Cimetidine-Based Cationic Amphiphiles for In Vitro Gene Delivery Targetable to Colon Cancer

Shireesha Manturthi, Dwaipayan Bhattacharya, Kalyani Rajesh Sakhare, Kumar Pranav Narayan,* and Srilakshmi V. Patri*
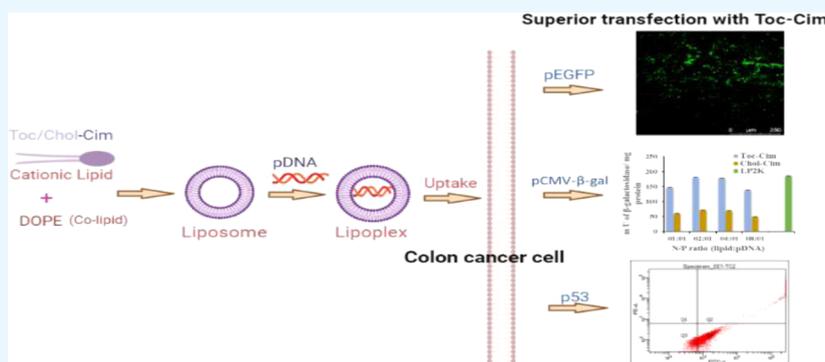
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ABSTRACT: Cimetidine, a histamine-2 (H2) receptor antagonist, has been found to have anticancer properties against a number of cancer-type cells. In this report, we have demonstrated that cimetidine can acts as a hydrophilic domain in cationic lipids and targetable to the gastric system by carrying reporter genes and therapeutic genes through in vitro transfection. Two lipids, namely, Toc-Cim and Chol-Cim consisting cimetidine as the main head group and hydrophobic moieties as alpha-tocopherol or cholesterol, respectively, were designed and synthesized. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) is a well-known co-lipid employed to produce liposomes as uniform vesicles. The liposomes and lipoplexes were structurally and functionally evaluated for global surface charges and hydrodynamic diameters, and results found that both liposome and lipoplex size and surface charges are optimal to screen the transfection potentials. DNA-binding studies were analyzed as complete binding at all formulated N/P ratios. The liposomes and lipoplexes of both the lipids Toc-Cim and Chol-Cim show minimal cytotoxicity even though at higher concentrations. The results of the transfection experiments revealed that tocopherol-based cationic lipids (Toc-Cim) show finer transfection efficacy with optimized N/P ratios (2:1 and 4:1) in the colon cancer cell line. Toc-Cim lipoplexes show higher cellular uptake compare to Chol-Cim in the colon cancer cell line at 2:1 and 4:1 N/P ratios. Toc-Cim and Chol-Cim lipids showed highly compatible serum, examined up to 50% of the serum concentration. To evaluate the apoptotic cell death in CT-26 cells, exposed to Toc-Cim:p53 and Chol-Cim:p53 lipoplexes at 2:1 N/P ratios, superior results showed with Toc-Cim:p53. An effect of TP53 protein expression in CT-26 cell lines assayed by western blot, transfected with Toc-Cim:p53 and Chol-Cim:p53 lipoplexes, demonstrated the superior efficacy of Toc-Cim. All of the findings suggest that Toc-Cim lipid is relatively secure and is an effective transfection agent to colon cancer gene delivery.

1. INTRODUCTION

The positive outcome of gene therapy is primarily regulated by the expansion of effective and secure gene delivery vectors.[1−3] Two routes were chosen primarily to transfer bare DNA into cells using non-viral and viral vectors.[4] Non-viral vector evolution has already been one of the key research areas due to several notable factors, namely, more carrier ability, healthy, large-scale elegance development, rationality, indefinite vector size, and capability to include targeting ligands.[5,6] These advanced transmission vectors, however, are less competent than vectors for the transfection of viral genes.[7] Because of great transfection efficacy and specific characteristic behavior, cationic liposomes and polymer-based vectors have been of

interest in non-viral vectors.[8,9] Most of the specific studies have addressed the cationic lipids. The common cationic lipid structural base consists of following essential components, a hydrophilic group in polar phase exposure toward an aqueous phase, resting hydrophobic group in the non-polar phase, both
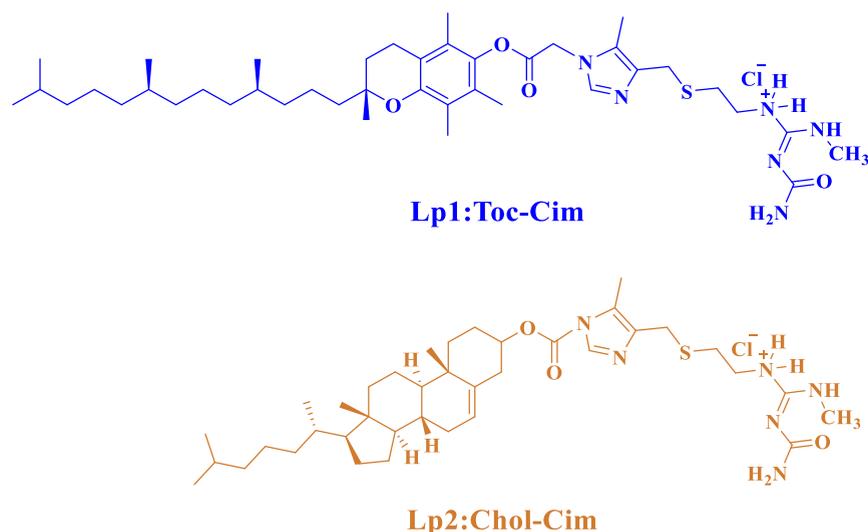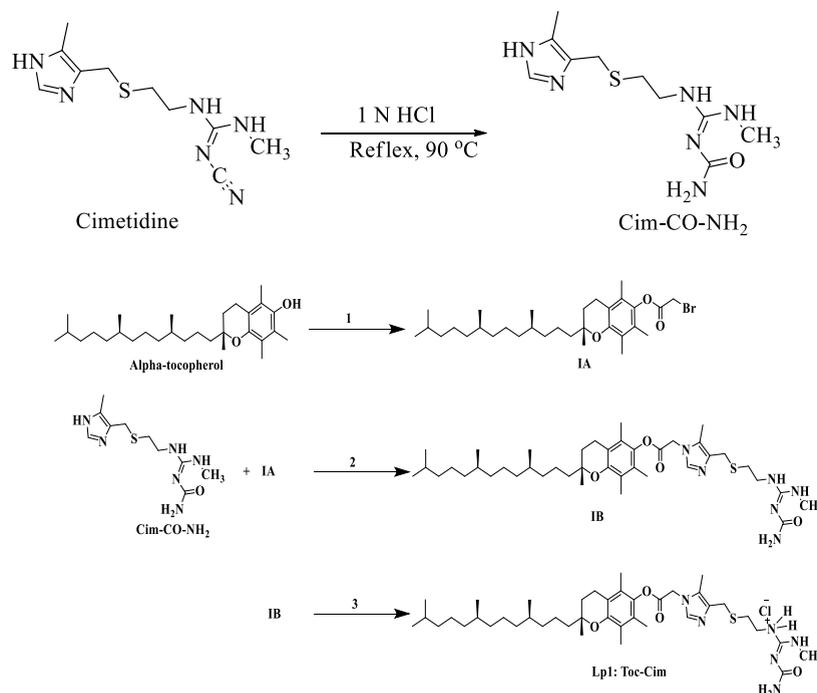
Figure 1. Synthesized cationic lipid's chemical composition.

**Scheme 1. Synthesis Route of Toc-Cim Lipid**[a]

[a]Reagents and conditions: (1) bromo acetyl bromide, pyridine, dichloromethane (DCM), 0 °C, 2 h. (2) Potassium carbonate (K₂CO₃), potassium iodide (KI), dry acetone, room temperature, 2 h. (3) 6 N HCl, methanol, room temperature, 12 h.

linked either by a specific part referred to as a linker or directly with a covalent bond.[10,11] Important attempts were made by various scientists to improve the transfection potential of cationic lipids by designing new molecules through systematic alteration of particular domains, especially the domains that are hydrophilic and hydrophobic.[12−14]

Cimetidine is an antihistamine (H2) and is used to treat peptic ulcers, stomach acidity, acid reflux, and hypersecretory states.[15−17] It has been proven to help patients with colorectal cancer survive better.[18−21] According to a study published in 1988, cimetidine post-operative medication enhanced the prognosis in stomach cancer patients of all stages.[22] Ranitidine and famotidine, two more H2 receptor antagonists, have no

such effects.[23−25] According to these research studies, cimetidine decreased the rapid increasing of human colorectal cancer cells and promoted apoptosis in in vitro.[26,27]

In human cancer, one of the most frequently mutated genes is the tumor suppressor TP53 or p53.[28,29] It generates the p53 protein, which has many antiproliferative properties by regulating the transcription of several target genes and interacting with other proteins. TP53 is a nucleus activator that transactivates various selected genes implicated in cell cycle arrest and/or apoptosis induction.[30−32] In order to source cell cycle arrest and/or apoptosis, an appropriate collection of target genes involved in it are transactivated by functionally active p53, which is proportional to the quantity

