Gemini Lipopeptide Bearing an Ultrashort Peptide for Enhanced Transfection Efficiency and Cancer-Cell-Specific Cytotoxicity

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ABSTRACT: Cationic gemini lipopeptides are a relatively new class of amphiphilic compounds to be used for gene delivery. Through the possibility of incorporating short peptides with cell-penetrating functionalities, these lipopeptides may be advantageous over traditional cationic lipids. Herein, we report the design, synthesis, and application of a novel cationic gemini lipopeptide for gene delivery. An ultrashort peptide, containing four amino acids, arginine–cysteine–cysteine–arginine, serves as a cationic head group, and two α-tocopherol moieties act as hydrophobic anchoring groups. The new lipopeptide (ATTA) is incorporated into the conventional liposomes, containing 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), at different molar ratios. The formulated liposomes are characterized and screened for better transfection efficiency. Transfection activity in multiple human cell lines from cancerous and noncancerous origins indicates that the inclusion of an optimal ratio of ATTA in the liposomes substantially enhances the transfection efficiency, superior to that of a traditional liposome, DOTAP–DOPE. Cytotoxicity of ATTA-containing formulations against multiple cell lines indicates potentially distinct activity between cancer and noncancer cell lines. Furthermore, lipoplexes of the ATTA-containing formulations with anticancer therapeutic gene, plasmid encoding tumor necrosis factor-related apoptosis-inducing ligand (pTRAIL), induce obviously more cytotoxicity than conventional formulations. The results indicate that arginine-rich cationic lipopeptide appears to be a promising ingredient in gene delivery vector formulations to enhance transfection efficacy and cell-selective cytotoxicity.

INTRODUCTION

Gene therapy offers a promising strategy for the treatment of several cancers by suppressing the cancer-associated gene expression and/or by increasing the therapeutic gene expression.1,2 However, naked genes cannot accomplish the desired results for the reason that their rapid clearance, low cellular internalization, and nonspecific biodistribution hinder their effectiveness. Delivery vectors are essential to carry these genes safely to the desired site. Development of biosafe delivery vectors is the major challenge for implementing gene therapy at the clinic level. Over the years, several viral and nonviral delivery vectors have been reported. Nonviral vectors such as lipid-based and polymer-based vectors have been frequently preferred in recent times due to their easily tunable chemistry structures, low risk of immunogenicity, complexation capability with a wide range of oligonucleotides, high flexibility, and large-scale production.3,4 Cationic lipids such as 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and DOTAP are amphiphilic structures that were developed during the early research on lipid-based gene delivery. These lipids typically possess quaternary ammonium head groups; thus, enormous research has been inspired by these lipids, and several cationic lipids have been developed over the years.5–7 Most of these traditional cationic lipids extensively studied by numerous researchers are largely limited by the challenges during the several steps of gene delivery. Transfection efficiency and dose-dependent cytotoxicity have been major barriers that hinder the expansion of cationic lipid-based gene delivery beyond research purposes.8 Therefore, a certain focus on the improvement of the efficacy and safety of the delivery vectors that carry the therapeutic genes to a particular target tissue is direly needed. Toward this end, a number of previous reports including ours have demonstrated that amino acid- or peptide head-based lipids help improve biosafety while still providing desired transfection efficiency.9–13 Among several amino acids that have been studied, arginine-based gene delivery systems have been widely accepted for their transfection efficiency.14 Lipid-based delivery vectors with stimuli-responsive linker moieties that are enzyme-cleavable15 and pH16 or redox-responsive17 have
grabbed the certain interest of researchers for their obvious beneficial results. Owing to the high levels of glutathione (GSH), which is an intracellular reducing agent in the tumor microenvironment, redox-responsive delivery vectors have been efficiently utilized to target tumors.\(^{18,19}\) Most recently, gemini-like cationic lipids have been sought after, attributed to their excellent complexation ability with DNA and RNA, facilitating better transfection efficacies while being low toxic.\(^{20−23}\) We have recently reported that the transfection efficiency in cationic gemini lipids with heterocyclic head groups is spacer-dependant.\(^{23}\) Despite several efforts that have been made to develop efficacious liposomal formulations for gene delivery, there still exists a strong and persistent need for novel gene carriers that address the issues affecting safe and efficient cellular delivery. Cationic gemini lipopeptides are a relatively new class of amphiphilic structures, where two monomeric peptide−lipid conjugates are connected by a spacer.\(^{24,25}\) These new types of molecules along with other conventional lipopeptides have been utilized for antimicrobial testing,\(^{26,27}\) but their use in gene delivery is very rare. Damen et al. utilized an ultrashort (serine−proline−lysine−arginine) peptide containing a lipopeptide to deliver DNA and siRNA.\(^{28}\) Zheng et al. synthesized a lysine-based gemini lipopeptide for siRNA delivery.\(^{29}\) In spite of these examples, the cationic gemini lipopeptides are less explored for their gene delivery and anticancer properties.

