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A novel method for conjugating the terminal amine of peptide ligands to cholesterol: synthesis iRGD-cholesterol

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Aim: Conventional conjugation reactions often involve the use of activated PEG as a linker, but concerns about PEG-mediated reduction in intracellular delivery and enhanced immunogenicity have generated interest in developing methods that eliminate the need for a PEG linker. **Materials & methods:** Reaction conditions were identified that specifically couples the terminal amine of a cyclic iRGD peptide (CRGDRG-PDC) to the hydroxyl moiety of cholesterol through a short carbamate linker. **Results & conclusion:** Using this method for synthesizing iRGD-cholesterol, peptide ligands can be incorporated into lipid-based delivery systems, thereby eliminating concerns about adverse reactions to PEG. Toxicity and stability data indicate low toxicity and adequate serum stability at low ligand levels.

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It is widely recognized that drug delivery to tumors *in vivo* represents a significant barrier to successful cancer treatment [1–3], and a recent analysis has shown that delivery systems generally deliver less than 1% of the injected dose to the tumor [4]. Although delivery to tumors in animal models can be increased due to the enhanced permeation and retention effect [5–8], the predominant clinical advantage offered by contemporary nanoparticles are reduced toxicity and simultaneous delivery of drugs at precise ratios [9–13]. Studies have shown that targeting ligands can be employed to further enhance delivery to tumors by promoting the retention of particles that distribute to the tumor [14–16]. However, previous studies have shown that delivery vehicles primarily interact with the vascular endothelium and thus only a small fraction of injected particles are likely to directly access receptors on cancer cells [17–19]. It follows that ligands that interact with the tumor-associated endothelium and facilitate access to cancer cells offer a distinct advantage over traditional ligands that target proteins overexpressed on tumor cells. Recently, it has been shown that a family of related peptide ligands, collectively referred to as iRGD, bind specifically to the $\alpha_v\beta_3/\beta_5$ integrin on tumor vasculature and promote transcytosis across tumor vasculature [20–23]. After translocation across the tumor vasculature, the iRGD ligand is cleaved by an endogenous protease to yield a peptide that serves as a ligand for the neuropilin-1 receptor that is present on a wide variety of cancer cells [20–23]. In this way, the iRGD ligand both enhances uptake of circulating nanoparticles from the blood, and promotes internalization by the tumor cells.

While many studies have demonstrated the ability of targeting ligands to promote delivery to specific tissues, it is generally not appreciated that the adsorption of proteins ('corona') can mask/cover ligands on the surface of nanoparticles, thereby compromising their targeting ability [24–26]. Although the use of large proteins (e.g., antibodies) as ligands and/or conjugation to a PEG linker can reduce the potential for masking by adsorbed proteins, these strategies raise concerns regarding the potential for commercial development and immunogenicity [27–31]. Accordingly, the use of small molecules and peptides as ligands that may circumvent these issues is gaining in popularity despite their potential for being masked/covered by adsorbed proteins [26,32–35].

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Our previous work has incorporated cholesterol nanodomains into lipoplexes to enhance serum stability and delivery to tumors *in vitro* and *in vivo* [26,36–39]. Of particular relevance to the current study, we have demonstrated that these membrane domains adsorb undetectable levels of protein, making them ideal locations for targeting ligands [37]. Because these domains are predominantly composed of cholesterol, ligands conjugated to cholesterol will preferentially partition into the domain, whereas ligands conjugated to lipid anchors are excluded from this region. Consistent with the ability of cholesterol nanodomains to provide a region wherein ligands can avoid being masked/fouled by adsorbed proteins, we have demonstrated that small-molecule ligands that are localized within a domain enhance delivery, in contrast to nanoparticles decorated with the identical ligand that is excluded from the domain [26,37,40]. However, one of the drawbacks of many previous studies was that PEG was employed as a linker in the conjugation reaction, and our experiments demonstrate that even these very low levels of PEGylation (0.4%) dramatically reduce intracellular delivery [26,27,40]. Accordingly, the goal of the current study was to identify methods that would allow direct conjugation of cholesterol to peptide ligands through their terminal amine moiety. Because RGD-based peptides contain other nitrogen-containing functional groups that can participate in conjugation reactions, we attempted to develop methodology that would allow conjugation to cholesterol specifically through the terminal amine of the peptide.

