Protein Delivery

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Combinatorially Designed Lipid-like Nanoparticles for Intracellular Delivery of Cytotoxic Protein for Cancer Therapy**

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Abstract: *An efficient and safe method to deliver active proteins into the cytosol of targeted cells is highly desirable to advance protein-based therapeutics. A novel protein delivery platform has been created by combinatorial design of cationic lipid-like materials (termed "lipidoids"), coupled with a reversible chemical protein engineering approach. Using ribonuclease A (RNase A) and saporin as two representative cytotoxic proteins, the combinatorial lipidoids efficiently deliver proteins into cancer cells and inhibit cell proliferation. A study of the structure–function relationship reveals that the electrostatic and hydrophobic interactions between the lipidoids and the protein play a vital role in the formation of protein–lipidoid nanocomplexes and intracellular delivery. A representative lipidoid (EC16-1) protein nanoparticle formulation inhibits cell proliferation in vitro and suppresses tumor growth in a murine breast cancer model.*

Protein therapy has been considered as the safest and most direct approach to manipulate cell function and treat human disease since the early 1980s, when insulin began to be used as the first human recombinant protein therapeutic.[1] A majority of protein pharmaceuticals (for example, cytokines, growth factors, and monoclonal antibodies) elicit their biological activity by targeting cell surface ligands or extracellular domains.[2] Nevertheless, advancements in molecular biology have suggested that proteins that target intracellular biological activity could be potent therapeutics.[3] The delivery of proteins safely and efficiently through the cell membrane to reach their intracellular targets remains a challenge for the success of protein therapy.[3b] As such, the development of methods for intracellular protein delivery is needed. Over the past few decades, the most thoroughly studied protein delivery approach has been fusing target protein cargos with protein transduction domains (PTD) or membrane transport signals. The delivery efficiency of PTD-protein fusions vary with protein type[4] and lack the capability to target a specific tissue or organ. More recently, nanoparticle drug delivery systems have offered alternative approaches for spatially and temporally controlled protein delivery. A number of synthetic nanomaterials, including liposomes,[5] polymers,[6] and inorganic nanoparticles,[7] have been designed for this purpose. These nanoparticules, however, are still of limited utility for protein therapy owing to the low delivery efficiency and/or complicated nanoparticle fabrication processes. Thus, a facile and convenient approach to develop novel nanomaterials for efficient intracellular protein delivery has yet to be developed.

We report herein a novel and efficient protein delivery platform that uses combinatorially designed cationic lipid-based nanoparticles combined with a reversible protein modification approach. Pioneered by Anderson, Langer, et al.,[8] the combinatorial library strategy has recently been used to generate cationic lipid-like materials (termed "lipidoids") for siRNA delivery. We have further extended this class of materials for use in DNA and mRNA delivery.[9] We hypothesize that lipidoids can be used as a novel protein delivery platform, as the charge–charge and hydrophobic interactions between lipidoids and proteins can load proteins into lipidoid nanoparticles. In turn, the hydrophobic nature of lipidoid nanoparticles allows easy protein transport through the cell membrane. In an attempt to strengthen the charge–charge binding of proteins and lipidoids, we modified the lysine residues of proteins with *cis*-aconitic anhydride in this investigation. The conjugation reaction between the amine groups of lysine and *cis*-aconitic anhydride converts the positively charged lysines into negatively charged carboxylate groups, thus increasing the negative charge density of protein and its binding with cationic lipidoids. Moreover, the *cis*-aconitic anhydride modification is reversible in the slightly acidic intracellular environment (for example, the pH of endosome and lysosome is in the range of 5–6),[10] leading to the restoration of the biological activity of the modified proteins.