In this contribution, we report the synthesis and application of a novel gemini-type cationic lipopeptide bearing a reduction-responsive and ultrashort peptide for gene delivery. Taking the abovementioned criteria into consideration, the molecular structure of this gemini lipopeptide contains an Arg−Cys−Cys−Arg peptide in the head group region with a reducible disulfide linker. Two α-tocopherol moieties act as hydrophobic tails of the gemini lipid. The new lipopeptide is formulated with a conventional lipid−colipid pair, DOTAP−DOPE, with the aim of enhancing the transfection efficiency of conventional formulations. The results indicate a remarkable enhancement in the transfection efficiency using the newly developed gemini lipopeptide as compared with the conventional liposome system, DOTAP−DOPE. Moreover, the new formulation also shows potential for cancer cell-specific cytotoxicity while being nontoxic to normal cells. Further mechanistic studies may help elucidate the mechanism behind its cell specificity.

**Reagents and Solvents.** The following reagents and solvents are used: NaOH, sodium hydroxide; DMF, N,N-dimethylformamide; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; (BOC-Cys-OH)\(_2\), Nα-Nα′-di-Boc-l-cysteine; EDC, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide.

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**Scheme 1. Synthesis of Cationic Gemini Lipopeptide (ATTA)**

[Diagram showing the synthesis process]

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**BPATTA**

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**ATTA**

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mide hydrochloride; 1-HOBT, hydroxybenzotriazole; TEA, triethylamine; DCM, dichloromethane; and TFA, trifluoroacetic acid.

**RESULTS AND DISCUSSION**

**Design and Synthesis of the Dimeric Lipid.** The dimeric cationic lipopeptide in the current study was synthesized using the bioconjugation strategy, as shown in Scheme 1. In tandem with our recent experience with arginine–tocopherol conjugates that have shown significant transfection and selectivity,12 the current dimeric cationic lipid was hypothesized to be an effective mediator for selective gene therapy. The newly developed lipopeptide molecule contains multiple intracellularly labile bonds that may help in the release of genetic material intracellularly. The reduction-responsive disulfide bond can be cleaved by intracellular reducing agents such as GSH, while the ester bonds can be broken by the enzymatic action of esterase to facilitate gene delivery.29 The role of the disulfide bridge in nanovesicles appears to be prominent as reported previously, where an increased release of siRNA by lipid nanovesicles with two lysine moieties bridged by a disulfide linker was observed.29,31 The arginine–tocopherol conjugate (APT) was synthesized initially as reported previously,12 with the guanidine group still protected. The free amine on APT reacted with the two carboxylic acid groups of the cysteine molecule to make a peptide linker. The resultant dimeric lipid with acid-labile protecting groups was deprotected using trifluoroacetic acid (TFA) to yield the final lipid/lipopeptide ATTA. Two α-tocopherol moieties serve as membrane-anchoring groups, while a short peptide Arg–Cys–Arg functions as a multicationic head group to interact with DNA. All of the reaction intermediates and the final lipid were produced in good yields (69−93%). The successful synthesis of the final lipopeptide, ATTA, was confirmed by 1H nuclear magnetic resonance (NMR), 13C NMR, and mass spectral data (Supporting Information, Figures S1−S4).

**Optimization and Characterization of Liposome Formulations.** Lipid components in required molar ratios were mixed in aqueous solutions as described in the Experimental Section to form a series of liposomal formulations. The chemical structures of lipid components used in this study are depicted in Figure 1. Cationic lipid DOTAP and helper lipid DOPE at 1:1 lipid/DOPE molar ratio form a well-known transfection reagent.

In the current study, DOTAP/DOPE at a lipid/DOPE ratio of 1:1 was formulated, labeled as “DTD 11”, and used to compare the transfection efficiency of new formulations. Initially, a peptide containing multicationic lipid ATTA was formulated with DOPE at various lipid/DOPE ratios. However, this formulation resulted in nonhomogeneous aggregations. Nevertheless, mixing ATTA at various concentrations to the DOTAP/DOPE formulation resulted in homogeneous colloidal solutions. Keeping the DOTAP/DOPE molar ratio at 1:1, various ratios of ATTA ranging from 0.5 to 4 were mixed to form liposomal solutions. The formulations, namely, DTD 11, DTD 110.5, DTD 111, DTD 112, and DTD 114, were characterized and optimized with regard to their size, zeta potential, stability, and transfection efficiency. As shown in Figure 2A, the sizes of liposome particles were in the range of 100−350 nm with DTD 11, DTD 110.5, and DTD 111 attaining a favorable size below 150 nm.

The storage stability of the particles was assessed for a week (Figure 2B), where all of the formulations exhibit high stability. An increase in the zeta potential was noted as the ratio of ATTA increased from DTD 11 to DTDA 114, as shown in Figure 2C. Owing to their favorable low particle sizes and moderate zeta potential values, DTD 11, DTDA 110.5, and DTDA 111 were presumed to show maximum gene delivery efficacy. Transmission electron microscopy observation confirmed the vesicular formation of lipid particles of DTD 11 and DTDA 111, as shown in Figure 2D. The reduction responsiveness of the liposome vesicles was analyzed by the treatment of liposomes with dithiothreitol (DTT). Disulfide-possessing liposomes are predicted to destabilize the liposomal structure by the action of reducing agents such as DTT and GSH. This may result in the variation of the liposome size.32

Slight increase in the size of ATTA-containing liposomes was noted upon incubation with 10 mM concentration of DTT. This is probably due to the disintegration of lipid components by the action of DTT. Although it is a common reducing agent used in demonstrating disulfide breakage, DTT is highly unstable in aqueous solution.33 Reverse oxidation may occur as the intermediates formed by DTT reduction are nonstable.33 In the current study, a maximum increase in the liposomal size was seen at 3 h incubation time with DTT. Thus, 3 h incubation time was considered to further examine DNA release from liposomes using the gel retardation assay.