Materials & methods

All chemical reagents and solvents were purchased from commercial sources and used without further purification with the exception of iRGD, which was purchased from Peptides International (KY, USA). NMR spectroscopy was performed on Bruker Avance-III (300 MHz) and Varian Inova (500 MHz) NMR spectrometers, operating at 300.13 and 499.60 MHz (respectively) for ^1H NMR observation. All 2D and diffusion ordered spectroscopy (DOSY) NMR experiments were performed using the Varian Inova 500 (500 MHz) instrument. DOSY experiments were performed using bipolar gradients with convection compensation and using correction for nonuniform-gradient amplitudes over the volume of the sample. 2D heteronuclear experiments (gradient heteronuclear multiple bond correlation and gradient heteronuclear single quantum coherence) were acquired using pulsed-field-gradient coherence selection, and with adiabatic pulses to achieve uniform coherence transfer between ^1H and ^{13}C .

Acryloyl-cholesterol

Toluene (20 ml) was added to a 3-neck round-bottom flask containing dry cholesterol (0.98 g, 2.54 mmol). Triethylamine (50 μl) was added and the contents were stirred in a closed nitrogen atmosphere. Acryloyl chloride (229 μl , 2.79 mmol) was added in a dropwise manner. The reaction was stirred at 25°C for 24 h. The solvents were removed in vacuo, and the product was extracted into hexanes. The product was further purified using column chromatography (hexanes/ethyl acetate [EtOAc] 95/5, 57% yield). ^1H NMR (Supplementary Figure 1, 300 MHz, chloroform-*d*) δ 6.39 (dd, J = 1.6, 17.3 Hz, 1H), 6.10 (dd, J = 10.4, 17.3 Hz, 1H), 5.80 (dd, J = 1.6, 10.4 Hz, 1H), 5.43–5.35 (m, 1H), 4.69 (ddt, J = 4.3, 8.1, 16.5 Hz, 1H), 2.36 (d, J = 7.9 Hz, 2H), 2.12–1.74 (m, 5H), 1.72–0.94 (m, 24H), 1.03 (s, 3H), 0.91 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H), 0.68 (s, 3H).

Cholesterol-*p*-nitrophenylcarbonate

THF containing 10% pyridine was added to a 3-neck round-bottom flask-containing cholesterol (1.03 g, 2.67 mmol). 4-Dimethylaminopyridine (0.098 g, 0.80 mmol) was added and the solution was stirred while 4-nitrophenyl chloroformate (0.840 g, 4.17 mmol) was added to the solution. The reaction was stirred overnight. The solvents were removed in vacuo, and the product was purified using column chromatography (hexanes/EtOAc 95/5, 84% yield). ^1H NMR (Supplementary Figure 2, 300 MHz, chloroform-*d*) δ 8.28 (d, J = 9.2 Hz, 2H), 7.39 (d, J = 9.2 Hz, 2H), 5.43 (d, J = 5.1 Hz, 1H), 4.70–4.52 (m, 1H), 2.49 (d, J = 7.8 Hz, 2H), 2.19–1.65 (m, 6H), 1.65–0.94 (m, 20H), 1.05 (s, 3H), 0.92 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 6.6 Hz, 3H), 0.69 (s, 3H).

Cholesterol arginylcarbamate

DMSO (3 ml) was added to a flask containing dry cholesterol *p*-nitrophenylcarbonate (0.100 g, 0.181 mmol) and arginine (0.035 g, 0.199 mmol). DIEA (600 μl) was added and the reaction mixture was heated to 50°C for 12 h. Disappearance of the cholesterol containing starting material was monitored by thin layer chromatography. Upon reaction completion, the solvents were removed in vacuo and the remaining solid was washed with diethyl ether

(3 × 10 ml) to remove the by-product 4-nitrophenol. The remaining solid was extracted with dichloromethane to provide 0.081 g, (76%). ¹H NMR (Supplementary Figure 3; 500 MHz, DMSO-*d*₆) δ 7.51 (t, *J* = 5.7 Hz, 1H), 7.36 (d, *J* = 7.9 Hz, 1H), 7.5–6.6 (m, 3H), 5.33 (d, *J* = 5.0 Hz, 1H), 4.31 (tt, *J* = 4.8, 11.2 Hz, 1H), 3.90 (td, *J* = 4.8, 8.4 Hz, 1H), 3.08 (q, *J* = 6.7 Hz, 2H), 2.33–2.16 (m, 2H), 2.01–1.63 (m, 6H), 1.61–0.93 (m, 25H), 0.97 (s, 3H), 0.89 (d, *J* = 6.4 Hz, 3H), 0.85 (d, *J* = 6.6 Hz, 3H), 0.85 (d, *J* = 6.6 Hz, 3H), 0.65 (s, 3H). Correlation spectroscopy (COSY, Supplementary Figure 4) and gradient heteronuclear single quantum coherence (Supplementary Figures 5 & 6) are included in the Supplementary Information. Supplementary Figure 7 displays the expanded ¹H NMR spectrum. Time-of-flight mass spectrometry (Supplementary Figures 8–11; *m/z*): C₃₄H₅₈N₄O₄, 588 [M + H]⁺.