Scheme 2. Synthesis Route of Chol-Cim Lipid[a]
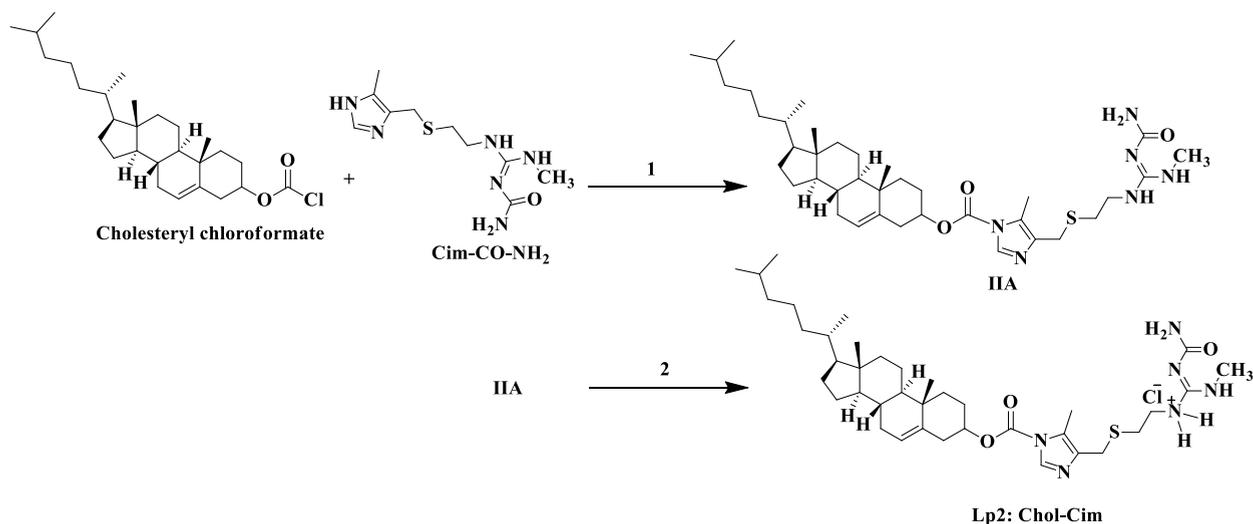
[a]Reagents and conditions: (1) potassium carbonate (K$_2$CO$_3$), potassium iodide (KI), dry acetone, room temperature, 2 h. (2) 6 N HCl, methanol, room temperature, 12 h.

and kind of DNA damage.[33] In reaction to DNA damage, the tumor suppressor protein p53 causes cell cycle arrest or apoptosis and activates both Bcl-2 and Bax. Overall, the combination of these molecules is essential for directing a cell nucleus life and death.[34−37]

We developed two cationic lipids to show how the cimetidine moiety regulates the p53 expression in colon cancer cells. Two cationic lipids were synthesized, each with a different hydrophobic domain: one is alpha-tocopherol, a type of vitamin E,[38] and the other is cholesterol. Many studies have shown that alpha-tocopherol[9,39−41] and cholesterol-related cationic lipids are effective gene delivery agents.[42−44] We used adapted cimetidine as the hydrophilic domain in this analysis after semi-oxidation of its cyanide group to an amide group to avoid the toxicity due to the cyanide group. As a result, cationic lipid toxicity will decrease once they reach the cell. The transfection studies of cationic lipids with a modified cimetidine hydrophilic group and two varying hydrophobic moieties are shown here. There are no reports of using cimetidine as the hydrophilic head group in cationic amphiphile-based non-viral gene delivery.

2. RESULTS AND DISCUSSION

2.1. Chemistry. In this analysis, Toc-Cim and Chol-Cim were synthesized as two mono cationic lipids (Lp1 and Lp2) with varying hydrophobic domains (Figure 1). The hydrophilic moiety, modified cimetidine, is obtained by converting the cyanide group of cimetidine to amide. 1 N hydrochloric acid is used to hydrolyze partially the cyanide group to convert to an amide group. After obtaining cyanide-free cimetidine with a high yield, the hydrophobic domain was linked to the hydrophilic domain by the N-alkylation process. Using the bromo acetyl bromide, in the presence of pyridine, alpha-tocopherol was converted to α-tocopheryl-2-bromoacetate in intermediate **IA** (Scheme 1).[45] In addition, **IB** and **IIA** were prepared by N-alkylation to 1*H*-imidazole (cimetidine) using α-tocopheryl-2-bromoacetate and cholesterol chloroformate (commercially available), respectively, in the presence of base potassium carbonate (K$_2$CO$_3$) and solvent dry acetone. To obtain the final lipid with a quaternized shape, **IB** and **IIA** were

treated with 6 N hydrochloric acid when dry methanol is present (Schemes 1 and 2). As a counter ion, one of the amines in the arginine group has quaternized (Toc-Cim and Chol-Cim). Modified cimetidine (Cim−CO−NH$_2$), intermediate (**IA**), compounds IB and IIA, and target lipids (Toc-Cim and Chol-Cim) were validated through ^{1}H NMR, ^{13}C NMR, and ESI-HRMS spectra (as described in Supporting Information Figures S1−S16). Reverse-phase HPLC analysis was used, and methanol (65%) and water (35%) were mobile phases to confirm the purity of the target lipids Toc-Cim and Chol-Cim. The target lipids were found to be of 100% purity (Figures S17 and S18). Fluorescence spectroscopy of Toc-Cim and Chol-Cim was analyzed with excitation and emission wavelengths (Figures S22 and S23).

Cationic lipids (Toc-Cim and Chol-Cim) can typically self-assemble to create nano-aggregates known as liposomes.[46,47] Usually, liposomes are prepared in an aqueous solution by formulating the cationic lipids with some of the beneficial lipids called DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, cholesterol, and so forth to improve the transfection potentials of the lipids. In this study, the liposomes are prepared by formulating the cationic lipids synthesized using 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) as co-lipid, which is regularly used in gene delivery.[48,49] Small unilamellar liposomes were prepared using the thin-film hydration method with two different molar ratios of synthesized lipid and co-lipids being 1:1 and 2:1. The prepared aqueous dispersions can be stored at 4 °C, and it is observed that up to 3 months, the liposomal solution is clear and homogeneous. The prepared vesicles were designated in terms of hydrodynamic diameter, charge potential, and DNA binding.

2.2. Hydrodynamic Size and Zeta Potentials. For the physicochemical characterization of synthesized cationic lipids (Toc-Cim and Chol-Cim), the nano sizes and charge potentials were measured in an aqueous medium with a dynamic laser light scattering (DLS) instrument. The magnitude of the suspended particle hydrodynamic diameter was calculated using photon correlation spectroscopy. In light of its fusogenic environment and inverted hexagonal phase behavior at low pH, DOPE is taken as a helper lipid as it helps
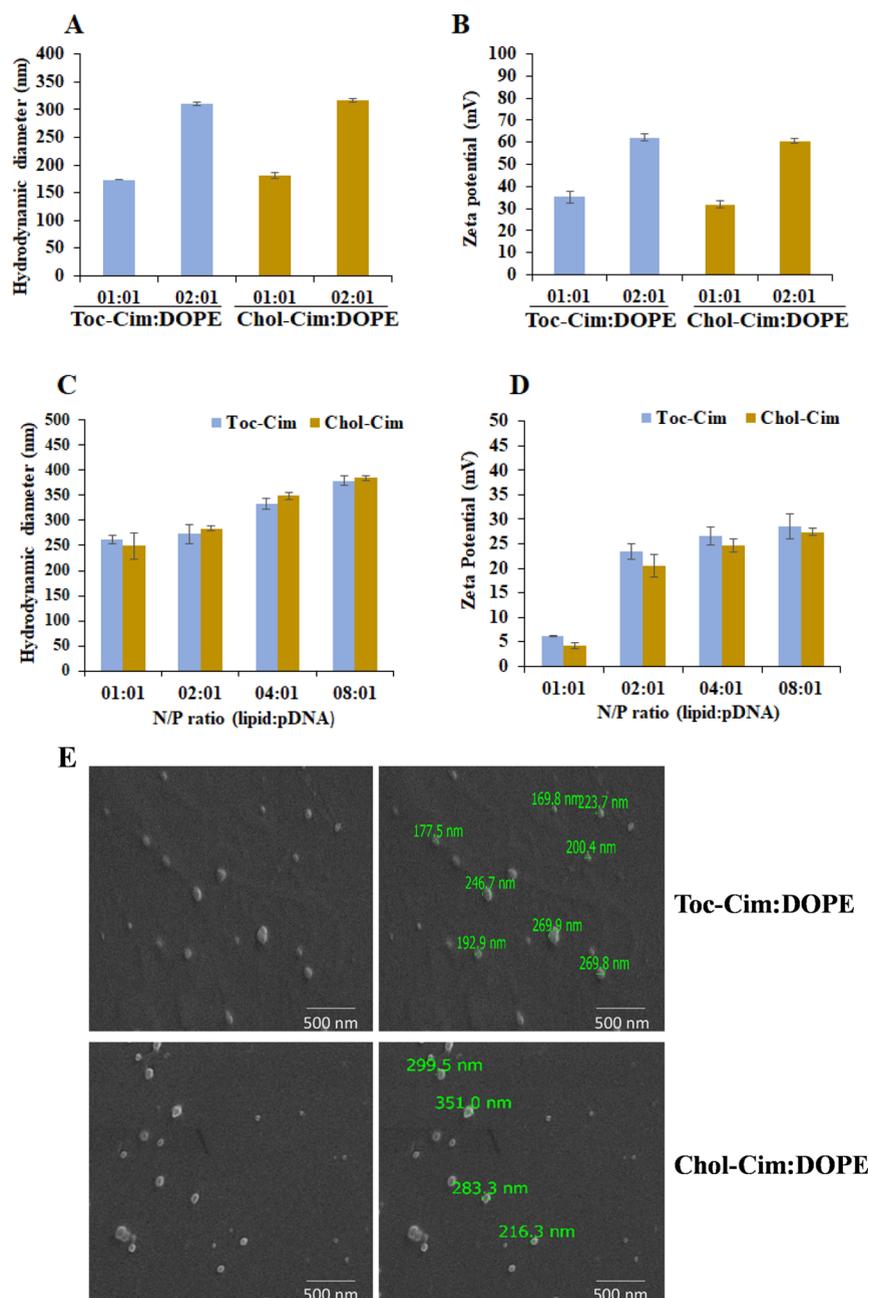
Figure 2. (A) Liposome hydrodynamic diameters (nm) at various lipid/DOPE ratios; (B) liposomes zeta potential at various lipid/DOPE ratios; (C) hydrodynamic diameter (nm) of lipid/pDNA complexes at (1:1−8:1) N/P ratios using 500 ng pCMV-β-gal DNA; (D) zeta-potentials (mV) of lipoplexes made from lipid/pDNA with constant volume of pCMV-β-gal DNA (500 ng) in Milli-Q at various N/P ratios (1:1−8:1); and (E) liposome nano-sizes at a lipid/DOPE molar ratio (1:1) by scanning electron microscopy (scale 500 nm).