**DNA Binding and Reduction-Responsive Release.** The surface charge of lipid vesicles measured by the zeta potential in turn determines the DNA-binding ability of the same. As depicted in Figure 3A, the electrophoretic mobility of DNA was completely inhibited by both liposomes DTD 11 and DTDA 111 at various N/P ratios ranging from 1 to 9. Due to the high net positive charge and stability of the liposome particles, it was expected to show good DNA binding by the tested lipid particles. In addition, the arginine-rich periphery is reported to be favoring greater DNA binding and transfection even at low N/P ratios.11 However, the compromise in the...
lipid/DNA complex is essential for the release of DNA at the targeted site. This destabilization can be sometimes brought by stimuli-responsive linkers in the lipid vesicles. Disulphide bonds in the lipid structure can act as redox-responsive sites as they can be cleaved by intracellular reducing agents such as GSH, helping in the release of genetic material that they are carrying. In the present study, the redox-responsive release of DNA was monitored by the gel electrophoresis assay of DTDA 111/DNA complexes incubated in DTT-containing solution at pH 8.0. As represented in Figure 3B, the discrete fluorescent DNA bands observed in DTT-treated complexes indicate a slight release of DNA by the action of DTT. Although several factors influence intracellular DNA release, the current results indicate that reduction-sensitive bonds may reinforce the DNA-releasing effect intracellularly. **Transfection Efficiency.** Selected liposomal formulations, DTD 11, DTDA 110.5, DTDA 111, and DTDA 112, were further optimized in terms of transfection efficiency. HEK-293 (a human embryonic kidney cell line) cells were treated with lipoplexes derived from the above liposomal formulations and pEGFP-C1 plasmid. Out of all of the formulations and the N/P ratios ranging from 1 to 9, DTDA 111 at N/P ratio 5 shows maximum transfection efficiency as observed by fluorescence microscopy (Figure S5). It was also confirmed that among the ATTA-containing formulations, DTDA 111 has the superior potential to deliver pDNA. DTD 11, containing commercial lipids DOTAP and DOPE, exhibited maximum transfection at

**Figure 2.** Physicochemical characterization and the reduction-responsive stability test of liposomal formulations. (A) Hydrodynamic diameters of liposomes at different lipid molar ratios (n = 3). (B) Stability of hydrodynamic diameters of liposomes over a period of 7 days (n = 3). (C) Zeta potentials of liposomes at different lipid molar ratios (n = 3). (D) TEM images of DTD 11 and DTDA 111. (E) Time-dependent relative size variations of DTDA 110.5, DTDA 111, DTDA 112, and DTD 11 with 10 mM dithiothreitol (DTT) treatment (n = 3).

**Figure 3.** (A) Gel retardation images of lipoplexes at different N/P ratios ranging from 1 to 15. (B) Reduction-responsive DNA release from DTDA 111 lipoplexes with DTT (10 or 50 mM) treatment.
N/P ratio 3. Thus, a further comparison of transfection efficiencies of DTD 11 and DTDA 111 with another most common transfection reagent, Lipofectamine2000, is presented in Figure 4. The ratio of the fluorescence intensity of DTDA 111 to the fluorescence intensity of DTD 11 at a given N/P ratio was calculated. The ratio of the fluorescence intensity of lipofectamine to the fluorescence intensity of DTD 11 at N/P ratio 3 was considered for the lipofectamine value. (C) Fluorescence intensities of pEGFP-transfected cells by various lipid formulations as quantified using ImageJ software ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$).

Figure 4. In vitro pEGFP gene transfection efficiency mediated by DTDA 111 compared with that by DTD 11 and lipofectamine in HEK-293 cells. (A) Representative fluorescence microscopy images after pEGFP gene transfection mediated by DTD 11 and DTDA 111 at various N/P ratios ranging from 3 to 9. The scale bar is 200 μm. (B) Relative transfection efficiencies of various lipid formulations quantified from the fluorescence intensities of images (A) using ImageJ software. The ratio of the fluorescence intensity of DTDA 111 to the fluorescence intensity of DTD 11 at a given N/P ratio was calculated. The ratio of the fluorescence intensity of lipofectamine to the fluorescence intensity of DTD 11 at N/P ratio 3 was considered for the lipofectamine value.