As a proof-of-concept for developing cationic lipid-based nanoparticles for protein delivery, we designed and synthesized a library of lipidoids through the ring-opening reaction of 1, 2-epoxyhexadecane and aliphatic amines with diversified chemical structures (Figure 1). Using RNase A and saporin, two representative cytotoxic proteins, along with the *cis*-aconitic anhydride modified versions (RNase A-Aco and saporin-Aco), we demonstrate that the lipidoid nanoparticles can deliver protein into cancer cells and inhibit cell proliferation, for potential applications such as cancer therapy. RNase A can cleave intracellular RNA and induce cytotoxic effects when taken up by cells,[11] while saporin irreversibly inhibits protein synthesis in eukaryotic cells by rending the 28S subunit of ribosomes.[12] RNase A and saporin have both

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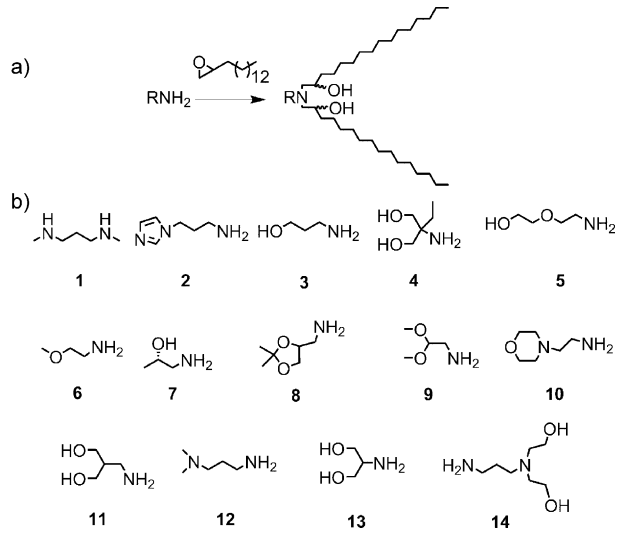

Figure 1. a) Route of synthesis for lipidoids. b) The chemical structures of the library of amines used for lipidoid synthesis (The lipidoids are named EC16, for 1, 2-epoxyhexadecane, followed by the amine number).

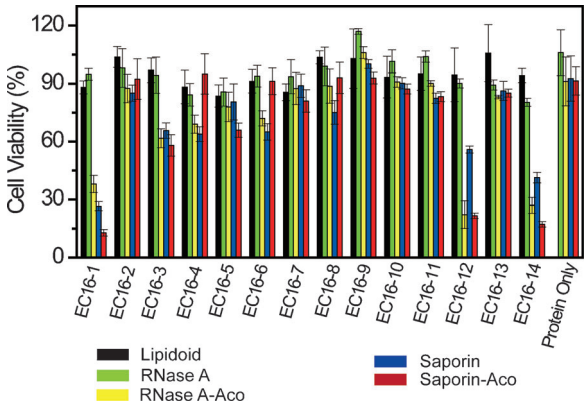

Figure 2. Evaluation of lipidoid-facilitated protein delivery on B16F10 cell line via a cytotoxicity assay. Black: lipidoid controls (4 $\mu g\,mL^{-1}$); green: RNase A (3.3 $\mu g\,mL^{-1}$); yellow: RNase A-Aco (3.3 $\mu g\,mL^{-1}$); blue: saporin (0.17 $\mu g\,mL^{-1}$); red: Saporin-Aco (0.17 $\mu g\,mL^{-1}$). Cytotoxicity was determined by MTT assay. Data are presented as mean $\pm$ SD ($n = 4$).

been used in clinical trials in cancer patients that are refractory to traditional chemotherapy.[13] In an in vivo study, we demonstrate that the administration of a representative lipidoid/saporin nanoparticle formulation suppresses tumor growth in a 4T1 murine breast cancer model by accumulating saporin at tumor sites.

The library of lipidoids was synthesized through the ring-opening reaction between 1,2-epoxyhexadecane and amine under mild conditions according to the methods of previous reports.[9a,b] The crude products were used directly for initial identification of protein delivery materials, and subsequently purified for detailed formulation studies. The lipidoids are named EC16 followed by the amine number in the library (Figure 1), where EC16 indicates 1,2-epoxyhexadecane. The reversible chemical modifications of RNase A or saporin were achieved by reacting proteins with excessive amounts of *cis*-aconitic anhydride, followed by a dialysis purification process. RNase A-Aco was selected for detailed study to confirm the acid-labile nature and chemical reversibility of the *cis*-aconitic anhydride modification of the proteins. The treatment of RNase A-Aco with an acidic buffer solution (NaOAc, pH 5.2) restored the protein back to RNase A, as confirmed by SDS-PAGE analysis (Supporting Information, Figure S1 A). Moreover, the acid-treated RNase A-Aco significantly enhances ribonuclease activity compared to that of neutral PBS treated RNase A-Aco (Figure S1 B). These results suggest that protein modification using *cis*-aconitic anhydride is acid-labile and chemically reversible. Such a reversible protein modification approach has the potential to boost electrostatic binding of protein with cationic lipidoids while having minor effect on their intracellular function.