breach the endosomal membrane in the wake of the cellular uptake of liposomes into cytoplasm.[50] In prior research, alpha-tocopherol and cholesterol-based cationic lipids utilized DOPE as co-lipids have been shown to be promising gene transfection efficacies.[51,52] The Toc-Cim and Chol-Cim liposome hydrodynamic sizes at a 1:1 M ratio with DOPE were shown to be 173 ± 1 nm and 180 ± 2 nm, respectively. However, at a 2:1 M ratio, hydrodynamic sizes were found to be around 300 nm for both lipids (Figure 2A). The zeta potential which is roughly equal to the transfection capacity is a specification used to dictate the stability of developed lipoplexes with pDNA.[45] The zeta potential of liposomes Toc-Cim and Chol-Cim at a 1:1 M ratio with DOPE was found to be 35 ± 3 mV and 31 ± 2 mV,

respectively, while at a 2:1 M ratio, it was 62 ± 2 and 60 ± 2 mV, respectively (Figure 2B). The molar ratio is 1:1 (lipid/DOPE), where optimal size and zeta potential observed are considered for further experiments. Lipoplexes of various N/P ratios (1:1, 2:1, 4:1, and 8:1) are prepared by complexing respective amounts of liposomes (lipid/DOPE,1:1 M ratio) with pCMV-β-gal DNA. The hydrodynamic diameters and surface charge potential of lipoplexes Toc-Cim:pDNA and Chol-Cim:pDNA (1:1, 2:1, 4:1, and 8:1) were measured. It is observed that with increasing lipid/pDNA N/P ratios, the nano-sizes of lipoplexes prepared were increased in a similar manner for both the lipids. The sizes of lipoplexes of both the lipids at 1:1, 2:1, 4:1, and 8:1 N/P ratios are ranging from
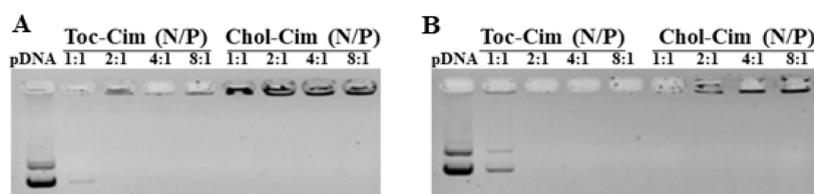
Figure 3. Electrophoretic gel patterns for lipid/pDNA were observed. (A) N/P ratios of Toc-Cim and Chol-Cim to pDNA are shown at the top of each section; (B) with heparin displacement assay for Toc-Cim and Chol-Cim, electrophoretic gel patterns for the lipid/pDNA complex were observed.
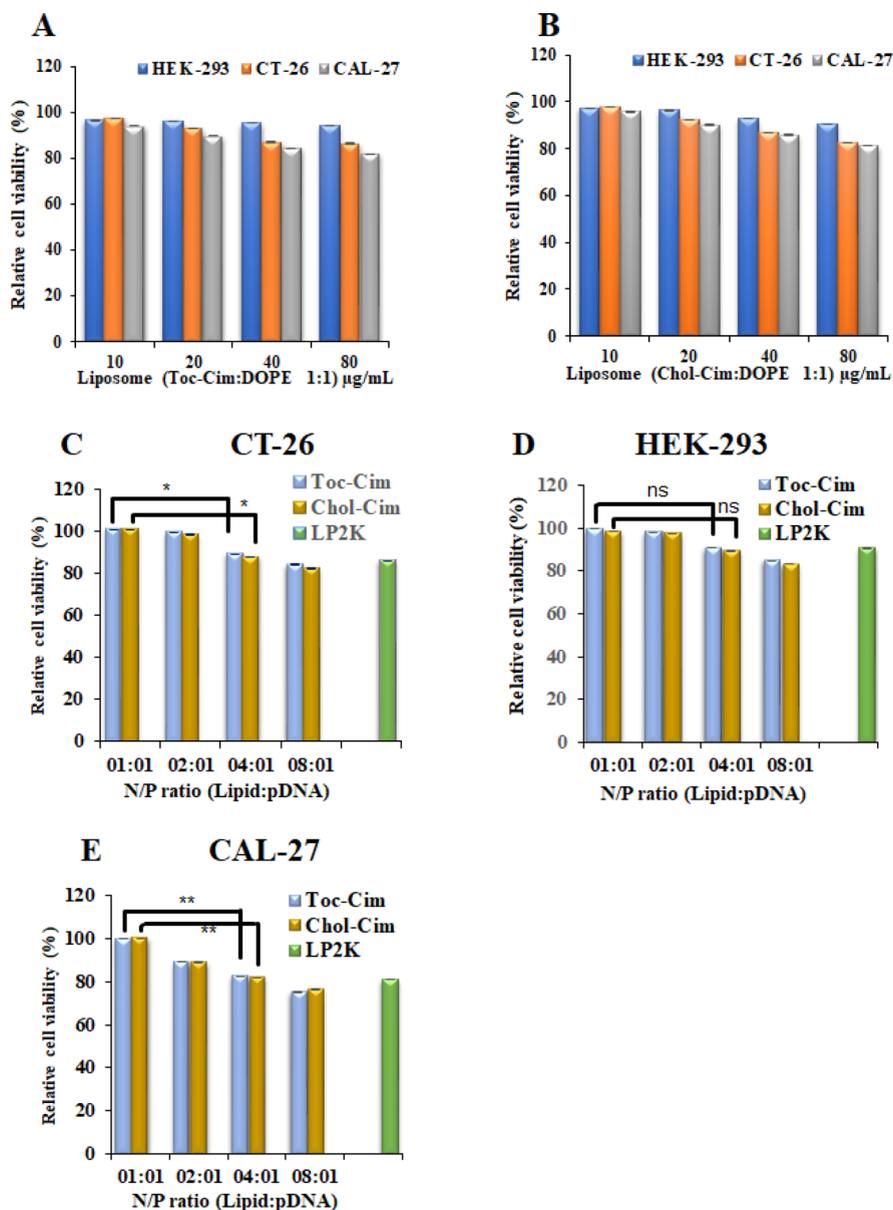

Figure 4. Amount of cell viability determined by an MTT assay. (A) Toc-Cim liposomes, (B) Chol-Cim liposomes at concentrations of 10, 20, 40, and 80 μg/mL in HEK-293, CT-26, and CAL-27 cells; lipoplexes derived from Toc-Cim and Chol-cim with pDNA at various N/P ratios (1:1, 2:1, 4:1, and 8:1) compared with LP2K in (C) CT-26, (D) HEK-293, and (E) CAL-27 cell lines ($n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ns = not significant).

approximately 260−380 nm (Figure 2C). The charge potentials of lipid/pDNA complexes at different N/P ratios is shown in Figure 2D. At an initial N/P ratio 1:1, the lipoplexes of Toc-Cim and Chol-Cim showed the least zeta potential. As the N/P ratios of lipoplexes increased from 2:1 to 8:1, the positive surface charge is increased. Also, the rate of increased zeta potential of both Toc-Cim and Chol-Cim lipoplexes is nearly identical (Figure 2D). Scanning electron microscopy images indicated that the hydrodynamic diameters of the dehydrated liposome vesicles slightly differed from the
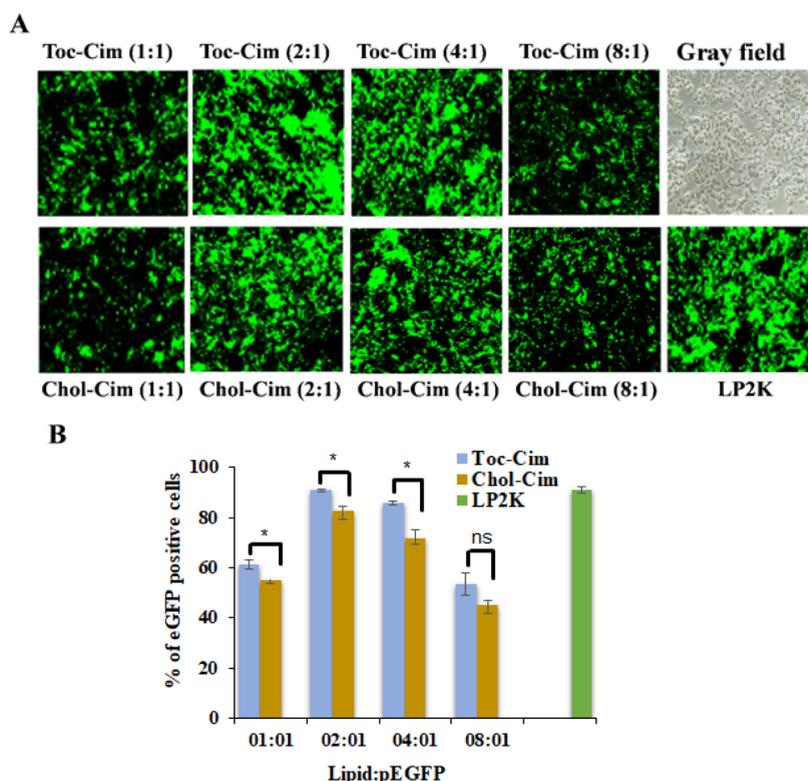
Figure 5. (A) eGFP expression in HEK-293 cell line-treated Toc-Cim:pEGFP and Chol-Cim:pEGFP at N/P ratios 1:1−8:1. For visual comparison, images were collected under a fluorescence microscope after 48 h of transfection. As a positive control, lipofectamine/pEGFP was used; (B) quantitative analysis by flow cytometry ($n = 3$, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and ns = not significant).

sizes of the same liposomes in the liquid form by DLS. The diameters of both liposomes are ranging from 177 to 350 nm (Figure 2E).