N/P ratio 3. Thus, a further comparison of transfection efficiencies of DTD 11 and DTDA 111 with another most common transfection reagent, Lipofectamine2000, is presented in Figure 4. The ratio of the fluorescence intensity of DTDA 111- and lipofectamine-treated groups to the fluorescence intensity of DTD 11-treated groups was determined as relative transfection efficiency. The relative transfection efficiencies of the formulations (Figure 4B) at their respective N/P ratios were quantified from the fluorescence intensity observed from Figure 4A. The fluorescence intensity of each group quantified from ImageJ software is given in Figure 4C. DTDA 111 has shown remarkable enhancement in transfection efficiency in comparison with DTD 11.

In the last two decades, cell-penetrating peptides (CPPs) have been utilized as coherent biological tools to attain better cellular uptake during cargo delivery. Several organic and inorganic biomaterials have been developed using CPPs, which proved that the peptide-based delivery vectors are highly efficient in achieving adequate transfection. Especially, guanidinylation of the delivery vector’s surface has proved to impact drug or gene delivery significantly. The ATTA lipid in DTDA 111 contains two guanidine cations on arginine moieties and two ammonium cations on cysteine moieties. The guanidine moiety in arginine plays a pivotal role in improving the transfection ability of arginine-based delivery systems. Arginine has been thought to be a key component of CPPs that involves binding to many cellular receptors. The guanidine moiety in arginine is especially known to interact with phosphate groups on the cell membrane, enabling the internalization of arginine-containing CPPs. The mechanism of interaction of CPPs and the cell membrane involves bidentate hydrogen bonding among guanidine moieties and phosphate groups on the surface of the cell, helping in improved uptake inside the cell. Similarly, in the case of the present study, the improved transmembrane function of lipopeptide, ATTA, through guanidine-rich arginine periphery, is believed to enhance transfection of DTDA 111 over DTD 11. To further evaluate the enhanced transfection efficiency...
and to confirm the effective N/P ratio, another noncancer cell line HEL-299 (human embryonic lung cell line) was employed. Notably, very low transfection was detected by DTD 11-based lipoplexes (Figure 5A) in HEL-299 cells. On the other hand, DTDA 111-containing ATTA exhibited superior transfection efficiency at all given N/P ratios (Figure 5B). At an N/P ratio of 5, DTDA 111 proved to be better than lipofectamine as evidenced by their fluorescence intensity values (Figure 5C). These results demonstrate that the inclusion of ATTA in the liposomal formulations significantly enhances the transfection efficiency of DTDA 111 liposomes.

After screening the effective N/P ratios of the liposomal formulations, we further evaluated the application of these formulations on various cancer cell lines where gene delivery may be essential. PC3 (a human prostate cancer cell line), U87 (a human glioblastoma cell line), and A549 (a human lung carcinoma cell line) were employed to compare the ability of DTD 11 and DTDA 111 in terms of transfection. Representative fluorescence images of three cancer cell lines treated by various formulations are presented in Figure 6A. Clearly, in these cancer cell lines, DTDA 111 showed greater transfection ability than DTD 11, substantiating the earlier results (Figure 6B). DTDA 111 demonstrated competitive transfection efficiency in comparison with commercial transfection reagent lipofectamine, except for the A549 cell line. On the other hand, the percentage of GFP-positive cells in the abovementioned cell lines revealed that DTDA 111 has transfected significantly more number of cells than DTD 11 and lipofectamine, except for the A549 cell line (Figure S6). In the case of the A549 cell line, although DTDA 111 was highly significant when compared with DTD 11, lipofectamine was dominant in transfection. It is to be noted that lipofectamine has yielded intense transgene expression in transfected cells, which sometimes is the reason for its cytotoxicity, irrespective of the cell line. The practical effectiveness of the transfecting reagent can be improved by enabling a moderate but uniform expression of the transgene with a high percentage of transfected cells.38

Contrary to lipofectamine, the DOTAP-
based transfection reagent, DTD 11, has transfected a much lower number of cells with less gene expression. The results suggest that the short peptide bearing multicationic lipid ATTA plays a crucial role in the enhancement of transfection efficiency.