The capability of lipidoids to deliver protein was evaluated by co-culturing murine melanoma cancer cells (B16F10) with lipidoid–protein complexes of RNase A, saporin, RNase A-Aco, or saporin-Aco. As the successful transduction of RNase A or saporin into cell induces cytotoxicity and inhibits cell proliferation,[11,13b] the protein delivery efficiency by various lipidoid-based nanoparticles was compared by measuring the viability of differently treated cells. As shown in Figure 2, all lipidoids displayed low carrier cytotoxicity, with cell viabilities greater than 90% following exposure to each of the lipidoids at a concentration of 4 $\mu g\,mL^{-1}$. Similarly, we observed no detectable toxicity to B16F10 cells following exposure to the four proteins (3.3 $\mu g\,mL^{-1}$ for RNase A and RNase A-Aco, 0.17 $\mu g\,mL^{-1}$ for saporin and saporin-Aco). This can be attributed to the naked protein lacking an efficient mode of entry to cells. Lipidoid–protein complex treated cells, however, showed distinct changes in cell viability, depending on the lipidoid and protein type. No appreciable viability decrease was observed for lipidoid/RNase A treated cells, indicating a low RNase A delivery efficiency by all lipidoids in the library. This is most likely because of the high intrinsic positive charge, and hydrophilic nature of RNase A, which prevented the formation of stable nanocomplexes for membrane penetration. In contrast, RNase A-Aco, saporin, and saporin-Aco can be delivered using seven of the lipidoids in the library (EC16-1, EC16-3, EC16-4, EC16-5, EC16-6, EC16-12, and EC16-14). A notable example of a lipidoid with high protein delivery efficiency is EC16-1, which facilitates the delivery of RNase A-Aco, saporin, and saporin-Aco, and reduces B16F10 cell viability down to 30%. It is also noteworthy that EC16-1 delivered both chemically modified and non-modified saporin with comparable efficiency. This indicates that the charge–charge interaction is not the only driving force facilitating lipidoid and protein binding, and that hydrophobic interactions may also contribute to the complexation between protein (saporin) and lipidoid. The significance of hydrophobic interaction between lipidoid and protein in facilitating protein delivery was further demonstrated by delivering RNase A-Aco and saporin with lipidoids of varied tail length.

Two lipidoids with shorter hydrophobic tails, EC14-1 and EC14-12, delivered RNase A-Aco and saporin and subse-

quently inhibited cell proliferation less efficiently than EC16-1 and EC16-12 (Figure S2). This suggests that lipidoids with longer hydrophobic tails have higher protein delivery efficiency. Moreover, these findings demonstrate the advantage of a combinatorial approach for the discovery of novel nanocarriers for protein delivery and the investigation of structure–function relationships. The complexation between EC16-1 and proteins was characterized by dynamic light scattering (DLS) analysis. The complexation between negatively charged RNase A-Aco or saporin-Aco and EC16-1 increased the size and decreased the zeta-potential of the EC16-1 particles, while the addition of unmodified RNase A or saporin into the EC16-1 solution had only minor effect on the size and surface charge of EC16-1 (Table S1). The representative nanoparticle structures of EC16-1 and EC16-1/RNase A-Aco complexes were further characterized by TEM imaging (Figure S3). The dispersed EC16-1 solution formed nanoparticles with a size of about 200 nm, while typical lipsome-like structures were observed for the EC16-1/RNase A-Aco complexs.