iRGD-cholesterol carbamate

DMSO (200 μl) and DIEA (15 μl) were added to a flask containing iRGD (5 mg, 0.0051 mmoles). Dry nitrophenyl-cholesterol (2.9 mg) was added as a solid and the stirred reaction mixture was heated to 50°C for 12 h. Disappearance of the cholesterol containing starting material was monitored by the thin layer chromatography. Upon reaction completion, the solvents were removed in vacuo and the remaining solid was purified by trituration with CHCl₃ to provide 3.3 mg (46%) of product. This material contained approximately 25% unconjugated peptide as determined by ¹H NMR integration. Product COSY (Supplementary Figure 12). ¹H NMR (Supplementary Figure 13, 500 MHz, DMSO-*d*₆) δ 12.38 (s), 8.78 (s), 8.55–6.33 (m), 5.34 (s), 4.75–4.10 (m), 3.82 (d, *J* = 17.0 Hz), 3.69–3.44 (m), 3.23–2.55 (m), 2.3–2.04 (m), 2.00–1.64 (m), 1.63–0.94 (m), 0.90 (d, *J* = 6.4 Hz), 0.84 (dd, *J* = 2.4, 6.6 Hz), 0.65 (s). Product DOSY (Supplementary Figure 14) and pure i-RGD peptide ¹H NMR (Supplementary Figure 15) and COSY (Supplementary Figure 16) are included in supplemental information. Time-of-flight mass spectrometry (Supplementary Figures 17–21) (*m/z*): C₆₃H₁₀₁N₁₅O₁₆S₂, 1388 [M + H]⁺.

Lipoplex preparation & toxicity evaluation

Sphingosine, cholesterol and 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (AL, USA) and used to prepare liposomes at a 3:2:5 mole ratio (respectively) as previously described [41]. Varying amounts of the iRGD-cholesterol conjugate preparation were incorporated into the lipid mixture to achieve different mole ratios of ligand to sphingosine prior to liposome formation. The resulting liposome suspension was mixed with a modified (CMV removed, ROSA26 added, based upon Watcharanurak *et al.* [42]) pSelect-LucSh (InvivoGen, CA, USA) plasmid-encoding luciferase at a ± charge ratio of 0.5 [41,43,44]. Both the liposome suspension and DNA solution were prepared in distilled water without buffer components. For cell culture experiments, murine mammary carcinoma cells (4T1) were cultured in Minimal Essential Medium containing 10% fetal bovine serum (FBS) as previously described [43]. To simulate serum protein conditions in blood, lipoplexes were incubated with an equal volume of 100% FBS for 30 min prior to exposure to cells in culture. Our previous studies have shown that the effects of serum on particle instability/aggregation are complete within this timeframe [38]. After 4 h, cells were washed, and incubated in fresh, serum-containing media. Cell viability was assessed after 40 h with the Invitrogen MTT assay as per the manufacturer's instructions as detailed in our earlier publication [41].

Particle size determination

Lipoplexes prepared with different concentrations of iRGD-cholesterol were subjected to dynamic light scattering, and sizes were determined on a Malvern ZetaSizer Nano Series Nano-ZS (Malvern Instruments, Worcestershire, UK). Lipoplex samples of 2 μg DNA were preincubated 1:1 v/v in water or FBS for 30 min prior to making the size measurements. Incubated samples were then diluted 1:100 prior to measurement to reduce light scattering. Malvern Nano Software Version 6.12 was used to take the measurements and analyze the data. Three individual measurements of 300 μl samples were made, and each measurement is the average of 12 replicates over 120 s. Settings for the measurement files were as follows: material: lipid, RI: 1.497, dispersant: water, temperature: 25°C.

Statistical evaluation

The effect of ligand concentration on viability and particle size were quantified as described above, and values were compared via an unpaired, two-tailed t-test.

Results & discussion

Our previous work has established that cholesterol domains do not adsorb detectable levels of serum proteins, making them ideal locations for displaying targeting ligands [37]. Furthermore, we have demonstrated that ligands conjugated

Figure 1. Attempted reaction of arginine with acrylated cholesterol.

to cholesterol preferentially partition into the cholesterol domain and thereby possess greater abilities to target and transfect cells *in vitro* and *in vivo*, as compared with lipoplexes possessing the identical ligand that is excluded from the domain [26,40]. While many studies utilize an activated PEG linker to conjugate ligands to nanoparticle components (e.g., lipids), recent concerns about the ability of PEG to inhibit intracellular delivery [27,45], elicit an immune response [28,30,31,46–48], and promote tumor growth [49,50] have motivated us to develop conjugation methods that avoid the use of PEG linkers. As described above, direct conjugation to cholesterol allows ligands to partition into the cholesterol domain on the nanoparticle, which has proven advantageous for the intracellular delivery of genes [26,37,40]. Accordingly, we intended to develop chemical methods of reliably conjugating peptides to cholesterol for use in targeting delivery vehicles.

