′), 20.7 (C-11′), 22.3 (C-26′), 22.5 (C-27′), 23.5 (C-23′), 24.0 (C-15′), 27.7 (C-16′), 27.9 (C-2′, C-25′), 28.1 (Me of *t*Bu), 31.5 (C-7′, C-8′), 35.5 (C-20′), 39.5 (C-22′), 36.2 (C-10′), 36.7 (C-1′), 38.3 (C-24′), 39.3 (C-4′), 39.4 (C-12′), 39.7 (C-1), 42.0 (C-13′), 47.9 (C-4), 48.2 (C-2), 49.7 (C-9′), 55.9 (C-17′), 56.3 (C-14′), 61.2 (C-14), 69.5 (C-5), 70.0 (C-7, C-8), 70.1 (C-10, C-11), 72.3 (C-13), 73.7 (C-3′), 79.5 (C-Me_3), 122.1 (C-6′), 139.5 (C-5′), 155.9 (2×NCOO); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3611, 2947, 2871, 1698, 1652, 1456, 1265 cm^{-1}; FABMS: *m/z*: 771 $[M+Na]^+$, 749 $[M+H]^+$, 649 $[M-Boc+H]^+$, 369 [Chol]$^+$; HRMS: calcd for $C_{43}H_{76}N_2O_8$ $[M+H]^+$: 749.5680, found: 749.5708.

N^1-*Cholesteryloxycarbonyl-3-aza*-N^3-tert-*butoxycarbonyl-6,9,12-trioxa-1-amino-15-O^{15}-4‴-nitrophenyloxycarbonyltetradecane* (**8**): Alcohol **7** (50 mg, 0.067 mmol) was dissolved in dry DCM (3.5 mL), then DMAP (16 mg, 0.13 mmol), NEt_3 (19 μL, 0.13 mmol) and *p*-nitrophenyl chloroformate (41 mg, 0.2 mmol) were added under nitrogen. The reaction was stirred for 10 h, the solvent removed under reduced pressure, and the crude product was purified by column chromatography (ethyl acetate/hexane (3:2)) to give carbonate **8** as a viscous white solid (56 mg, 92 %). R_f = 0.65 (DCM/MeOH 95:5); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.69 (s, 3H; H-18′), 0.87 (d, J = 6.5 Hz, 6H; H-26′, H-27′), 0.91 (d, J = 6.5 Hz, 3H; H-21′), 1.00 (s, 3H; H-19′), 1.01–1.72 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.46 (s, 9H; Me of *t*Bu), 1.78–2.14 (m, 5H; H-2′, H-7′, H-8′), 2.21–2.44 (m, 2H; H-24′), 3.26–3.52 (m, 6H; H-1, H-2, H-4), 3.56–3.78 (m, 10H; H-5, H-7, H-8, H-10, H-11), 3.80 (m, 2H; H-13), 4.42–4.54 (m, 3H; H-3′, H-14), 5.35 (m, 1H; H-6′), 5.63 (br m, 1H; CholOCON*H*), 7.38 (m, 2H; H-2″, H-6″), 8.27 (m, 2H; H-3″, H-5″); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.5 (C-18′), 18.4 (C-21′), 19.0 (C-19′), 20.7 (C-11′), 22.3 (C-26′), 22.5 (C-27′), 23.5 (C-23′), 24.0 (C-15′), 27.7 (C-16′), 27.9 (C-2′, C-25′), 28.1 (Me of *t*Bu), 31.5 (C-7′, C-8′), 35.5 (C-20′), 39.5 (C-22′), 36.2 (C-10′), 36.7 (C-1′), 38.3 (C-24′), 39.2 (C-4′), 39.4 (C-12′), 39.7 (C-1), 42.1 (C-13′), 48.0 (C-4), 48.2 (C-2), 49.7 (C-9′), 55.8 (C-17′), 56.3 (C-14′), 69.3 (C-5), 69.9 C-13), 70.2 (C-7, C-8, C-10, C-11),70.4 (C-14), 73.9 (C-3′), 79.3 (C-Me_3), 122.2 (C-6′), 124.9 (C-2″, C-6″), 125.7 (C-3″, C-5″), 139.6 (C-5′), 145.0 (C-4″), 152.1 (OCOO), 156.5 (2×NCOO), 163.0 (C-1″); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3348, 2942, 1770, 1696, 1616, 1593 cm^{-1}; FABMS: *m/z*: 936 $[M+Na]^+$, 914 $[M+H]^+$, 814 $[M-Boc+H]^+$, 369 [Chol]$^+$; HRMS: calcd for $C_{50}H_{79}N_3O_{12}$: 914.5742 $[M+H]^+$, found: 914.5772.