**Cell Viability.** The most effective liposomal formulations DTD 11 and DTDA 111 were assayed for their in vitro cytotoxicity against multiple normal and cancer cell lines (Figure 7). Through the MTT-based cell viability assay, it was interesting to note that DTDA 111 has shown no cytotoxicity against normal cells HEK-293 even at 100 μg/mL concentration (Figure 7A). Also, the cell viability of DTDA 111 against normal cell line HEK-293 is much greater than lipofectamine (Figure S7). To validate this result, several cancer cell lines from various cancer tissues (PC3, U87, A549, and MDA-MB (a human breast cancer cell line)) and another noncancer cell line HEL-299 were tested against the same formulations. Similar to HEK-293, significantly high cell viabilities were observed in HEL-299 cells treated with DTDA 111, compared with DTD 11. Nevertheless, some cytotoxicity was noted at high concentrations (100 μg/mL) of DTD 111 with 68.83 ± 10.1 cell viability (Figure 7B). It was anticipated that, similar to HEK-293 and HEL-299, DTD 111 may show better cell viabilities than DTD 11 and lipofectamine in other cell lines tested. However, against all of the tested cancer cell lines, PC3 (Figure 7C), U87 (Figure 7D), A549 (Figure 7E), and MDA-MB-231 (Figure 7F), the newly developed liposomal formulation DTDA 111 has apparently exhibited higher cytotoxicity than DTD 11. As the x-axis of Figure 7 represents the total concentrations of liposomes, it is understood that at any given concentration, the amount of DOTAP is lower in DTDA 111 than that in DTD 11. It clearly demonstrates that DOTAP in DTD 11 has nonselective cytotoxicity. Thus, minimizing the DOTAP concentration and increasing the ATTA concentration favor the transfection as well as selective cytotoxicity. Although DOTAP helps in keeping liposomal integrity, excess concentration of DOTAP in the liposomes negatively affects transfection and cytotoxicity. The data also infers that the cell-selective cytotoxicity of DTDA 111 has arrived from the lipopeptide, ATTA but not from DOTAP. Ultrashort peptides containing lipopeptides have been investigated and demonstrated for their antimicrobial activity in the literature. However, due to the nonselective cytotoxicity of these lipopeptides, the studies on their anticancer activities are very rare. The effect of DTDA 111 being more cytotoxic in cancer cells was particularly seen at the concentration of 50 μg/mL or above, irrespective of the cell line. Further investigations may be required to determine the mechanism behind the cancer cell-specific cytotoxicity of DTDA 111. Several naturally occurring lipopeptides known to show toxicity against mammalian cells are, however, noncell-selective. Most recently, there is a report on arginine-rich synthetic cationic lipopeptides showing selective cytotoxicity against cancer cells. Studies also informed that a combination of cationic and amphiphilic structures is critical to exhibit potent anticancer activity. Typically, in a single molecule, a ratio of 1:2 for a cationic group and hydrophobic residues was summarized to display potent cytotoxicity against cancer cells. Gemini lipopeptides typically contain two hydrophobic groups per cationic peptide, which may help these compounds to achieve cancer-specific cytotoxicity. Moreover, a short tetrapeptide, hydrophobicity, and disulfide linkers are found to be favoring factors for cancer-specific cytotoxicity.

The cytotoxicity of pEGFP-C1-derived lipoplexes against HEK-293 and HEL-299 was in accordance with the cytotoxicity of liposomes (Figure S7). Higher cell viabilities were noted by the DTDA 111/pEGFP system compared to DTD 11 and lipofectamine in HEK-293 and HEL-299 cell lines. The corresponding lipoplexes of DTD 11 and DTDA 111, with either a control pEGFP-C1 gene or an anticancer pTRAIL gene, were tested for their cytotoxicity against selected cancer
cell lines (Figure 8). In comparison with DTD 11-based lipoplexes, either with pEGFP-C1 or pTRAIL gene, there was a significant decrease in cell viability of the cells treated with DTDA 111-based lipoplexes. In the case of PC3 cells, the DTDA 111/pTRAIL system effectuated 42.7 ± 2.1% cell viability, whereas the DTD 11/pTRAIL system could cause 52.2 ± 3.1% cell viability (Figure 8A). Similarly, the DTDA 111/pTRAIL system caused nearly 49% cell viability in U87
cells, while the DTD 11/pTRAIL system resulted in ~60% viable cells, as shown in Figure 8B. A large variation in the cell viability of pTRAIL-derived lipoplexes of DTD 11 and DTDA 111 was witnessed in the case of A549 cells. In the case of the A549 cell line, no obvious variation in cell viability was detected in cells treated with either the DTD 11/pTRAIL or DTD 11/pEGFP system with more than 80% of viable cells in both treatments (Figure 8C). This was probably due to the very poor transfection ability of DTD 11 in the A549 cell line as evidenced by transfection results (Figure 6). In contrast, A549 cells treated with the DTDA 111/pTRAIL system resulted in 51.4 ± 0.9% cell viability, indicating the high cytotoxicity-inducing ability caused by transfection. In each of these cancer cell lines, even the control DTDA 111/pEGFP system could induce significantly higher cytotoxicity than the DTD 11/pEGFP system (Figure 8). Nevertheless, as expected, lipofectamine was more cytotoxic irrespective of cell lines tested. In the current study, the optimal N/P ratio for transfection of DTDA-based lipoplexes was 5, where the concentration of liposomes was calculated to be ~35 μg/mL. Data shown in Figure 8A–C indicate that the control DTDA 111/pEGFP lipoplexes at N/P 5 have induced a certain level of cytotoxicity that is significant as compared to DTD 11/pEGFP lipoplexes. It also infers that further increasing the N/P ratio may result in enhanced selective cytotoxicity but may affect the transfection efficiency. The cytotoxicity results were also substantiated through optical imaging of U87 cells displayed in Figure 8D. Normal cell morphologies and good confluence were observed in control naked DNA-treated U87 cells. However, DTDA 111/pTRAIL-treated cells showed significantly fewer cell numbers and their disrupted cell morphology was similar to that of lipofectamine-treated cells. Anticancer activity of lipopeptides is often determined by their cell membrane interactions as membrane profiles of cancer cells differ from normal cells. Several anticancer peptides or lipopeptides are shown to be inducing cytotoxicity primarily by disrupting the plasma membrane of the cells. Membranolytic nature of cationic, amphiphilic peptides was reported previously, demonstrating that the accumulation of these peptides on the cell membrane leads to their internalization, either by making pores on the membrane or by completely dissolving the membrane through detergent-like activity. Although these membrane activities of cationic lipopeptides and peptoids are establishing them as promising anticancer