As a cost-effective way to investigate the chemistry necessary to conjugate iRGD to cholesterol, initial reactions were carried out using arginine as a peptide analog. Arginine contains similar functional groups as are present in the iRGD peptide and successful conjugation of cholesterol through the amine present in arginine could be translated to the synthesis of iRGD analogs.

The first attempt at the conjugation of cholesterol with arginine utilized an approach similar to the reported conjugation of paclitaxel and survivin shRNA to iRGD [51]. Functionalizing the secondary alcohol on cholesterol by reaction with acryloyl chloride yielded the acrylate. However, further reaction of the functionalized cholesterol with arginine failed to provide the desired product (Figure 1). ^1H NMR of the worked up product mixture revealed a large amount of acrylic acid and none of the desired product. Instead of attempting to optimize conditions for this reaction pathway, we chose to pursue activation of cholesterol with 4-nitrophenyl chloroformate.

Activation of cholesterol with 4-nitrophenyl chloroformate followed by reaction of the isolated product with arginine (Figure 2) produced the desired product. This chemistry is well known and has been successfully employed in coupling alcohols with peptide amines [52,53]. The desired reaction product was confirmed using MS and NMR. Assignments and connectivity were determined using ^1H , HSQC and ^1H COSY NMR. This experiment served as proof of synthetic concept and confirmed that under appropriate conditions, cholesterol can be conjugated to arginine through the amine functional group. The successful conjugation of arginine to cholesterol prompted us to apply like chemistry to the cyclic peptide, iRGD (CRGDRGPDC). Cholesterol was activated with 4-nitrophenyl chloroformate and reacted with iRGD to provide the desired product (Figure 3). The final preparation contained 25% unreacted iRGD peptide. The product was confirmed using NMR, 2D NMR and MS. Diffusion-ordered NMR spectroscopy was supportive in confirming cholesterol to peptide connectivity. The NMR data and explanation of assignments are included in the Supplemental Information.

Figure 2. The conjugation of cholesterol to arginine.

Table 1. Effect of incorporation of iRGD-cholesterol on lipoplex size (nm) and polydispersity index in saline and serum. Asterisks indicate statistically larger particles sizes as compared with lipoplexes lacking ligand under each condition ($p < 0.05$).

iRGD concentration (%)	PBS		FBS	
	Diameter (nm)	PDI	Diameter (nm)	PDI
0	280.93 $\pm$ 10.80	0.26 $\pm$ 0.01	328.83 $\pm$ 61.48	0.79 $\pm$ 0.02
0.05	222.8 $\pm$ 17.52	0.33 $\pm$ 0.01	271.53 $\pm$ 12.44	0.74 $\pm$ 0.01
0.1	231.55 $\pm$ 26.16	0.30 $\pm$ 0.01	331.73 $\pm$ 18.75	0.64 $\pm$ 0.02
0.24	231.27 $\pm$ 24.70	0.42 $\pm$ 0.01	446.53 $\pm$ 12.89*	0.63 $\pm$ 0.01
0.5	275.93 $\pm$ 22.46	0.40 $\pm$ 0.01	522.53 $\pm$ 27.25*	0.71 $\pm$ 0.01
1	307.28 $\pm$ 12.44	0.31 $\pm$ 0.01	606.37 $\pm$ 21.12*	0.63 $\pm$ 0.06
2	231.57 $\pm$ 18.23	0.35 $\pm$ 0.02	897.87 $\pm$ 56.60*	0.64 $\pm$ 0.01
3	322.5 $\pm$ 10.97*	0.32 $\pm$ 0.02	704.50 $\pm$ 67.72*	0.72 $\pm$ 0.05

FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; PDI: Polydispersity index.