To investigate lipidoid facilitated protein delivery in detail, RNase A-Aco, and saporin were selected along with the lipidoid EC16-1 as representative proteins and lipidoid for the remainder of the studies. Protein delivery conditions were initially optimized by treating B16F10 cells with lipidoid–protein complexes mixed at varied EC16-1 to protein ratio, while the concentrations of RNase A-Aco and saporin that the cells were exposed to was fixed at 3.3 $\mu g\,mL^{-1}$ and 0.17 $\mu g\,mL^{-1}$, respectively (Figure S4). Optimal results were achieved at mass ratio of 2: 5 for EC16-1/RNase A-Aco, and 20:1 for EC16-1/saporin complex, and further increases in lipidoid to protein ratio did not improve the delivery efficiency. Furthermore, the cytotoxicity of EC16-1/RNase A-Aco and EC16-1/saporin complexes against B16F10 cells is dependent on protein dose. As shown in Figure 3, when used as stand-alone agents, RNase A-Aco and saporin show low cytotoxicities at all of the studied concentrations. EC16-1/RNase A-Aco and EC16-1/saporin complexes prepared at optimized delivery conditions, however, had significantly enhanced protein cytotoxicity and displayed protein-concentration dependence. The half-maximal growth inhibitory concentration (IC_{50}) of EC16-1/RNase A-Aco and EC16-1/saporin complexes against B16F10 cells was determined to be 64 nM and 5.3 nM respectively, which was greatly improved compared to RNase A-Aco and saporin alone.

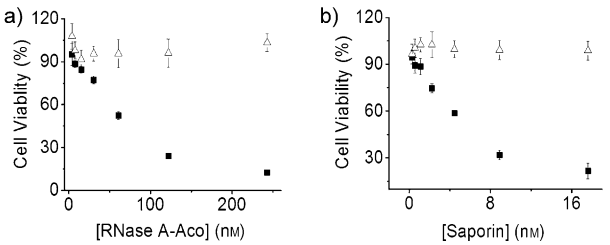

Figure 3. Protein concentration-dependent cytotoxicity of EC16-1 mediated RNase A-Aco (a) and saporin (b) delivery on B16F10 cells (Protein only: △; Lipidoid/protein nanoparticle: ■). Data are presented as mean ± SD ($n = 4$).

Whether lipidoids such as EC16-1 are able to deliver proteins to a panel of cancer cell lines was also investigated. An efficient protein delivery platform for cancer therapy must be able to transfect various cell lines, as each consists of different cell surface environments which may affect the internalization of nanoparticles and the protein delivery. We selected human breast cancer cells (MCF-7 and MDA-MB-231), human liver hepatocellular carcinoma cells (HepG 2), human prostate cancer cell lines (PC-3 and LNCaP), human cervical carcinoma cells (HeLa), and murine breast cancer cells (4T1) as target cell lines. These cells were treated with EC16-1/RNase A-Aco or EC16-1/saporin complexes at previously optimized delivery conditions. Table 1 summarizes the IC_{50} values that were determined following the treatments of EC16-1/RNase A-Aco or EC16-1/saporin complexes to all of the cancer cell lines. The delivery of EC16-1/RNase A-Aco or EC16-1/saporin complexes resulted in significant cytotoxicity in all cell lines, suggesting the general applicability of lipidoid EC16-1 for protein delivery.

Table 1: IC_{50} values of RNase A-Aco and saporin delivered by EC16-1 against various cancerous cell lines.

Cell line	Cancer type	RNaseA-Aco [nM]	Saporin [nM]
MCF-7	breast	40.6	2.9
MDA-MB-231	breast	57	2.5
B16F10	melanoma	64	5.3
HepG 2	liver	28	3.9
PC-3	prostate	125	2.3
LNCaP	prostate	243	1.4
HeLa	cervix	48	3.2
4T1	breast	412	0.9