Class A lipopeptide (**9**): The tenascin peptidoresin **1** (50 mg, 0.03 mmol) was swelled in DMF (7 mL, 30 min), the terminal Fmoc group was deprotected by using 25 % piperidine in DMF (2×5 min, 5 mL), and the product was washed with DMF (5×2 min, 5 mL). A Kaiser test was performed and produced a positive result. Dry DMF (1.5 mL) and NEt_3 (11 μL, 0.075 mmol) were transferred to the resin and shaken for 5 min. A premixed solution containing carbonate **8** (54 mg, 0.06 mmol) and NEt_3 (11 μL, 0.075 mmol) in dry DMF (1 mL) was also transferred into the reaction vessel under argon (including 2 mL of washings) and shaken for 18 h. The resin was washed with DMF (5×2 min, 10 mL), and a Kaiser test was performed. The colourless beads produced a negative result; this indicated that the lipid had been successfully coupled. The resin was washed further with MeOH (3×2 min, 5 mL) and with DCM (3×5 mL, 2 mins). The resin was air-dried, then cleaved for 1.5 h by using TFA/water (95:5, 2 mL). The crude lipopeptide was precipitated in ice cold *tert*-butyl methyl ether (MTBE; 10 mL), centrifuged (3600 rpm, 4 °C, 2×5 min) and freeze-dried to produce a white powder. The crude lipopeptide was purified by reversed-phase HPLC (Vydac C4 colum at a flow rate of 1 mL min^{-1}, 214 nm) with a gradient of 30–100 % acetonitrile in water over 40 min. Lipopeptide **9**: R_t = 32.6 min; yield: 5.8 mg, 9.7 % (99 % purity); ESI-MS *m/z*: calcd for $C_{96}H_{162}N_{16}O_{28}$: 1986 $[M+H]^+$, found: 1985.8 $[M+H]^+$, 1574.8 $[M-CholOCO]^+$, 1311.7 [tenascin+H]$^+$.

Control peptide (**10**)*, deprotected*: This peptide was synthesised by using exactly the same procedure as for the peptide tenascin (**1**). Fmoc-Glu-Wang resin (0.7 g, 0.38 mmol, 0.55 mmol g^{-1}) was used instead of Fmoc-Ala-Wang and the amino acid coupled in the desired scrambled sequence. 50 mg batches were deprotected and cleaved for characterisation. The crude peptide was purified by re-

versed-phase HPLC (Vydac C4 column at a flow rate of 1 mL min^{-1}, 214 nm) with a gradient of 30–100% acetonitrile in water over 40 min. G_3IGALPIEDALE: R_t = 6.24 min; yield: 10 mg, 28% (98% purity); ESI-MS *m/z*: calcd for $C_{57}H_{95}N_{14}O_{21}$: 1311 $[M+H]^+$, found 1311 $[M+H]^+$.

Class A lipopeptide ***11***: The synthesis was carried out the same as described for lipopeptide **9** except the following quantities were used; control peptidoresin **10** (60 mg, 0.03 mmol), carbonate **8** (61 mg, 0.06 mmol), 2×NEt_3 (12 μL, 0.08 mmol) in the same volumes of dry DMF. The crude lipopeptide was purified by reversed-phase HPLC (Vydac C4 colum at a flow rate of 1 mL min^{-1}, 214 nm) with a gradient of 30–100% acetonitrile in water over 40 min. Lipopeptide **11**: R_t = 32.6 min; yield 10.1 mg, 16.8% (99% purity); ESI-MS *m/z*: calcd for $C_{96}H_{162}N_{16}O_{28}$: 1986 $[M+H]^+$, found: 1986.2 $[M+H]^+$, 1575.1 $[M-CholOCO]^+$, 1311.7 [Control tenascin+H]$^+$.