2.3. Strength of Lipid/pDNA Binding and Heparin Displacement Assays. The electrostatic interactions between lipids (Toc-Cim and Chol-Cim) and pDNA defined as a result of the lipid/pDNA N/P ratio. Electrophoretic mobility retardation assay was carried out by standard gel electrophoresis.[53] The results of gel electrophoresis experiment showed that 70% pDNA mobility was retarded at a low N/P ratio 1:1 and at higher N/P ratios 2:1 to 8:1. Almost 100% of pDNA mobility was deferred with Toc-Cim:DOPE. In the case of Chol-Cim:DOPE, pDNA retardation was totally initiated even at initial N/P ratios and continued 100% of pDNA mobility retardation at higher N/P ratios (Figure 3A). The electrophoretic mobility of Toc-Cim and Chol-Cim lipoplexes was studied in the presence of an anticoagulant, negatively charged biomolecule, heparin to further investigate their ability to retain the plasmid under systemic conditions. The maximum stability of lipids was determined through monitoring the sensitivity of lipoplexes formed with lipids followed by treatment with heparin.[9] The DNA-binding efficacies of Toc-Cim and Chol-Cim lipoplexes with heparin were in agreement with that of the DNA gel electrophoretic mobility retardation, as shown in Figure 3B.

2.4. Cytotoxicity of Liposomes and Lipoplexes. One of the major disadvantages of synthetic non-viral cationic lipid-based systems is toxicity.[54] To evaluate the synthesized lipid's safety for use in gene delivery, we performed the MTT-based cell viability assay of Toc-Cim and Chol-Cim cationic liposomes and their complexes in three different cell lines: HEK-293, CT-26, and CAL-27. The cell viability is carried out

using the increased concentration of liposomes (lipid/DOPE), namely, 10, 20, 40, and 80 μg/mL and N/P ratios of lipoplexes (lipid:pCMV-β-gal), ranging from 1:1 to 8:1. The results demonstrate that both the lipids have shown similar cell viability in all the cell lines studied. The results also showed that at all the liposomal concentrations and N/P ratios, the synthesized lipids exhibited limited cytotoxicity (more than 80% of cell viability) except at 80 μg/mL liposomal concentration and 8:1 N/P ratio (Figure 4A−E). Lipofect-amine-2000 (LP2K), a commercial transfection reagent, was used as the control. Both the lipid formulations are less cytotoxic than LP2K in all the three cell lines studied except at higher concentrations and N/P ratios (80 μg/mL and 8:1) (Figure 4). The variable transfection efficacy of Toc-Cim and Chol-Cim lipids may not be due to their cytotoxic effects, as evidenced by the cell viability results.

2.5. Transfection Biology: In Vitro Transfection Studies. After establishing low cytotoxicity, Toc-Cim and Chol-Cim-based liposomes were initially tested for transfection effectiveness using pEGFP expression. Flow cytometry was used to quantify eGFP expression in addition to qualitative analysis. The positive control is the commercial formulation LP2K. Toc-Cim and Chol-Cim based liposomes were complexed with pEGFP (plasmid DNA encoding green fluorescent protein) at various N/P ratios of 1:1, 2:1, 4:1, and 8:1. It is observed that at 4:1 and 2:1 lipid/pEGFP N/P ratios, higher eGFP expression of Toc-Cim:pEGFP and Chol-Cim:pEGFP was detected, which could be related to ideal outcomes generated by nano size, DNA binding, and cytotoxicity.[52] Furthermore, in all the three cell lines studied, Toc-Cim:pEGFP exhibited slightly better transfection at a 2:1 N/P ratio and relatively similar activity at a 4:1 N/P ratio
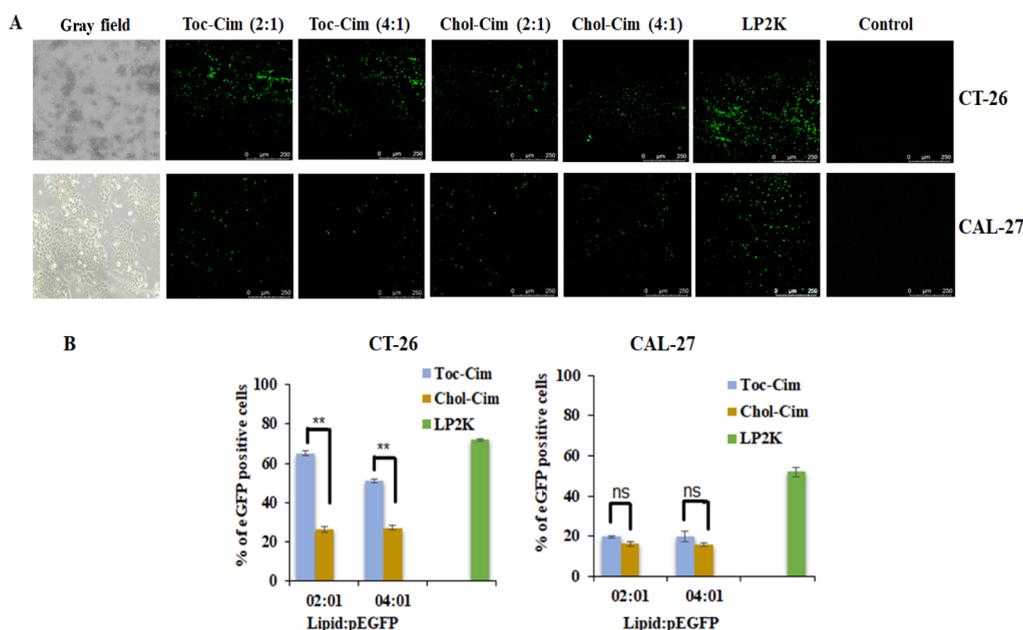
Figure 6. (A) eGFP expression in CT-26 and CAL-27 cancer cell lines treated with Toc-Cim:pEGFP and Chol-Cim:pEGFP with 2:1 and 4:1 N/P ratios. For visual comparison, images were collected under a fluorescence microscope after 48 h of transfection. As a positive control, lipofectamine/pEGFP has been used; (B) quantitative analysis by flow cytometry ($n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ns = not significant).
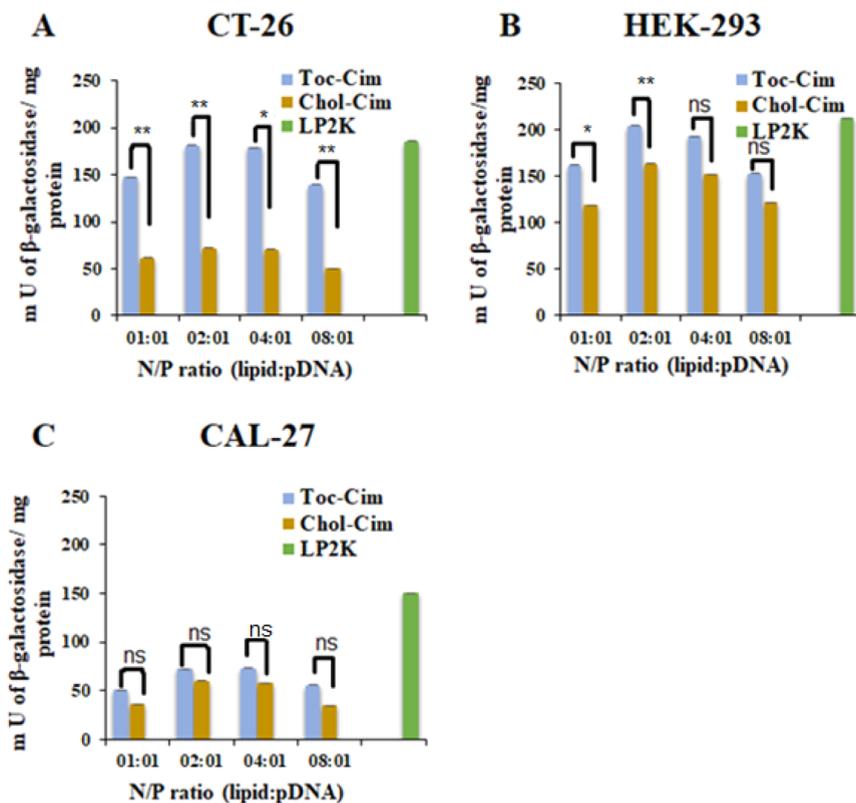
Figure 7. Toc-Cim and Chol-Cim lipids, in vitro gene transfer efficiency in (A) CT-26, (B) HEK-293, and (C) CAL-27 cells. The varied lipid:pCMV-β-gal N/P ratios (1:1−8:1) are plotted against the units of β-galactosidase activity. The lipid transfection efficiencies were compared to lipofectamine:pCMV-β-gal; Toc-Cim:pCMV-β-gal and Chol-Cim:pCMV-β-gal results represented the average of three tests. The standard error is shown by the error bar ($n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ns = not significant).

compared to LP2K in HEK-293 cells (Figure 5A,B). Prior studies have shown that tocopherol hydrophobic-based cationic lipids express higher transfection in human embryonic kidney cell lines.[55] Toc-Cim:pEGFP lipoplexes irrespective of the N/P ratio demonstrated significantly greater eGFP expression than Chol-Cim:pEGFP especially much significant