Having demonstrated the high efficiency and generality of EC16-1 for protein delivery, we next developed a general protein delivery formulation that comprises protein (RNase A-Aco or saporin), lipidoid EC16-1, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cholesterol, and *N*-(palmitoyl)sphingosine[succinyl{methoxy(polyethylene glycol)2000}] (C16-mPEG-ceramide) for in vivo protein delivery. We further post-modified the lipidoid/protein formulations with DSPE-PEG2000-biotin to target tumor cells and tissues. Biotinylated polymers or nanoparticles can be selectively taken up by cancer cells and accumulate in the tumor tissue, improving therapeutic efficacy.[14] The EC16-1/RNase A-Aco and EC16-1/saporin nanoparticle formulation and also empty EC16-1 formulated nanoparticles are about 120 nm in size, as determined by dynamic light scattering (DLS) analysis (Figure 4a). The typical nanoparticular structure of EC16-1/saporin formulation was visualized by TEM (Figure 4b). Subsequent zeta-potential analysis of the nanoparticles revealed the positively charged nature of EC16-1 (7.6 ± 0.3 mV) and EC16-1/saporin formulations (5.6 ± 0.2 mV), while EC16-1/RNase A-Aco formulation had a reduced zeta-potential of −13.5 ± 1.4 mV (Figure 4a). The surface charge measurements of EC16-1/protein nanoformu-

a)

	Size (nm)	Zeta potential (mV)
EC16-1	129.4 ± 5.4	7.65 ± 0.25
EC16-1/RNase A-Aco	126.0 ± 8.7	-13.5 ± 1.45
EC16-1/saporin	131.5 ± 9.7	5.64 ± 0.23

b)

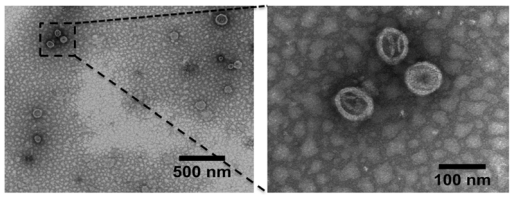

Figure 4. Characterization of EC16-1/protein nanoformulations: a) size and zeta-potential measurements; b) TEM images of EC16-1/saporin nanoformulation.

lations further confirmed the different complexation modes of lipidoids with RNase A-Aco and saporin. The charge–charge interaction between RNase A-Aco and EC16-1 neutralizes the positive charge of the lipidoid nanoparticles, while saporin is encapsulated into the lipidoid primarily by hydrophobic interactions and thus has a minor effect on the surface charge of EC16-1 nanoparticles.

The cellular uptake and intracellular trafficking of EC16-1/protein nanoparticles were studied by formulating FITC-labeled RNase A-Aco (FITC-RNase A-Aco) with EC16-1 and exposing to B16F10 cells. A confocal laser fluorescence microscopy (CLSM) imaging study (Figure 5a) reveals that

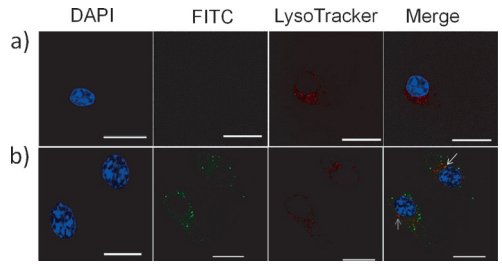

Figure 5. Confocal laser scanning microscopy (CLSM) images of B16F10 cells incubated for 4 h with free FITC-RNase A-Aco (a) and EC16-1/FITC-RNase A-Aco nanoformulations (b). Cells were counterstained with DAPI (for nuclei) and LysoTracker Red (for endosome/lysosomes). Scale bar: 20 μm.

free FITC-RNase A-Aco had a low efficiency of entry of protein. In contrast, significant cellular uptake and intracellular accumulation of EC16-1/protein nanoparticles were observed as green fluorescent pinpoints in the CLSM images of cells treated with the EC16-1/FITC-RNase A-Aco nanoformulation (Figure 5b). The different uptake of free protein and EC16-1/FITC-RNase A-Aco nanoparticles was further confirmed by a flow cytometry analysis (Figure S5). FITC-RNase A-Aco treated cells had comparable mean fluorescence intensity to that of untreated cells, while the cells exposed to EC16-1/FITC-RNase A-Aco nanoformulation had significantly enhanced fluorescence intensities, suggesting that the EC16-1 formulation is capable of delivering proteins. Moreover, B16F10 cells incubated with EC16-1/FITC-RNase A-Aco nanoparticles at a lower temperature (4 °C) have a significantly reduced nanoparticle uptake compared to that at 37 °C (Figure S5). This is an indicative of endocytosis process of EC16-1/FITC-RNase A-Aco nanoparticles, as endocytosis of nanoparticles is known as an energy-dependent process. The endocytosized EC16-1/FITC-RNase A-Aco nanoparticles efficiently escape from endosome/lysosome after entering cells. The CLSM imaging studies by counterstaining endosome/lysosome reveals the co-localization of EC16-1/FITC-RNase A-Aco nanoparticles within endosome/lysosome after 4 h of incubation (white arrows in Figure 5b). However, significant amounts of protein had already escaped from the endosomal compartment, as indicated by the green fluorescence throughout the cell.