O^{1}*-4′-Nitrophenyloxycarbonyl-3,6,9-trioxa-1-oxyundecane (****12****)*: A flask was charged with TEG (predried with $MgSO_4$, 1 g, 5.3 mmol), pyridine (0.5 mL, 6.5 mmol), dry DCM (100 mL), placed under nitrogen and cooled to 0 °C. *p*-nitrophenyl chloroformate (1.05 g, 5.3 mmol) was dissolved in a minimum quantity of dry DCM (1 mL), transferred into the reaction mixture and left stirring over night allowing the ice bath to warm to room temperature. Once TLC indicated that the reaction had finished (20 h), the solvent was removed by reduced pressure and purified by column chromatography (ethyl acetate/hexane/dioxane 67:22:11) to produce alcohol **12** (0.75 g, 45%) as a yellow oil. R_f = 0.15 (ethyl acetate/hexane/dioxane 67:22:11); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.76 (br, 1H; OH), 3.59 (m, 2H; H-10), 3.58–3.67 (m, 10H; H-2, H-4, H-5, H-7, H-8), 3.78 (m, 2H; H-11), 4.41 (t, *J* = 4.6 Hz, 2H; H-1), 7.36 (m, 2H; H-2′, H-6′), 8.25 (m, 2H; H-3′, H-5′); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 61.5 (C-11), 68.1 (C-2), 68.4 (C-4, C-8), 70.3 (C-5, C-7), 70.5 (C-1), 72.3 (C-10), 121.7 (C-2′, C-6′), 125.1 (C-3′, C-5′), 145.2 (C-4′), 152.3 (OCOO), 155.4 (C-1′); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3429, 3072, 2875, 1767 cm^{-1}; FABMS: *m/z*: 360 $[M+H]^+$; HRMS: calcd for $C_{15}H_{21}NO_9$ $[M+H]^+$: 360.1295, found: $[M+H]^+$ 360.1297.

N^{1}*-Cholesteryl-*N^{2}*-(11″-hydroxy-3″,6″,9″-trioxaundecanyloxycarbonyl)-1,2-diaminoethane (****13****)*: **12** (0.12 g, 3.3 mmol) was charged into the flask and dissolved in DCM (25 mL) under nitrogen, and NEt_3 (0.14 mL, 10 mmol) was added. This mixture was stirred vigorously for 5 min, amine **2** (0.19 g, 0.4 mmol) was added, and the mixture was stirred for a further 12 h. The solvent was removed under vacuo, and the solid on purification by chromatography (DCM/ethyl acetate/MeOH 80:16:4→90:10:0) gave alcohol **13** (0.21 g, 91%). R_f = 0.2 (DCM/ethyl acetate/MeOH 80:16:4); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.63 (s, 3H; H-18′), 0.81 (d, *J* = 6 Hz, 6H; H-26′, H-27′), 0.85 (d, *J* = 6 Hz, 3H; H-21′), 0.95 (s, 3H; H-19′), 1.01–1.70 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.75–2.02 (m, 5H; H-2′, H-7′, H-8′), 2.16–2.35 (m, 2H; H-24′), 3.22 (m, 4H; H-1, H-2), 3.35 (br m, 1H; OH), 3.55 (m, 2H; H-10″), 3.57–3.64 (m, 10H; H-2′, H-4″, H-5″, H-7″, H-8″), 3.67 (m, 2H; H-11″), 4.15 (m, 2H; H-6), 4.45 (m, 1H; H-3′), 5.31 (m, 1H; H-6′), 5.62 (br m, 1H; OCN*H*), 5.88 (br m, 1H; CholOCON*H*); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.7 (C-18′), 18.5 (C-21′), 19.1 (C-19′), 20.8 (C-11′), 22.4 (C-26′), 22.7 (C-27′), 23.7 (C-23′), 24.1 (C-15′), 27.8 (C-16′), 28.0 (C-2′, C-25′), 31.7 (C-7′, C-8′), 35.6 (C-20′), 36.0 (C-22′), 36.3 (C-10′), 36.8 (C-1′), 38.4 (C-24′), 39.3 (C-4′), 39.5 (C-12′), 40.8 (C-1), 40.9 (C-2), 42.1 (C-13′), 49.8 (C-9′), 55.9 (C-17′), 56.5 (C-14′), 61.3 (C-11″), 63.7 (C-1″), 69.5 (C-2″), 70.1 (C-4″), 70.2 (C-5″), 70.3 (C-7″), 70.4 (C-8″), 72.4 (C-10″), 74.1 (C-3′), 122.3 (C-6′), 139.6 (C-5′), 156.3 (NCOO), 156.8 (NCOO); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3443, 3054, 2949, 2867, 1713, 1452 cm^{-1}; FABMS: *m/z*: 715 $[M+Na]^+$, 693 $[M+H]^+$, 369 $[Chol]^+$; HRMS: calcd for $C_{39}H_{68}N_2O_8$ $[M+H]^+$: 693.5054, found: $[M+H]^+$ 693.5087.