Figure 8. Rhodamine-PE tagged Toc-Cim:pCMV-β-gal and Chol-Cim:pCMV-β-gal were treated to CT-26 cells. (A) Confocal microscopy images of cells transfected by Toc-Cim and Chol-Cim stained with rhodamine-PE; (B) quantitative analysis of uptake by using flow cytometry ($n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ns = not significant).
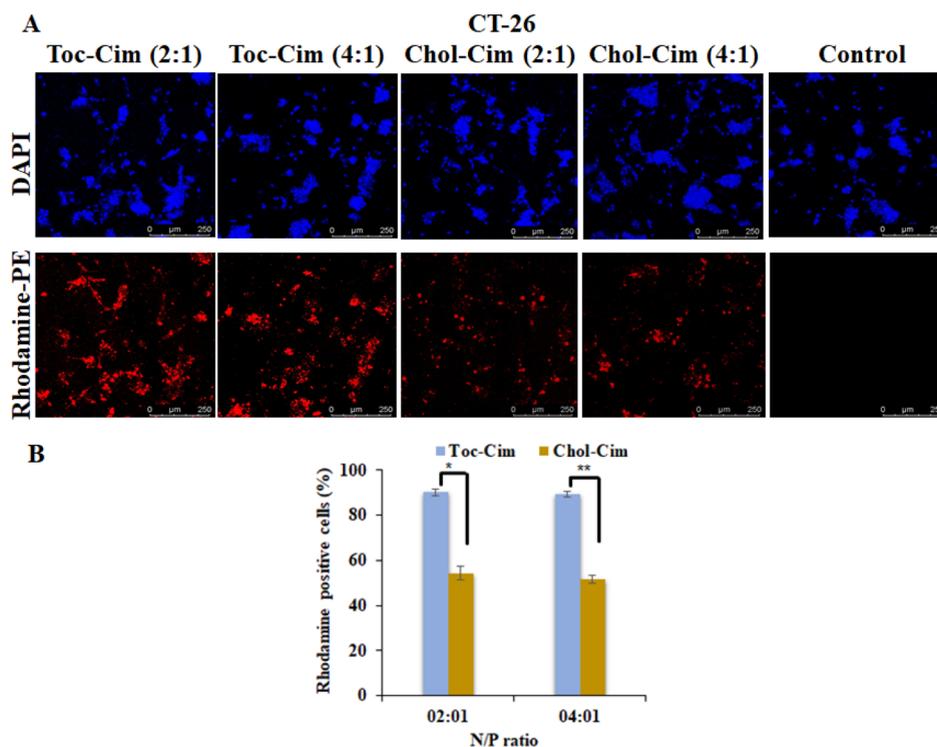
in the case of CT-26 cell lines (Figure 6A,B). Also in CT-26 cell lines at 2:1 (Toc-Cim:pEGFP) N/P ratio, eGFP expression was nearly equal to commercial composition lipofectamine/pEGFP (Figure 6A). The pEGFP transfection results indicate that the inclusion of cimetidine in tocopherol-based cationic lipid helped the transfer of cimetidine and its analogues to colon cancer cells by targeting gene delivery, resulting in significant transfection activity of Toc-Cim in CT-26 cells.[26]

In transient transfection assay, we tested the effectiveness of transfection with a second plasmid vector containing the β-galactosidase reporter gene. The relative in vitro gene delivery effectiveness of Toc-Cim and Chol-Cim lipids was assessed at four formulations in CT-26, HEK-293, and CAL-27 cells at lipid:pCMV-β-gal N/P ratios 1:1 to 8:1 and compared to the commercial formulation lipofectamine:pCMV-β-gal, which was employed as a reference standard. This analysis revealed that at 4:1 and 2:1 N/P ratios, the Toc-Cim:pCMV-β-gal were produced the most efficient as closely to lipofectamine:pCMV-β-gal in the HEK-293 cell line compared to Chol-Cim:pCMV-β-gal. Furthermore, Toc-Cim:pCMV-β-gal at a 2:1 N/P ratio is as effective against the CT-26 cell line as compared to the CAL-27 cell line (Figure 7). This lipid efficiency corresponds to the precise N/P ratios, where lower particle size, higher zeta potential, and maximal binding efficiency are achieved. β-galactosidase assay revealed that the activity pattern of the cimetidine-based formulations is identical to eGFP expression levels. As a result, the comparative examination of gene expression using two alternative plasmid vectors, pEGFP and pCMV-β-gal, complements and reinforces the reported activity profiles trustworthiness.

2.6. Cellular Uptake. From the above transfection results, it is clear that the greatest transfection potential of Toc-Cim

and Chol-Cim was found at 2:1 and 4:1 lipid/pDNA N/P ratios. In order to verify the targetability of Toc-Cim lipid compared to Chol-Cim toward colon cancer, the cellular uptake study is carried out in CT-26 cell lines using both the lipids at their higher transfection N/P ratios. The cellular absorption of rhodamine-PE-labeled complexes was detected under a confocal microscope. It is observed from the confocal images that significantly higher cytoplasmic fluorescence is observed in case of the cells transfected with rhodamine-PE labelled Toc-Cim compared to that of Chol-Cim in CT-26 (Figure 8A). On the other hand, nearly similar cytoplasmic fluorescence is observed for both the lipid formulations in HEK-293 and CAL-27 cell lines (Figures S19A and S20A). The percentages of rhodamine positive cells were quantified using flow cytometry, and quantitative results concur with that of qualitative images (Figures 8B, S19B and S20B). Both these quantitative and qualitative cellular uptake analyses clearly demonstrate the specificity of Toc-Cim liposomes toward the CT-26 cell lines compared to Chol-Cim liposomes. As a result of these observations in the cellular uptake analysis, the superior transfection profiles of the Toc-Cim compared to Chol-Cim in CT-26 arise due to the specific uptake of Toc-Cim by CT-26 cell lines. Therefore, it supports our hypothesis that the tocopherol-based lipids having the cimetidine head group may be useful for targeted colon cancer treatment.

2.7. Serum Compatibility Study. Gene transfection potentials of catalogue relating novel cationic lipids are typically assessed either by without serum or with merely 10% (v/v) serum, as described in several previous studies.[56,57] Unfortunately, serum incompatibility is still one of the primary roadblocks to cationic transfection lipid clinical effectiveness.[58] The adhesion to positively charged cationic liposome sites by negatively charged serum proteins is thought to be the cause of
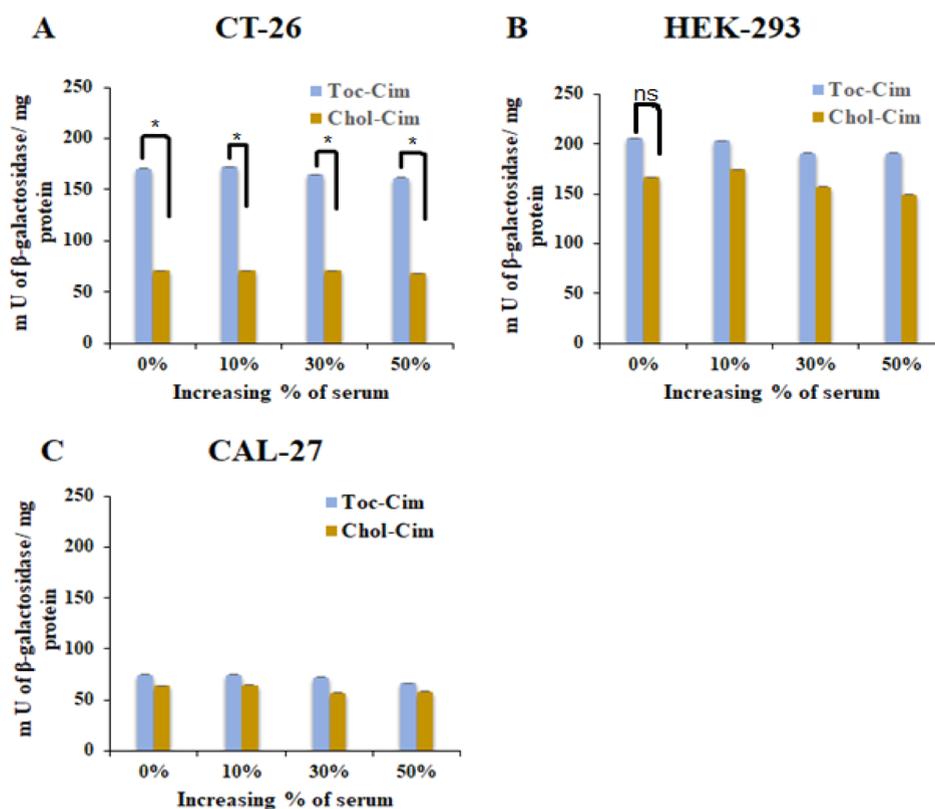
Figure 9. Cationic lipids (Toc-Cim and Chol-Cim) transfection efficacies in the environment of higher serum concentrations. Lipid:pCMV-β-gal complexes prepared reporter gene at a 2:1 N/P ratio against (A) CT-26, (B) HEK-293, and (C) CAL-27 cells. The standard error is shown by the error bar ($n = 3$, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ns = not significant).

cationic transfection lipid's normal serum incompatibility. This makes it difficult for them to engage with the cell surface and/ or internalize them. To acquire the real systemic capability of in vitro transfection effective cationic lipids, the measurement of pDNA transfer potentials across the lipid/pDNA N/P ratios in numerous grown cells with increasing doses of supplemented serum is clearly required. In this approach, extensive serum compatibility analysis for lipids Toc-Cim and Chol-Cim as carried out with increasing quantities of additional serum (10−50%, v/v) in all three cell lines utilized for transfection (CT-26, HEK-293, and CAL-27) across lipid:pCMV-β-gal N/P ratio is 2:1, where both the lipids found highest transfection ability. Up to 50% additional serum, the in vitro transfection potentials of the Toc-Cim:pCMV-β-gal and Chol-Cim:pCMV-β-gal were found to be unaltered, as demonstrated in Figure 9. Across the serum concentrations, both lipids were shown to be the most serum compatible. The improved sheltering of the lipid/pDNA complexes caused by the additional hydrogen bonding of lipid and pDNA due to numerous amine functionalities at their headgroup domains may be related to the extremely serum compatible transfection capabilities of lipid Toc-Cim and Chol-Cim (up to 50%).