It is well recognized that the size of delivery vehicles can play a major role in biodistribution and cell uptake [54–57]. In addition, many studies have documented the ability of serum proteins to adsorb to intravenously injected nanoparticles and promote aggregation and/or dissociation [38,58–61]. Accordingly, it is critical that delivery vehicles are capable of resisting serum-induced aggregation in order to maximize delivery upon intravenous administration. Consistent with early studies on liposomes, our previous work with lipoplexes has demonstrated the ability of cholesterol to stabilize particles in the presence of serum [37,38,41,62,63]. As shown in Table 1, incubation in full-strength (50%) serum for 30 min does not significantly alter particle size in our lipoplex formulation, although the polydispersity index is increased. However, the incorporation of iRGD-chol into the lipoplex at concentrations above 0.1% dramatically increases particle size in the presence of serum. This should not be surprising considering the cationic nature of the iRGD peptide and the known effects of charge on serum stability [38,43,62], but the destabilizing effects of low amounts of iRGD may compromise its ability to serve as an effective targeting ligand after intravenous administration.

In addition to serum stability, it is well known that the toxicity of the vehicle is an important consideration for clinical development. This aspect is especially important for gene delivery systems due to the cationic components that are required for complexation. Our previous work has demonstrated that substitution of sphingosine for conventional cationic lipids greatly reduces the liver toxicity and allows the recipient cell to maintain its viability

Figure 3. Synthesis of an iRGD/cholesterol conjugate.

and expression of the exogenous gene [41,44]. To test the effects of iRGD on toxicity, lipoplexes were prepared with different mole percentages (relative to sphingosine) of the iRGD–cholesterol conjugate preparation, and were used to assess the effect of iRGD content on the viability of 4T1 cells in culture. As shown in Figure 4, cell culture experiments indicate that the incorporation of the iRGD-cholesterol conjugate at levels up to 1% (mol/mol) did not reduce the viability of 4T1 cells as compared with vectors lacking ligand. However, the incorporation of 2% iRGD-cholesterol did result in a significant decrease in cell viability as determined by the MTT assay ($p < 0.0001$; Figure 4).

Conclusion

This manuscript describes methods that allow for the specific conjugation of the terminal amine of peptide ligands to the hydroxyl moiety of cholesterol. More specifically, we demonstrated that iRGD could be directly conjugated to cholesterol with a yield of approximately 50%. As mentioned earlier, the term ‘iRGD’ refers to a family of related RGD peptides that contain an internal CendR motif (R/KXXR/K) [21–23], and our studies only investigated the conjugation of a single cyclic sequence (CRGDRGPDC) to cholesterol. Therefore, conjugation of other iRGD peptides that possess slightly different sequences might require modifications to the methods described here. More

Figure 4. *In vitro* toxicity of lipoplexes incorporating different levels of iRGD. Each bar represents the mean $\pm$ one standard error of cells in eight individual wells. The asterisk indicates a statistically significant reduction in cell viability ($p < 0.0001$)

specifically, the most commonly used iRGD sequence contains a lysine residue instead of an arginine at position 5, and this sequence was avoided due to the potential for competing conjugation reactions with the primary amine in the lysine side chain. Our experiments with tumor cells in culture indicate that incorporation of the iRGD conjugate into lipoplexes at amounts up to 1% did not cause diminished viability. However, our serum stability experiments indicate that incorporation of iRGD into lipoplexes at levels exceeding 0.1% caused significant increases in particle size, which may limit the use of higher concentrations of this conjugate for intravenous administration.

Future perspective

The use of peptides as targeting ligands is receiving increased attention in the delivery literature. Accordingly, conjugation of peptides to hydrophobic molecules that anchor the ligand to self-assembling nanoparticles is commonly performed by utilizing activated PEG linkers. However, the evidence surrounding the immune response to PEG is overwhelming, and so alternative conjugation chemistries need to be developed. In this manuscript, we describe a novel conjugation strategy that specifically couples the terminal amine of a peptide ligand to the alcohol moiety of cholesterol. We believe that the methods described here will be useful to many in the delivery community.

Summary points

Background

- Incorporation of ligands into delivery vehicles typically involves conjugation reactions that require the use of PEG. Many studies have demonstrated an immune response to PEG that recently resulted in the termination of a clinical trial. Therefore, it would be advantageous to develop methodology for conjugating ligands that does not rely on PEG linkers.

Materials & methods

- A method was identified that allows conjugation of peptide ligands to cholesterol via the terminal amine of the peptide.

Results & discussion

- The incorporation of the iRGD-cholesterol conjugate up to 1% did not reduce the viability of murine cancer cells *in vitro*, but 2% ligand caused a significant decrease in viability.
- Incorporation of the iRGD-cholesterol conjugate at levels above 0.1% resulted in significant size increases upon incubation in serum.

Financial & competing interests disclosure

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/tde-2018-0057. 1D and 2D NMR spectra and mass spectra for the compounds described in the Experimental section; narrative explaining assignments of structure are available in the Supporting Information.

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