N^{1}*-Cholesteryloxycarbonyl-*N^{2}*-(11″-hydroxy-3″,6″,9″-trioxa-*O^{11}*-(4‴-nitrophenyloxycarbonyl)-undecanyldi(oxycarbonyl))-1,2-diamidoethane (****14****)*: A solution of alcohol **13** (2.1 g, 3 mmol) in dry DCM (100 mL) was placed under nitrogen. DMAP (0.73 g, 6 mmol) and NEt_3 (0.88 mL, 6 mmol) were added, and the mixture was stirred for 10 min. *p*-Nitrophenyl chloroformate (1.8 g, 9 mmol) was then added, and the mixture was stirred for 20 h. The resultant residue was purified by column chromatography (hexane/ethyl acetate 66:34→34:66) to give carbonate **14** (1.7 g, 66%). R_f = 0.18 (ethyl acetate/hexane 66:34); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.63 (s, 3H; H-18′), 0.82 (d, *J* = 6 Hz, 6H; H-26′, H-27′), 0.87 (d, *J* = 6 Hz, 3H; H-21′), 0.98 (s, 3H; H-19′), 1.00–1.65 (m, 21H; H-1′, H-4′, H-9′, H-11′, H-12′, H-14′, H-15′, H-16′, H-17′, H-20′, H-22′, H-23′, H-25′), 1.75–2.02 (m, 5H; H-2′, H-7′, H-8′), 2.16–2.38 (m, 2H; H-24′), 3.27 (m, 4H; H-1, H-2), 3.60–3.72 (m, 10H; H-2″, H-4″, H-5″, H-7″, H-8″), 3.80 (m, 2H; H-10″), 4.19 (m, 2H; H-1″), 4.40–4.44 (m, 3H; H-11″, H-3′), 5.28 (br m, 1H; OCN*H*), 5.33 (m, 1H; H-6′), 5.47 (br m, 1H; ChOCN*H*), 7.38 (m, 2H; H-2‴, H-6‴), 8.27 (m, 2H; H-3‴, H-5‴); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 11.7 (C-18′), 18.5 (C-21′), 19.2 (C-19′), 20.9 (C-11′), 22.4 (C-26′), 22.7 (C-27′), 23.7 (C-23′), 24.1 (C-15′), 27.8 (C-16′), 28.0 (C-2′, C-25′), 31.7 (C-7′, C-8′), 35.6 (C-20′), 36.0 (C-22′), 36.4 (C-10′), 36.8 (C-1′), 38.4 (C-24′), 39.3 (C-4′), 39.5 (C-12′), 40.8 (C-1), 41.0 (C-2), 42.1 (C-13′), 49.8 (C-9′), 56.0 (C-17′), 56.5 (C-14′), 63.8 (C-1″), 68.1 (C-2″), 68.5 (C-10″), 69.4 (C-4″), 70.3 (C-5″, C-7″), 70.4 (C-8″), 70.5 (C-11″), 74.4 (C-3′), 121.7 (C-6′), 122.4 (C-2‴, C-6‴), 125.1 (C-3‴, C-5‴), 139.6 (C-5′), 145.2 (C-4‴), 152.3 (OCOO), 155.3 (C-1‴), 156.5 (NCOO), 156.7 (NCOO); IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3439, 3054, 2982, 2867, 1717, 1216 cm^{-1}; FABMS: *m/z*: 880 $[M+Na]^+$ 858 $[M+H]^+$, 369 $[Chol]^+$.