Figure 8. Comparison of relative cell viabilities of lipoplexes derived from the control pEGFP plasmid and therapeutic pTRAIL plasmid using DTD 11, DTDA 111, and lipofectamine formulations against (A) PC3, (B) U87, and (C) A549 cells (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001). (D) Microscopy observation of variations in U87 cell morphologies upon transfection with various lipoplexes based on the control pEGFP plasmid and therapeutic pTRAIL plasmid. The scale bar is 200 μm.
agents, their intracellular activity must not be overlooked. The mechanism of action of lipopeptides against cancer cell lines was not fully understood so far. Structurally variant lipopeptides act differently to induce cytotoxicity in cancer cell lines.\(^{27,33}\) Apoptotic as well as nonapoptotic pathways are possibly taking part in the anticancer activities of lipopeptides.\(^{36}\) In the present work, ATTA is equipped with the structural characteristics as described by previous studies to destabilize the anionic cell membranes; these interactions of ATTA with cancer cells may have a role in its selective cytotoxicity. However, we understand that the intracellular pathways cannot be ruled out and further explorations will be focused in that direction. Most importantly, ATTA, with its high efficiency to transfer therapeutic genes, offers an attractive combinatorial effect to gene therapy.

The current results indicate that the newly synthesized lipopeptide may have the potential to induce cell-selective cytotoxicity. Further detailed mechanistic studies may help in understanding the specificity of the lipopeptide and to probe enhancement of cancer-specific cytotoxicity. Due to the nonselective cytotoxicity of lipofectamine and low transfection of DOTAP/DOPE in many cancer cell lines, the new lipopeptide, ATTA, may be advantageous for improving transfection and cytotoxicity properties. Moreover, ATTA can be formulated with other conventional liposomes to enhance the delivery of the therapeutic gene as well as to reinforce its therapeutic activity.

**CONCLUSIONS**

A short peptide containing cationic gemini lipopeptide (ATTA) was rationally synthesized using a bioconjugation strategy. Various formulations resulting from ATTA-mixed liposomes and N/P ratios of their corresponding lipoplexes were characterized and systematically optimized for better gene delivery properties. Optimal formulations with favorable sizes and zeta potentials as determined by dynamic light scattering were further utilized to analyze the transfection efficiency. Moreover, the reduction response of the optimal formulations was verified by the gel electrophoresis assay after incubation in reducing conditions. Substantial enhancement of transfection activity was observed with the ATTA-based formulation irrespective of cell lines. Distinct cytotoxicity of formulations was noted between various human cell lines due to the presence of ATTA in the formulations. Cationic lipopeptides are known to be cytotoxic toward mammalian cell lines. However, information on self-assembly, gene delivery, and cytotoxicity of new synthetic lipopeptides is scarce. Extensive studies on cationic gemini lipopeptides may help in designing efficient gene vectors with anticancer properties.

**EXPERIMENTAL SECTION**

**Materials.** \(\alpha\)-Tocopherol, (BOC-Cys-OH)\(_2\), 2-bromoethanol, Fmoc-Arg(pbf)-OH, DOPE, and DOTAP were purchased from Sigma-Aldrich Co., Ltd. Lipofectamine2000 was supplied by Invitrogen Life Technologies. If not mentioned otherwise, other reagents and organic solvents, i.e., trifluoroacetic acid (TFA), sodium hydroxide (NaOH), piperidine, \(N,N'\)-dicyclohexylcarbodiimide (DCC), methylene chloride (DMC), \(N\)-(3-dimethylaminopropyl)-\(N'\)-ethylcarbodiimide hydrochloride (EDC), 4-dimethylaminopyridine (DMAP), 1-hydroxybenzotriazole (HOBr), dimethylformamide (DMF), and methanol, were acquired from Sigma-Aldrich, Finar Alfa, and Aesor at their maximum purity, which were utilized without additional purification. Reaction progress was monitored on silica gel thin-layer chromatography (TLC) plates (0.25 mm). Silica gel (60–120 mesh, Acme Synthetic Chemicals, India) column chromatography was used for the separation of compounds. Spectral data (\(^1\)H and \(^13\)C NMR) were obtained using a Varian FT400 MHz NMR spectrometer. Mass spectrometry data were recorded on a commercial ion trap LCQ mass spectrometer (Thermo Finnigan, San Jose, CA) fitted with an ESI source. Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin–EDTA, and Roswell Park Memorial Institute (RPMI) 1640 Medium were procured from Invitrogen. 3-((4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was acquired from MP Biomedicals. pTRAIL plasmid was gifted by Prof. Chien-Wen Chang. Briefly, the pTRAIL vector was obtained by cloning the TRAIL gene (synthesis protocol by Genedirex) downstream of the CMV promoter of a pcDNA3 (Invitrogen) vector.