2.8. In Vitro TP53 Expression-Induced Cell Apoptosis. The therapeutic potential of Toc-Cim and Chol-Cim complexes was employed to establish their efficacy in eliciting apoptotic activity against CT-26 cells. Toc-Cim:p53 and Chol-Cim:p53 lipoplexes generated at N/P ratios, ranging from 1:1 to 8:1, and an impact of p53 transfection against cell viability was assessed using the MTT test. In case of Toc-Cim:p53 and Chol-Cim:p53 at an optimum 2:1 N/P ratio, cell viability was 65 and 80%, respectively. When CT-26 cells were transfected

with Toc-Cim:pCMV-β-gal and Chol-Cim:pCMV-β-gal, cell viability was 99 and 97%, respectively, at a 2:1 N/P ratio (Figure 10A). A bright-field microscope was use to examine the cellular morphology of CT-26 cells. When CT-26 cells were transfected with p53, shape was abnormal or curved, with a low cell density, relative to cells alone or treated with pCMV-β-gal, regardless of whether Toc-Cim or Chol-Cim or lipofectamine was used as the gene vector (Figure S21). Thus, p53 therapeutic gene is the source of cancer cell death.[59−61] Studies employing a cell line from a myeloid leukemia that expresses a thermal-sensitive constitutively functional mutant of p53 provided the first evidence that p53 can cause apoptotic cell death (i.e., at 37 °C, this protein functions like mutant p53, but at 32 °C, it adopts the form and function of WT p53).[62] Similar investigations, where a thermal-sensitive p53 or WT p53 also forcibly produced in the colon cancer cell line corroborated and expanded the monitoring that p53 can promote apoptosis.[63] The annexin V-FITC/PI dual staining test was used to assess the apoptotic activity of Toc-Cim and Chol-Cim with p53 (Figure 10B). The apoptotic cells percentage was calculated from the number of apoptotic cells labeled with annexin V-FITC in the lower right quadrant (Q_4), and the bar graph (Figure 10C) represents the apoptotic cell percentage associated with the lower right quadrant (Figure 10B). Results show that Toc-Cim:p53 treatment has induced higher level of apoptosis than Chol-cim:p53. This corroborates with our findings from transfection studies.

2.9. Western Blot Analysis. In CT-26 cells, an effect of p53 protein expression is evaluated through western blotting. CT-26 cells were treated with Toc-Cim:p53 and Chol-
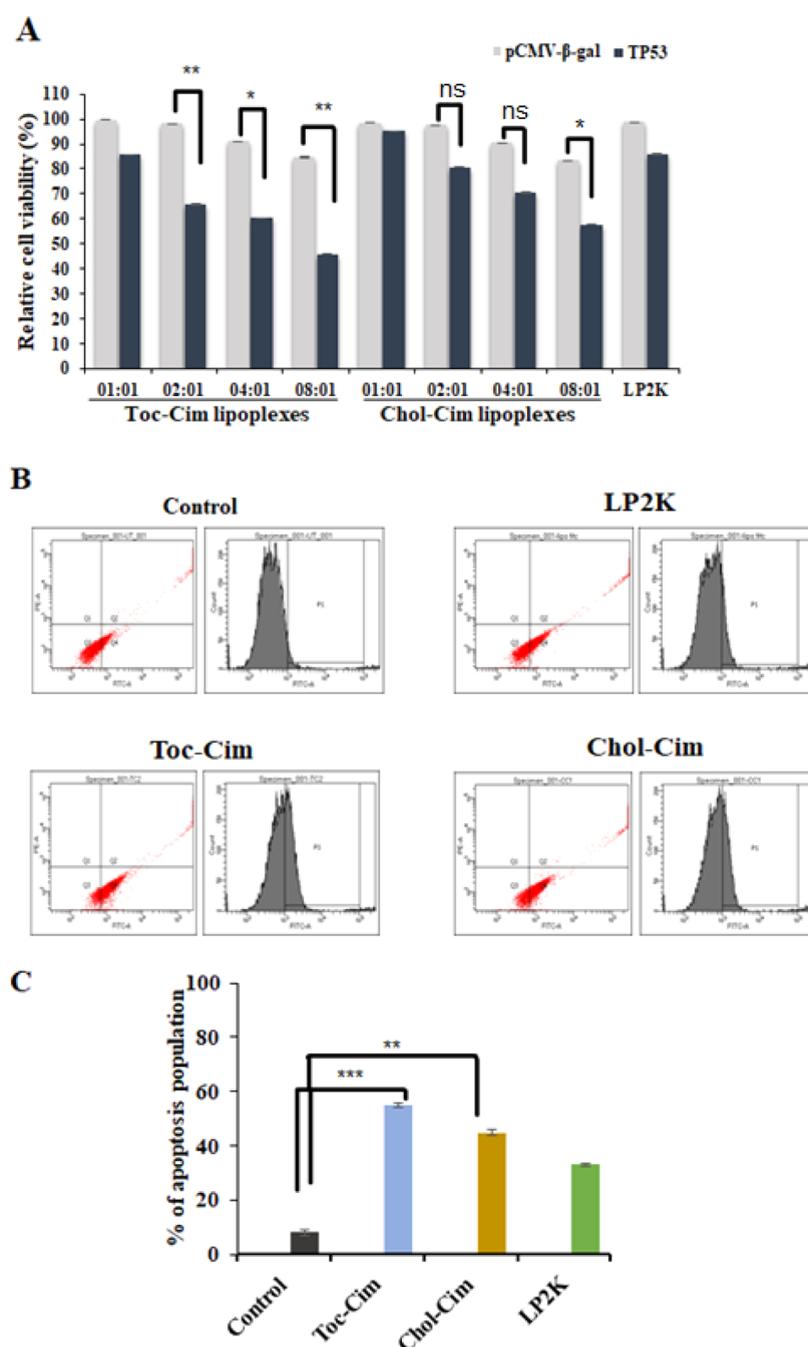
Figure 10. Apoptosis induction in CT-26 cells subjected to Toc-Cim:p53 and Chol-Cim:p53. (A) Cell viability analyzed by MTT assay, LP2K used as a positive control; (B) flow cytometry based study of apoptosis in CT-26 cells exposed to Toc-Cim:p53 and Chol-Cim:p53 using the annexin V-FITC/propidium iodide dual-staining technique; and (C) percentage of apoptotic positive cells (lower right quadrant) made as bar graph ($n = 3$, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; and ns = not significant).

Cim:p53 lipoplexes, and lipofectamine/p53 was used as a positive control. An effect of p53 transfection on protein levels of two apoptosis markers, Bax and Bcl-2, was evaluated. Previous research has linked p53 production to alterations in the levels of Bax and Bcl-2 two important players in the apoptosis signaling pathway.[64,65] In mammalian cells, there are two distinct but eventually convergent routes to apoptosis; the Bcl-2-regulated (also known as intrinsic, mitochondrial, or stress) pathway, which is activated by stress conditions like cytokine deprivation and DNA damage and the death receptor (also known as extrinsic) pathway, which is stimulated by the binding of participants of the tumor necrosis factor receptor

(TNFR) group carrying a subcellular death domain.[66] Previous research on other cancer cell lines has also shown that when p53 is delivered, there is a considerable decrease in the Bcl-2 expression.[67−69] When some additional proteins are not present, p53 activated the proapoptotic protein Bax to permeabilize the mitochondria and initiate the apoptotic process.[34] TP53 transcription-independent promotion of apoptosis needed Bax and entails cytochrome *c* release and caspase activation, all of which takes place in the absence of a nucleus, implying that p53 can initiate the apoptotic program directly from the cytoplasm.[70,71] In line with these facts, our current results suggest that Bcl-2 is an important factor in
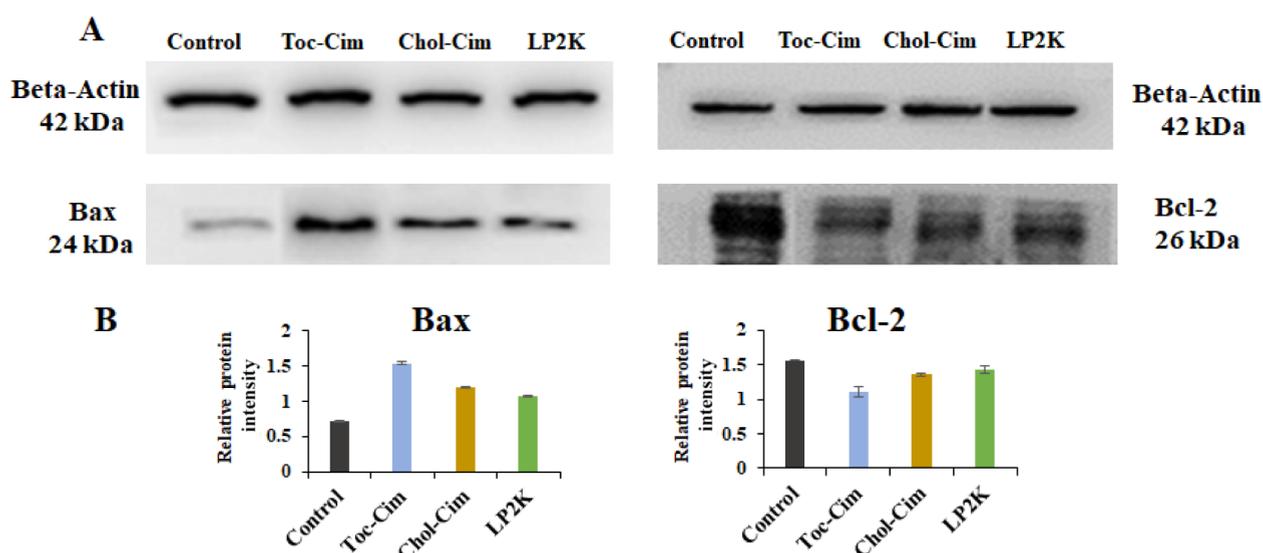
Figure 11. (A) Toc-Cim:p53 and Chol-Cim:p53 were transfected into CT-26 cells, and protein lysates were collected 48 h later. Western blot analysis used to assess the effects of Toc-Cim:p53, Chol-Cim:p53, and lipofectamine/p53 on the apoptosis signaling pathway. The protein levels were normalized using beta-actin; (B) ImageJ software was used to compute protein expression ratios compared to non-treated CT-26 cells from western blot data ($n = 2$).