Class B lipopeptide ***15***: The tenascin peptidoresin **1** (50 mg, 0.03 mmol) was swelled in DMF (7 mL, 30 min), the terminal Fmoc group was deprotected by using 25% piperidine in DMF (2×5 min, 5 mL), and the product was washed with DMF (5×2 min, 5 mL). A Kaiser test was performed and produced a positive result. Dry DMF (1.5 mL) and NEt_3 (11 μL, 0.075 mmol) were transferred to the resin, which was shaken for 5 min. A premixed solution containing carbonate **14** (76 mg, 0.088 mmol) and NEt_3 (11 μL, 0.075 mmol) in dry DMF (1 mL) was also transferred into the reaction vessel under argon (including 2 mL of washings), and the vessel was shaken for 18 h. The resin was washed with DMF (5×2 min, 10 mL), and a Kaiser test was performed. The colourless beads produced a negative result; this indicated that the lipid had been successfully coupled. The resin was washed further with MeOH (3×2 min, 5 mL) and with DCM (3×5 mL, 2 min). The resin was air-dried then cleaved for 1.5 h by using TFA/water (95:5, 2 mL). The crude lipopeptide was precipitated in ice-cold MTBE (10 mL), centrifuged (3600 rpm, 4 °C, 2×5 min) and freeze-dried to produce a white powder. It was then purified by reversed-phase HPLC (Vydac C4 colum at a flow rate of 1 mL min^{-1}, 214 nm) with a gradient of 30–100% acetonitrile in water over 40 min. Lipopeptide **15**: R_t = 33.2 min; yield: 2.2 mg, 3.7% (96% purity); ESI-MS *m/z*: calcd for $C_{97}H_{162}N_{16}O_{30}$: 2030 $[M+H]^+$, found: 2030 $[M+H]^+$, 1617.8 $[M-CholOCO]^+$, 1311.7 [tenascin+H]$^+$.

Class B lipopeptide ***16***: The synthesis was performed as described for lipopeptide **15**, except that the following quantities were used: control peptidoresin **10** (60 mg, 0.03 mmol), carbonate **14** (85 mg, 0.099 mmol), 2×NEt_3 (12 μL, 0.08 mmol) in the same volumes of dry DMF. The crude lipopeptide was purified by reversed-phase HPLC (Vydac C4 colum at a flow rate of 1 mL min^{-1}, 214 nm) with a gradient of 30–100% acetonitrile in water over 40 min. Lipopeptide

16: $R_t = 33.2$ min; yield: 2.5 mg, 3.7% (95% purity); ESI-MS *m/z*: calcd for $C_{97}H_{162}N_{16}O_{30}$: 2030 $[M+H]^+$, found: 2029.8 $[M+H]^+$, 1618.3 $[M-\text{CholOCO}]^+$, 1311.7 [tenascin+H]$^+$;.

Formulation materials: Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Plasmid DNA nis-pCMV β-galactosidase was produced by Bayou Biolabs (Harahan, LA, USA). DC-Chol, CDAN and Mu peptide were synthesised in our laboratory.[5] Hepes was purchased from Gibco (Invitrogen BV, Netherlands). Glucose was obtained from Sigma Aldrich (Poole, UK). A Coulter Delta N4 plus 440SX photon correlation spectrometer (PCS) was used to determine the particle size of the polyplex formulations.