**Synthesis.** A novel gemini cationic lipopeptide bearing ultrashort peptide was synthesized using the bioconjugation strategies described in Scheme 1. The arginine–tocopherol conjugate (APT) was synthesized initially as reported in our previous method.\(^{12}\) Intermediate APT was then conjugated to (BOC-Cys-OH)\(_2\) using the peptide chemistry approach. Intermediate compounds and the final lipopeptide were characterized by nuclear magnetic resonance (NMR) and mass spectrometry.

**Synthesis of Arginine–Tocopherol Lipopeptide (ATTA).** Synthesis of Bis[tocopheryloxyethyl] 2,2′-[[3,3′-Disulfanediylbis(2-(BOC)amino)propanoynyl]bis(azaelediy)]bis-(5-3′-(Pbf)guanidino)pentanate) (BPATTa). 2-Tocopheryloxyethyl 2-amino-5-[(3-pbf)guanidino]pentanate (APT, 2.2 equiv, 0.5 g) dissolved in 10 mL of dry DCM in a round-bottomed flask was added to (BOC-Cys-OH)\(_2\) (1.0 equiv, 0.113 g), HOBt (2.3 equiv, 0.082 g), EDC (2.3 equiv, 0.115 g), and trimethylamine (2.3 equiv, 0.06 g) at 0 °C. The mixture was stirred at 0 °C for half an hour followed by stirring for another 24 h at room temperature. The solvent was evaporated under reduced pressure using a rotary evaporator. Ethyl acetate (EA, 50 mL) was added to the concentrated residue, and the mixture was washed with brine (3 × 50 mL) and water (3 × 50 mL). The organic layer was collected, dried over Na\(_2\)SO\(_4\), concentrated to dryness, and triturated with methanol to yield a solid. The solid was redissolved in methanol (10 mL) and washed with brine (3 × 10 mL). Evaporation of the organic layer under reduced pressure left a white solid (2 g, 73%). Intermediate compounds and the final lipopeptide were characterized by NMR and mass spectrometry.

**Synthesis of 1,1′-(3,3′-Disulfanediylbis(2-ammonio propanoynyl)]bis(azaelediy)]bis-(tocopheryloxyethoxy)pentane-4,1-diyldiguanidinium (ATTA).** Deprotection of pbf and BOC groups from the above intermediate was done using 50% trifluoroacetic acid (TFA) in DCM. BPATTA (0.3 g, 0.14 mmol) was dissolved in 2 mL of DCM, and 2 mL of TFA was dropped slowly at 0 °C. After
complete addition of TFA, the reaction mixture was slowly warmed up to room temperature while stirring for 3 h. Then, TFA and DCM were removed under vacuum and the residue with the crude product was washed thrice with diethyl ether that yielded the pure target product, arginine–tocopherol-conjugated lipopeptide (ATTA), as a white solid. Yield: 0.25 g (93%). \[^{1}H\text{NMR}\ [\delta/\text{ppm}] (400 MHz, DMSO-\text{d}_{6} + \text{CDCl}_{3})\]
\[
\begin{align*}
\text{4.42-4.36} & \quad (\text{m}, 8\text{H}, 2\times \text{-COO-CH}_{2}), \\
\text{4.3} & \quad (4\times \text{-NH-CH}_{2}), \\
\text{3.81} & \quad (4\times \text{H}, 2\times \text{-CH}_{2}-O), \\
\text{3.13} & \quad (3\times \text{H}, 4\times \text{H} - \text{S-CH}_{2}), \\
\text{2.81} & \quad (2\times \text{H}, 24\times \text{-CH}_{2}-), \\
\text{2.05} & \quad (2\times \text{H}, 8\times \text{-CH}_{2} - \text{tocopherol}), \\
\text{1.51} & \quad (2\times \text{H}, 24\times \text{-CH}_{2} - \text{arginine}), \\
\text{1.24-1.18} & \quad (2\times \text{H}, 38\times \text{-CH}_{3} - \text{arginine}), \\
\text{0.88-0.85} & \quad (2\times \text{H}, 24\times \text{-CH}_{2} - \text{arginine}).
\end{align*}
\]
\[^{13}C\text{NMR}\ [\delta/\text{ppm}] (400 MHz, DMSO-\text{d}_{6} + \text{CDCl}_{3})\]
\[
\begin{align*}
\text{171.4} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{167.4} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{157.7} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{147.6} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{125.9} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{122.5} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{117.9} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{74.7} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{70.5} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{70.3} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{64.6} & \quad (8\times \text{CH}_{3} - \text{arginine}), \\
\text{64.4} & \quad (8\times \text{CH}_{3} - \text{arginine}).
\end{align*}
\]