The efficiency of EC16-1/protein formulations to inhibit cell proliferation was further determined using B16F10 cells. The treatment of cells with RNase A-Aco or saporin nanoformulation inhibited cell proliferation in a protein-dose-dependent manner (Figure S6). The potential cytotoxicity of empty EC16-1 nanoparicles was excluded in Figure S7. Meanwhile, B16F10 cells treated with varying concentrations of RNase A-Aco or saporin formulations, without EC16-1 caused only a minor decrease in cell viability, compared to that of similar formulations containing EC16-1 (Figure S8). This indicates the vital role that lipidoids play in the formulation of protein nanoparticles for intracellular delivery. The IC_{50} of RNase A-Aco and saporin nanoformulation against B16F10 cells was determined to be 36.5 nM and 4.2 nM, respectively, with an improvement compared to that of EC16-1/protein complexes without formulation processes (Table 1).

Finally, in vivo protein delivery using EC16-1/saporin nanoparticles as a representative protein formulation was conducted to assess the potential of intracellular delivery of EC16-1/protein nanoparticles for cancer therapy. First, the accumulation of EC16-1/saporin nanoformulation at tumor sites was investigated in a murine breast cancer model. Balb/c mice brearing 4T1 tumors were intravenously injected with EC16-1/saporin nanoparticles or free saporin (310 $\mu g\,kg^{-1}$ of saporin). The tumors were harvested 4 h post-injectection for saporin analysis. Immunohistochemical studies (Figure 6a) showed significant amounts of saporin accumulation at tumor sites with EC16-1/saporin nanoformulation injection (as indicated by the dark regions), while no protein accumulation was observed for mice treated with free saporin. The in vivo protein delivery ability of EC16-1/saporin nanoparticles and saporin accumulation at tumor sites were also observed in a B16F10 murine melanoma cancer model. As shown in Figure S9, the intravenous injection of EC16-1/saporin nanoparticles into C57BL/6J mice bearing B16F10 melanoma tumors resulted in similar accumulation of saporin at the tumor site.

The accumulation of EC16-1/saporin nanoformulations at the tumor sites could be ascribed to the enhanced permeability and retention (EPR) effect of the leaky vascular structure of the tumor tissue.[15] Having confirmed the successful and efficient delivery of saporin into tumors, a comparative tumor growth suppression study was performed in the 4T1 murine breast cancer model. 4T1-tumor-

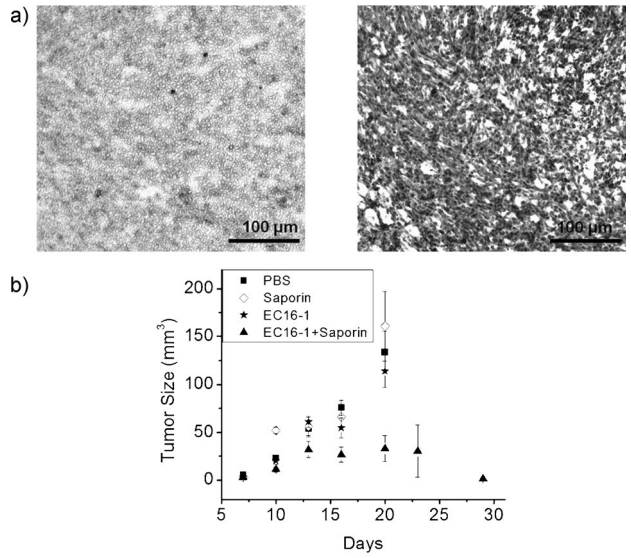

Figure 6. In vivo delivery of EC16-1/saporin nanoformulations into 4T1 breast tumor bearing mice: (a) immunohistochemistry (IHC) studies of saporin accumulation at tumor sites. Saporin in an uncomplexed form (left) and in EC16-1/saporin nanoparticles (right), administrated by tail-vein injection. (b) EC16-1/saporin nanoparticle delivery suppresses tumor growth on a 4T1 breast cancer model. Tumor sizes reported as mean ± SEM.