initiating apoptosis via Bax activation. In comparison to Chol-Cim:p53 and commercial reagent lipofectamine/p53, Toc-Cim:p53-treated cells showed a considerable increase in Bax and a concomitant decrease in the Bcl-2 expression (Figure 11A). These results corroborate with our flow cytometry-based study of apoptosis, where significant results were obtained in the case of Toc-Cim:p53. Western blot results highlighted that the apoptosis in CT-26 cells caused by the Toc-Cim and Chol-Cim systems is mostly attributable to the supplied p53 therapeutic gene.

3. CONCLUSIONS

Two new cationic amphiphiles were synthesized by antihistamine receptor cimetidine conjugated with alpha-tocopherol or cholesterol. Storage durable co-liposomes have optimal nano-size and charge potential for transfection were obtained by combining cationic lipids to fusogenic helper lipid, DOPE in liposomal formulations. After effective DNA binding with liposomes, positively charged nanosized complexes were generated. In various cell lines, all formulations as liposomes and lipoplexes paired with pEGFP or pCMV-β-gal showed good cell viability, indicating that nano vectors are harmless for delivery systems. Transfection rates were adjusted using pEGFP or pCMV-β-gal with multiple N/P ratios, and qualitative and quantitative measurements were made using fluorescence microscopy and flow cytometry, respectively. Tocopherol-based Toc-Cim lipoplexes achieved significantly high internalization in CT-26 colon cancer cells, according to transfection and cellular uptake tests. Both lipids are serum compatible even at higher serum concentrations. The therapeutic gene p53 was also complexed and delivered into CT-26 cancer cell lines using Toc-Cim and Chol-Cim liposomes. The initial transfection results were backed up by the fact that apoptotic activity via p53-mediated cytotoxicity was much higher in CT-26 cells. Annexin V-FITC/PI dual labeling experiments confirmed that Toc-Cim:p53 and Chol-Cim:p53 lipoplexes induced apoptosis in CT-26 cells. Western blot studies have proven that p53 binding to Bcl-2 and Bax activates the apoptotic pathway. The findings show that the

Toc-Cim:p53 and Chol-Cim:p53 complexes preferentially penetrated colon cancer cells, significant with Toc-Cim:p53 and causing cell death after cellular p53 delivery. We believe that Toc-Cim:p53 bio macromolecular assembly have capability as an effective and defended non-viral therapeutic candidates for colon cancer gene therapy and the biocompatible nanocarrier system.

4. EXPERIMENTAL SECTION

4.1. Synthesis. The procedures presented in Schemes 1 and 2 were used to synthesize two cationic amphiphiles. To characterize the final lipids and their precursor intermediates, 400 MHz ^{1}H NMR and 126 MHz ^{13}C NMR and mass spectrometry were used (Supporting Information).

4.1.1. Synthesis of Cim−CO−NH₂ from Cimetidine. To a powdered form of cimetidine (4.0 g, 15.86 mmol), 10 mL of 1 N HCl was added and put for stirring about 12 h at reflex conditions. After consumption of the starting materials, the obtained reaction mixture was dissolved in 120 mL of ethyl acetate and washed twice with (2× 100 mL) water in sodium bicarbonate to remove any excess HCl. The organic portion separated and dried with sodium sulphate (Na_2SO_4) and evaporated the solvent on a vacuum evaporator. A white solid was obtained without impurities. Hence, it is used for the next steps without any further purification. Yield: 3.8 g, 12.24 mmol (95%); ^{1}H NMR (solvent DMSO-d_6) [δ/ppm]: 11.79 (s, 1H, imidazole N−H), 7.46 (s, 1H, imidazole=C−H), 7.14 (s, 2H, O=C−NH₂), 3.64 (s, 2H, imidazole−CH₂−S−), 3.33−3.28 (m, 2H, −CH₂−NH−), 2.70 (d, J = 4.8 Hz, 3H, −NH−CH₃), 2.56 (t, J = 7.6 Hz, 2H, −S−CH₂), 2.13 (s, 3H, imidazole−CH₃); ^{13}C{^{1}H} NMR (Solvent DMSO) [δ/ppm]: 160.41, 149.87, 133.84, 130.32, 125.44, 31.13, 30.43, 28.70, 26.25, 10.17; ESI-HRMS m/z: calcd 270.1263; found, 271.1343 [M + H]$^+$.

4.1.2. Synthesis of Toc-Cim Lp1, Scheme 1. 4.1.2.1. Synthesis of Intermediate α-Tocopheryl 2-Bromoacetate (IA). In 5 mL of dichloromethane, 2-bromoacetyl bromide (0.42 g, 2.08 mmol) was applied gradually for about 10 min to the cooled composition of alpha-tocopherol (1.5 g, 3.48 mmol) in

pyridine (0.41 g, 5.10 mmol). The reaction mixture was allowed to stir at 0 °C for 3 h. Then, the content was diluted by the transfer with 1 N HCl (50 mL) in a separating funnel and extracted three times (3× 20 mL) into dichloromethane. Then, fractions were washed through saturated NaHCO$_3$ (2× 30 mL), water (30 mL), and brine (30 mL), and the organic portion was isolated, water droplets were removed using anhydrous Na$_2$SO$_4$, followed by deletion of the solvent under vacuum and purification of the residue using petroleum ether as the eluent by column chromatography. Product: pale yellow oily liquid, yield: 1.3 g, 2.36 mmol (86.66%); ^{1}H NMR (solvent CDCl$_3$) [δ/ppm]: 4.18 (s, 2H, O−CO−CH$_2$−Br), 2.61 (t, J = 6.8 Hz, 2H, −CH$_2$−tocopheryl), 2.15 (d, J = 18.8 Hz, 9H, (CH$_3$)$_3$−tocopheryl), 1.81−1.72 (m, 2H, −CH$_2$−tocopheryl), 1.60−1.05 (m, 24H, tocopheryl−chain), 0.88−0.83 (m, 12H, (CH$_3$)$_4$ tocopheryl); ^{13}C{^{1}H} NMR (solvent CDCl$_3$) [δ/ppm]: 149.77, 140.17, 126.55, 124.86, 123.29, 117.58, 75.20, 39.39, 37.47, 37.41, 32.80, 32.70, 29.71, 27.99, 25.08, 24.82, 24.46, 22.73, 22.64, 21.03, 20.58, 19.76, 19.70, 12.86, 12.01, 11.82; ESI-MS m/z: calcd 550.3022; found, 551.3087 [M + H]$^+$.

4.1.2.2. Synthesis of α-Tocopheryl 2-(Cimetidine)-acetate (IB) and Toc-Cim (Lp1). Alpha-tocopheryl-2-bromoacetate (5.45 g, 9.90 mmol), potassium carbonate (0.68 g, 4.92 mmol), and catalytic amount of potassium iodide were added to cimetidine (2.5 g, 9.90 mmol) in 3 mL of acetone and kept for stirring at normal temperature. After 2 h, the reaction progress was monitored by TLC. Then, the resultant reaction mixture was diluted in 100 mL of ethyl acetate and washed twice with 120 mL of water after the starting materials were consumed. Then, the organic layer was collected and dried with sodium sulfate, and the solvent was concentrated in a rotary evaporator. The resulting crude was purified by column chromatography with 60−120 mesh silica gel using methanol in chloroform as the eluent (R_f = 0.5, 2% methanol/chloroform), followed by quaternization with using anhydrous HCl, obtaining the final compound Lp1. Product: light yellow oily liquid, yield: 1.80 g, 2.49 mmol (72%).

α-Tocopheryl 2-(cimetidine)-acetate (IB): ^{1}H NMR (solvent CDCl$_3$) [δ/ppm]: 7.97 (s, 1H, imidazole═C−H), 5.01 (s, 2H, O−CO−CH$_2$−N−), 3.66 (s, 2H, imidazole−CH$_2$−S−), 3.37 (dd, J = 14.0, 6.4 Hz, 2H, −CH$_2$−NH−), 2.80 (d, J = 4.4 Hz, 3H, −NH−CH$_3$), 2.62 (t, J = 7.6 Hz, 2H, −CH$_2$ tocopheryl), 2.51 (t, J = 6.4 Hz, 2H, −S−CH$_2$), 2.20 (s, 3H, imidazole−CH$_3$), 2.09 (s, 1H, CH$_2$−NH−C═), 2.04 (s, 1H, ═C−NH−CH$_3$), 1.90 (m, 9H, (CH$_3$)$_3$−tocopheryl), 1.72−1.60 (m, 2H, −CH$_2$−tocopheryl), 1.48−1.10 (m, 24H, tocopheryl−chain), 0.81−0.77 (m, 12H, (CH$_3$)$_4$ tocopheryl); ^{13}C{^{1}H} NMR (solvent CDCl$_3$) [δ/ppm]: 169.65, 159.47, 148.98, 142.21, 140.78, 138.83, 128.87, 127.33, 126.47, 125.04, 123.42, 122.51, 74.33, 45.64, 40.34, 38.34, 36.52, 36.42, 36.37, 36.26, 31.76, 31.68, 28.69, 27.58, 26.96, 24.79, 23.79, 23.42, 21.71, 21.61, 19.99, 19.56, 18.73, 18.66, 13.11, 12.01, 11.17, 10.82, 7.23.