Preparation of liposomes for transfection: The liposomes contained varying percentages of targeting ligand. The range was a 100-fold increase from 0.05 to 5 mol% targeting compound. The lipopeptides A and B and their targeted controls were dissolved in 5% methanol in anhydrous DCM at a concentration of 1 mg mL^{-1} (0.5 mM solution). DOPE, DC-Chol and CDAN were all dissolved in anhydrous DCM (10 mg mL^{-1}). The liposomes were made up to a total volume of 0.5 mL.

Four different sets of liposomes were made for the transfection studies based on the first and second generation of LMD. The targeted liposomes incorporated lipopeptides **9** and **15** with their controls:

a) CDAN (50−*x* mol%), DOPE (50 mol%) and **9** (*x* mol%)
b) CDAN (50−*x* mol%), DOPE (50 mol%) and **15** (*x* mol%)
c) DC-Chol (60−*x* mol%), DOPE (40 mol%) and **9** (*x* mol%)
d) DC-Chol (60−*x* mol%), DOPE (40 mol%) and **15** (*x* mol%)

A nitric acid-treated 25 mL round-bottom flask was charged with distilled DCM (500 μL) and either a, b, c or d outlined above. The flask was swirled gently to ensure thorough mixing of the lipids, then placed onto the rotary evaporator and the solvent removed under reduced pressure. The solvent was removed slowly as an even lipid film was desired to form the liposomes. Once the film had formed (15 min) the vacuum was increased to full to remove any traces of methanol or DCM (15 min). The flask was then removed and sterile Hepes buffer (4 mM, pH 7.2, 500 μL) was added to the flask. The flask was then placed in the sonicator for 3–4 min, in order for the liposomes to form. All liposomal solutions were prepared at a concentration of 5 mg mL^{-1}. The pH of the liposomal suspension was checked by pH Boy (Camlab Ltd., Cambridgeshire, UK) and adjusted to pH 7.0 ± 0.1 with concentrated aqueous solutions of HCl and NaOH. The liposomes were extruded 10× (Extruder, Northern Lipids, Inc., Vancouver, BC, Canada), passing through two 0.1 μm polycarbonate filters (Isopore Membrane Filters, Millipore (UK) Ltd., Hertfordshire, UK), and the pH was maintained at 7. In order to check the size distribution of the liposomes, 10 μL of sample were diluted with Hepes buffer (190 μL) and measured by PCS. Liposomes were stored under argon at 4 °C.

Preparation of LD (Liposome:DNA): These lipoplexes used a ratio of 12:1 (*w/w*), liposomes to DNA. The pDNA containing the β-galactosidase gene (pNGVL1-nt-beta-gal; 7.53 kbp) was stored as frozen aliquots at −80 °C, at a concentration of 1.2 mg mL^{-1}. The liposomes were diluted with Hepes buffer (4 mM, pH 7), vortexed, and then pDNA added to the solution with continuous vortexing to ensure homogeneous complexation. Sucrose (65%, *w/v*) was then added to the LDs in order for them to be stored at −20 °C. The size distribution was measured by PCS, similar as for liposomes alone. Each formulation resulted in evenly distributed complexes of approximately 250 ± 50 nm. For DC-Chol/DOPE liposomes a ratio of 12:1 was used. Also, due to the difficult LD formulations, the maximum incorporation of targeting lipopeptide was 2%.

Preparation of LMD (Liposome:Mu:DNA):[5] LMD complexes were formulated in a liposome/mu peptide/pDNA 12:0.6:1 ratio. The volume of Hepes buffer (4 mM, pH 7) required for the formulation (68 μL) was split equally between the liposomes and mu peptide. To the diluted peptide, the DNA was added whilst vortexing, this was then transferred to the diluted liposomes in small aliquots, again with continuous vortexing to ensure homogeneous complexation. As described for the LD preparation, sucrose (65%, *w/v*) was added and vortexed. The size distribution was again measured by PCS. Each formulation produced evenly distributed complexes of approximately 120 ± 40 nm. Due to the difficult nature of DC-Chol/DOPE-based liposomes, the formulations were carried out at 12:0.6:1 LMD and stopped at a maximum 2% incorporation of targeted compound.