bearing mice were divided into four groups, and each group of mice received a different treatment (PBS, EC16-1, saporin, or EC16-1/saporin) by intravenous tail vein injection every three days for a total of three injections per mouse. Following each treatment, the tumor growth was monitored by direct caliper measurement. As shown in Figure 6b, EC16-1 and free saporin treated groups had similar tumor volumes to that of PBS control mice. EC16-1/saporin nanoparticle-treated mice, however, had significantly reduced tumor volumes (by 80%) compared to the control groups. Mouse body weight were also monitored during the treatments (Figure S10) to verify that administration did not result in significant toxicity. No significant body weight changes during the treatments were observed nor were a decrease in body condition. Furthermore, the immune response to EC16-1/saporin nanoparticle injections was monitored by measuring the TNF-α and IFN-γ level in the blood samples of mice (Figure S11) to confirm that reduction in tumor volume was not a result of immune stimulation. No significant increase in TNF-α or IFN-γ level were observed in EC16-1/saporin-nanoparticle treated mice compared to the levels in PBS or free saporin-treated mice. The above results demonstrate that EC16-1/saporin nanoparticles can deliver protein in vivo and suppress tumor growth. This suggests that lipidoids are a safe and efficient protein delivery platform for the delivery of cytotoxic proteins for cancer therapy.

In summary, we have reported that our combinatorially designed cationic lipidoid nanoparticles function as a novel protein delivery platform. We synthesized a library of lipidoids through the mild ring-opening reactions of 1,2-epoxyhexadecane with primary or secondary aliphatic amines. Using two representative cytotoxic proteins (RNase A and saporin), we have found that the intrinsic physical properties of the proteins (net negative charge and hydrophobicity) determine the protein–lipidoid interactions and affect the intracellular delivery efficiency. The lipidoid that we selected for primary study, EC16-1, is able to deliver cytotoxic RNase A-Aco and saporin to a panel of cancer cell lines and inhibit cell proliferation. Furthermore, we developed several lipidoid/protein formulations that are efficient for in vitro and in vivo protein delivery. EC16-1/saporin nanoparticles were shown to accumulate at the tumor site and they suppressed tumor growth in a murine breast cancer model. Taken together, these results suggest that combinatorially developed lipidoids can be a highly efficient and effective delivery platform for protein therapeutics. We believe the results disclosed herein will help advance and accelerate the clinical translation of protein pharmaceuticals for cancer therapy.