**Agarose Gel Electrophoresis.** The lipid–DNA complexation was assessed through a typical agarose gel electrophoresis assay. Cationic liposomes DOTAP/DOPC at a lipid molar ratio of 1:1 and DOTAP/DOPC/ATTA at a lipid molar ratio of 1:1:1 were added to 0.3 μg of pDNA. N/P ratios of lipoplexes ranging from 1 to 15 were formulated by mixing various amounts of the liposome and a constant amount (0.3 μg) of pEGFP-C1 plasmid in deionized Milli-Q water. After 30 min of incubation at room temperature, the lipoplexes were added to 0.8% agarose gel that was prestained with DNA safe stain. The gel was subjected to electrophoresis for 20 min at a current of 140 V in tris-borate EDTA (TBE) buffer. The DNA retention pattern was observed under UV light at 365 nm. To check the reduction-responsive release of DNA from lipoplexes, dithiothreitol (DTT) was used as the reducing agent. Lipoplexes at N/P ratios of 3 and 5 were initially prepared as mentioned above and incubated in PBS at pH 8.0 with 10 or 50 mM DTT. Then, the complexes were incubated for another 3 h at 37 °C. After the incubation, the lipoplexes were added to 0.8% agarose gel that was prestained with DNA safe stain. The gel was subjected to electrophoresis for 20 min at a current of 140 V in TBE buffer.

**Cell Experiments.** HEL-299 and U87 cell lines were cultured and preserved in a CO\textsubscript{2} incubator (5% CO\textsubscript{2}) at 37 °C under moisture conditions in MEM, complemented with 1% sodium pyruvate, 10% FBS, and 100 μg/mL penicillin–streptomycin. HEK-293 cells and MDA-MB-231 were cultivated in DMEM, supplemented with 10% FBS and 100 μg/mL penicillin–streptomycin. PC3 cells and A549 were cultivated in Roswell Park Memorial Institute medium-1640 (RPMI-1640), supplemented with 10% FBS and 100 μg/mL penicillin–streptomycin. The cells were continuously monitored for confluence throughout the duration of the experiments. The medium was changed every two or three days, and subculture was done when the cells reached 90% confluence.

**Cell Viability.** The cell viability of liposomes or lipoplexes was evaluated by the MTT assay. The cytotoxicities of liposomes were examined for incubation with various concentrations of TDD 11 liposomes or DTDA 111 liposomes (0.5–100 μg of liposome/mL). HEK-293, HEL-299, PC3, U87, A549, and MDA-MB-231 cells at a density of 5 × 10\textsuperscript{4} per well were seeded in tissue culture plates (96-well) in their respective medium consisting of 10% FBS. The relative cell viabilities of lipoplexes were examined at various N/P ratios.
(3–9) of DTD 11/DNA lipoplexes or DTDA 111/DNA lipoplexes at 48 h postincubation at 37 °C. Two different pDNAs were used in this study to make lipoplexes and their corresponding lipoplexes were compared for cell viability. A control pEGFP plasmid and pTRAIL plasmid with anticancer properties were used to make lipoplexes. Lipofectamine/DNA complexes were prepared as per the manufacturer’s protocol. The percentage of viable cells was estimated using the tetrazolium-based colorimetric technique by determining their mitochondrial reductase activity.57

In Vitro Transfection studies. In vitro transgene expression was done in HEK-293, HEL-299, U87, A549, and PC3 cells at a cell density of $5 \times 10^5$ per well in 24-well plates and incubated in their respective culture medium comprising 10% FBS in a CO$_2$ incubator for 24 h prior to transfection. Lipoplexes with N/P ratios ranging from 3 to 9 were made using different amounts of DTD- or DTDA-based liposomes and a fixed DNA (pEGFP-C1 plasmid) amount of 1 μg to a final volume of 100 μL. After 30 min of incubation, the formed lipid/DNA complexes were added to well plates containing cells in MEM/RPMI/DMEM medium. The cells were then incubated with lipoplexes for 4 h followed by the replacement of the medium with fresh complete medium comprising 10% FBS. The cells were further incubated for another 48 h of post-transfection. Expression of green fluorescent protein (GFP) was directly imaged using fluorescence microscopy (Leica DMi8; Leica Microsystems, Wetzlar, Germany). We have adopted a previously reported quantification method for the quantification of green fluorescence expression in various cell lines after transfection.36,58 Briefly, for individual experiments, at least three fields of view in each group were taken as fluorescent images. Subsequently, the fluorescence intensity of each corresponding image was semiquantitated using ImageJ software (v1.51a, National Institute of Health). The average and standard deviation of fluorescence intensity of each test group were calculated. The relative transfection efficiency was measured by comparing the fluorescence intensity of DTD 11 and that of lipofectamine-treated groups with DTD 111 transfection. Expression of green fluorescent protein (GFP) was directly imaged using fluorescence microscopy (Leica DMi8; Leica Microsystems, Wetzlar, Germany). We have adopted a previously reported quantification method for the quantification of green fluorescence expression in various cell lines after transfection.36,58

Statistical Analyses. Data were analyzed with SigmaPlot 11.0 software (Systat Software, Inc, San Jose, CA) and presented as the mean ± SD of a minimum of three individual experiments. Significant differences between individual groups were determined by performing the t-test. Statistical differences between two groups were considered significant as per the following criteria: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03620.

NMR and ESI-MS spectra of the cationic lipopeptide, optimization of transfection of various N/P ratio formulations, data of GFP-positive cell percentage in cancer cells, and cell viability data of noncancer cell lines treated with control lipoplexes (PDF)

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Notes

The authors declare no competing financial interest.

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