General biological testing: Luminescent and colorimetric assays were performed on a Lucy 1 luminometer (Labtech International, UK). Commercial kits included a β-Gal Standard Chemiluminescent Reporter Gene Assay (Roche Diagnostics GmbH, Mannheim, Germany). Foetal calf serum (FCS) was purchased from ICN Biochemicals; bovine serum albumine (BSA), Fibronectin, Crystal Violet and G418 were acquired from Sigma; PBS, OptiMEM, DMEM, trypsin-EDTA, SDS, penicillin and streptomycin were bought from Gibco-BRL (Invitrogen BV, Netherlands). Chicken tenascin C was ordered from Chemicon, UK. Tissue culture and nontissue culture-treated plastic ware was purchased from Falcon (Becton Dickinson, UK).

Growth and maintenance of cells: Cells were maintained in DMEM containing Glutamax supplemented with G418 (1 mg mL^{-1}), 10% FCS, penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}). Cells were routinely grown in T-75 or T-150 tissue culture flasks at 37 °C in a 5% CO_2 atmosphere. Once the cells had formed a subconfluent monolayer the growth medium was removed by aspiration, the cells washed with phosphate-buffered saline (PBS; 2×5–10 mL) and detached by treatment with trypsin–EDTA (2–5 mL, 0.25 M) at 37 °C for 5 min. If necessary, the flask was tapped gently to dislodge the cells. The trypsin was neutralised with an equal volume of media, and the cells appropriately diluted (1:3 and 1:5) with fresh media.

Competitive cell binding inhibition assay:[20,21] A solution (50 μL) of chicken tenascin C protein (20 μg mL^{-1}) and fibronectin (20 μg mL^{-1}) in PBS was placed in each of the appropriate wells of the nontissue culture-treated 96-well plate. The plate was left for 16 h at room temperature to allow the protein to bind to the bottom of the wells. The wells were then washed with PBS (3× 100 μL) and a solution of BSA in PBS (3%, *w/v*) was added to the wells (100 μL) and placed at 37 °C for 2 h. The wells were washed again with PBS (2×100 μL). DMEM containing 5×10^4 cells (100 μL) were combined together with either the peptide (20 and 40 μL) or lipopeptide (2 and 5 μL) and incubated together for 30 min at 37 °C. The concentrations of peptide and lipopeptide were 1 and 0.5 mM, respectively. The peptides were dissolved in PBS to give a concentration of 1 mM and lipopeptides in 2.5% DMSO in PBS to give a concentration of 0.5 mM. The cells and peptide/lipopeptide were then transferred into the treated wells of the 96-well plate and incubated at 37 °C for 1–1.5 h (until the control cells had flattened in appearance). The cells were then washed (PBS, 3× 100 μL), fixed (methanol, 50 μL) and stained (crystal violet, 50 μL). After air-drying, the cells were solubilised (10% SDS, 100 μL) and quantified by measuring the absorbance at 540 nm, A_{540}.

Transfection studies: The experiments were carried out in triplicate in 48-well tissue culture-treated plates seeded with 5×10^4 cells per well, these were grown until 50% confluent (12 h) in DMEM containing 10% FCS, G418 (1 mg mL^{-1}) and penicillin/streptomycin (5 mL in 500 mL media), and then replaced with OptiMEM (250 μL). 2 μL of construct (either LD or LMD) was then added to each well and swirled to ensure even dispersion, then incubated at 37 °C for either 15 min (LMD) or 4 h (LD). The solution was removed, and the cells were washed with medium (250 μL), the medium was replaced (250 μL), and the cells were incubated for a further 24 h. Before the luciferase activity was measured, the cells were washed (PBS, 500 μL) and harvested with lysis buffer (150 μL). To ensure the cells were all ruptured, the plates were subjected to −80 °C for 15 min and then defrosted. The luciferase activity was measured by using the assay kit from Roche Diagnostics and a luminometer.

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