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[1] D. V. Goeddel, D. G. Kleid, F. Bolivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, A. D. Riggs, *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 106–110.
[2] B. Leader, Q. J. Baca, D. E. Golan, *Nat. Rev. Drug. Discovery* **2008**, *7*, 21–39.
[3] a) G. Walsh, *Nat. Biotechnol.* **2010**, *28*, 917–924; b) Z. Gu, A. Biswas, M. X. Zhao, Y. Tang, *Chem. Soc. Rev.* **2011**, *40*, 3638–3655.
[4] Z. B. Mi, J. Mai, X. L. Lu, P. D. Robbins, *Mol. Ther.* **2000**, *2*, 339–347.
[5] a) S. K. Kim, M. B. Foote, L. Huang, *Biomaterials* **2012**, *33*, 3959–3966; b) O. Zelphati, Y. Wang, S. Kitada, J. C. Reed, P. L. Felgner, J. Corbeil, *J. Biol. Chem.* **2001**, *276*, 35103–35110.
[6] J. S. Lee, J. Feijen, *J. Controlled Release* **2012**, *161*, 473–483.
[7] P. Ghosh, X. Yang, R. Arvizo, Z.-J. Zhu, S. S. Agasti, Z. Mo, V. M. Rotello, *J. Am. Chem. Soc.* **2010**, *132*, 2642–2645.
[8] a) A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J. R. Dorkin, K. N. Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Y. Sah, J. Soutschek, I. Toudjarska, H. P. Vornlocher, T. S. Zimmermann, R. Langer, D. G. Anderson, *Nat. Biotechnol.* **2008**, *26*, 561–569; b) Y.-H. Huang, Y. Bao, W. Peng, M. Goldberg, K. Love, D. A. Bumcrot, G. Cole, R. Langer, D. G. Anderson, J. A. Sawicki, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 3426–3430; c) K. T. Love, K. P. Mahon, C. G. Levins, K. A. Whitehead, W. Querbes, J. R. Dorkin, J. Qin, W. Cantley, L. L. Qin, T. Racie, M. Frank-Kamenetsky, K. N. Yip, R. Alvarez, D. W. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1864–1869; d) D. N. Nguyen, K. P. Mahon, G. Chikh, P. Kim, H. Chung, A. P. Vicari, K. T. Love, M. Goldberg, S. Chen, A. M. Krieg, J. Chen, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E797–E803; e) C. A. Alabi, K. T. Love, G. Sahay, H. Yin, K. M. Luly, R. Langer, D. G. Anderson, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 12881–12886.

[9] a) M. Wang, S. Sun, K. A. Alberti, Q. Xu, *ACS Synth. Biol.* **2012**, *1*, 403 – 407; b) S. Sun, M. Wang, S. A. Knupp, Y. Soto-Feliciano, X. Hu, D. L. Kaplan, R. Langer, D. G. Anderson, Q. B. Xu, *Bioconjugate Chem.* **2012**, *23*, 135 – 140; c) S. Sun, M. Wang, K. A. Alberti, A. Choy, Q. B. Xu, *Nanomed-Nanotechnol.* **2013**, *9*, 849 – 854.

[10] a) Y. Lee, T. Ishii, H. Cabral, H. J. Kim, J. H. Seo, N. Nishiyama, H. Oshima, K. Osada, K. Kataoka, *Angew. Chem.* **2009**, *121*, 5413 – 5416; *Angew. Chem. Int. Ed.* **2009**, *48*, 5309 – 5312; b) Y. Lee, T. Ishii, H. J. Kim, N. Nishiyama, Y. Hayakawa, K. Itaka, K. Kataoka, *Angew. Chem.* **2010**, *122*, 2606 – 2609; *Angew. Chem. Int. Ed.* **2010**, *49*, 2552 – 2555; c) K. Maier, E. Wagner, *J. Am. Chem. Soc.* **2012**, *134*, 10169 – 10173.

[11] R. T. Raines, *Chem. Rev.* **1998**, *98*, 1045 – 1065.

[12] J. G. Beitz, P. Davol, J. W. Clark, J. Kato, M. Medina, A. R. Frackelton, D. A. Lappi, A. Baird, P. Calabresi, *Cancer Res.* **1992**, *52*, 227 – 230.

[13] a) M. S. Iordanov, O. P. Ryabinina, J. Wong, T. H. Dinh, D. L. Newton, S. M. Rybak, B. E. Magun, *Cancer Res.* **2000**, *60*, 1983 – 1994; b) J. G. Beitz, P. Davol, J. W. Clark, J. Kato, M. Medina, A. R. Frackelton, D. A. Lappi, A. Baird, P. Calabresi, *Cancer Res.* **1992**, *52*, 227 – 230; c) L. Polito, M. Bortolotti, M. Pedrazzi, A. Bolognesi, *Toxins* **2011**, *3*, 697 – 720.

[14] a) A. Taheri, R. Dinarvand, F. S. Nouri, M. R. Khorramizadeh, A. T. Borougeni, P. Mansoori, F. Atyabi, *Int. J. Nanomed.* **2011**, *6*, 1863 – 1874; b) J. Su, F. Chen, V. L. Cryns, P. B. Messersmith, *J. Am. Chem. Soc.* **2011**, *133*, 11850 – 11853.

[15] Y. Matsumura, H. Maeda, *Cancer Res.* **1986**, *46*, 6387 – 6392.