Toc-Cim (Lp1): ^{1}H NMR (solvent CDCl$_3$) [δ/ppm]: 7.82 (s, 1H, imidazole═C−H), 6.42 (s, 1H, CH$_2$−NH−C═), 5.03 (s, 2H, O−CO−CH$_2$−N−), 3.99 (s, 1H, ═C−NH−CH$_3$), 3.71 (s, 2H, imidazole−CH$_2$−S−), 3.43 (dd, J = 14.0, 6.4 Hz, 2H, −CH$_2$−NH−), 2.87 (d, J = 4.4 Hz, 3H, −NH−CH$_3$), 2.67 (t, J = 7.6 Hz, 2H, −CH$_2$ tocopheryl), 2.59 (t, J = 6.4 Hz, 2H, −S−CH$_2$), 2.26 (s, 3H, imidazole−CH$_3$), 2.09−1.95 (m, 9H, (CH$_3$)$_3$−tocopheryl), 1.82−1.74 (m, 2H, −CH$_2$−tocopheryl), 1.56−1.05 (m, 24H, tocopheryl-chain), 0.87−

0.84 (m, 12H, (CH$_3$)$_4$ tocopheryl); ^{13}C{^{1}H} NMR (solvent CDCl$_3$) [δ/ppm]: 168.99, 156.41, 149.73, 145.58, 144.59, 143.81, 127.76, 127.13, 125.13, 122.63, 121.13, 120.02, 74.55, 47.14, 39.93, 39.85, 39.42, 37.61, 37.51, 37.44, 37.34, 32.83, 32.74, 31.60, 31.54, 28.03, 24.86, 24.49, 23.83, 22.78, 22.68, 21.10, 20.81, 19.80, 19.74, 19.71, 19.65, 12.26, 11.87, 11.83, 11.32; ESI-HRMS m/z: calcd 741.5096; found, 741.5077 [M]$^+$.

4.1.3. Synthesis of Chol-Cim Lp2, Scheme 2. 4.1.3.1. Synthesis of Cholesteryl 1-(Cimetidine)-formate (IIA) and Chol-Cim (Lp2). To the solution of cimetidine (2.5 g, 9.90 mmol) in 3 mL of acetone, cholesterol chloroformate (4.44 g, 9.90 mmol), potassium carbonate (0.68 g, 4.92 mmol), and catalytic amount of potassium iodide were added and kept for stirring at room temperature. Upon the consumption of starting materials, the reaction mixture is dissolved in 100 mL of ethyl acetate and washed twice with 120 mL of water. The organic layer was separated and dried on sodium sulfate, and the solvent was evaporated on a vacuum evaporator. Then, crude was purified by column chromatography with 60−120 mesh silica gel using methanol in chloroform as the eluent (R_f = 0.5, 2% methanol/chloroform), followed by quaternization with anhydrous HCl, affording the title compound Lp2. Product: white solid, yield: 1.90 g, 2.85 mmol (76%).

Cholesteryl 1-(cimetidine)-formate (IIA): ^{1}H NMR (solvent CDCl$_3$) [δ/ppm] 8.05 (s, 1H, imidazole═C−H), 5.45 (d, J = 4.0 Hz, 1H, cholesteryl═C−H), 4.87−4.81 (m, 1H, imidazole═C−H), 3.64 (s, 2H, imidazole−CH$_2$−S−), 3.53 (dd, J = 12.4, 6.0 Hz, 2H, −CH$_2$−NH−), 2.93 (d, J = 4.4 Hz, 3H, −NH−CH$_3$), 2.88 (t, J = 6.8 Hz, 2H, −S−CH$_2$), 2.52 (d, J = 7.6 Hz, 2H, −CH$_2$−cholesteryl), 2.42 (s, 3H, imidazole−CH$_3$), 2.33 (s, 1H, CH$_2$−NH−C═), 2.25 (s, 1H, ═C−NH−CH$_3$), 2.06−1.74 (m, 6H, cholesteryl), 1.62−0.85 (m, 32H, cholesteryl), 0.69 (t, J = 2.4 Hz, 3H, cholesteryl); ^{13}C{^{1}H} NMR (solvent CDCl$_3$) [δ/ppm]: 160.59, 154.28, 148.44, 138.56, 137.29, 136.36, 123.84, 121.72, 78.91, 56.77, 56.66, 56.14, 49.96, 42.32, 39.68, 39.52, 37.95, 36.80, 36.56, 36.19, 35.79, 31.91, 31.82, 28.50, 28.23, 28.02, 27.72, 24.29, 23.84, 22.83, 22.57, 21.05, 19.32, 18.72, 11.87, 10.85.

Chol-Cim (Lp2): ^{1}H NMR (solvent CDCl$_3$) [δ/ppm]: 8.15 (s, 1H, imidazole═C−H), 6.46 (s, 1H, CH$_2$−NH−C═), 5.46 (d, J = 4.0 Hz, 1H, cholesteryl═C−H), 4.88−4.79 (m, 1H, H−C−O−C═O), 3.66 (s, 2H, imidazole−CH$_2$−S−), 3.51 (dd, J = 12.4, 6.0 Hz, 2H, −CH$_2$−NH−), 3.10 (s, 1H, ═C−NH−CH$_3$), 2.92 (d, J = 4.4 Hz, 3H, −NH−CH$_3$), 2.72 (t, J = 6.8 Hz, 2H, −S−CH$_2$), 2.50 (d, J = 7.6 Hz, 2H, −CH$_2$−cholesteryl), 2.42 (s, 3H, imidazole−CH$_3$), 2.05−1.76 (m, 6H, cholesteryl), 1.60−0.86 (m, 32H, cholesteryl), 0.69 (s, 3H, cholesteryl); ^{13}C{^{1}H} NMR (solvent CDCl$_3$) [δ/ppm]: 160.60, 155.04, 148.44, 138.57, 137.30, 136.35, 124.92, 123.82, 78.90, 56.66, 56.14, 49.97, 42.32, 41.52, 39.68, 39.52, 37.95, 36.80, 36.57, 36.18, 35.79, 31.91, 31.82, 28.49, 28.22, 28.02, 27.72, 26.48, 24.28, 23.83, 22.83, 22.57, 21.06, 19.32, 18.73, 11.87, 10.84; ESI-HRMS m/z: calcd 683.4677; found, 683.4681 [M]$^+$.

4.2. Transfection Biology. An in vitro transfection study was performed in HEK-293, CT-26, and CAL-27 cells to examine the transfection abilities of cationic liposomal formulations using a pEGFP-plasmid. A day before the transfection, a 24-well plate was seeded with the corresponding cells at a density of 5×10^4 cells per well. The lipoplexes were prepared with pEGFP-plasmid (0.9 μg per well) at four various N/P ratios, from 1:1 to 8:1 in serum-free medium and

incubated about 30 min. Following that, the lipoplexes were developed into the final transfection complex and treated to the cells. The culture plate was placed in a CO_2 incubator (37 °C) for 4 h. Then, complex medium was replaced with complete medium, and the incubation period was extended to 48 h. LP2K (commercially available formulation) was used as the positive control. The complete medium was withdrawn from each well after 48 h of incubation, and the cells were washed with 1× PBS (2 × 200 μL). Finally, 200 μL of 1× PBS was added to each well, and the cells producing the green fluorescent protein were observed using an inverted fluorescence microscope. Flow cytometry was used to perform fluorescence-activated cell sorting (FACS) analysis for quantification. By adding 100 μL of 0.1% trypsin/EDTA led the way to detach the cells, then added 400 μL of complete medium and allowed us to centrifuge at 4 °C to form the cell pellet. Then, the cell pellet was resuspended in 500 μL of cold PBS. Without delay, cells were analyzed using a FACS caliber system at 488 nm for excitation and at 530 nm for detection. Total 500 μL of PBS containing cells was analyzed for each sample using the BD FACSAria III Cell Sorter (BD Biosciences).

In addition, β-galactosidase assay performed in the same cells to endorse the transfection patterns obtained from pEGFP DNA. A day before transfection, cell lines were seeded in separate 96-well plates at a density of 10,000 cells per well. At the time of treatment, lipoplexes were made using cationic liposomes and pCMV-β-gal DNA (0.3 μg per well) at four different N/P ratios, ranging from 1:1 to 8:1 in serial dilutions with serum-free medium (100 μL) on the day of transfection. The complexes were diluted in serum-containing medium, after 30 min of incubation to form the final transfection complex. Following, complexes were added to the cell line-containing wells and incubated in the CO_2 incubator (37 °C) for around 4 h. The specific medium was exchanged with 10% serum medium (0.2 mL per well), and the incubation was continued for about 48 h. Then, analysis was assayed according to the following protocol. The cells were washed twice with 1× PBS (2× 100 μL each) and lysed in 50 μL of lysis buffer [0.25 M Tris-HCl (pH 8.0) and 0.5% NP-40]. The process was carefully monitored to achieve complete lysis. By adding 50 μL of 2×-substrate solution [1.33 mg/mL of o-nitrophenyl-β-D-galactopyranoside (ONPG), 0.2 M sodium phosphate (pH 7.3), and 2 mM magnesium chloride] to the lysate in a 96-well plate, the β-galactosidase activity per well was calculated, using a calibration curve built with pure commercial galactosidase enzyme, and the absorbance of the product ortho-nitrophenol at 420 nm was converted to β-galactosidase units.

4.3. Apoptosis Analysis. In a 12-well plate at a density of 10^5 per well, CT-26 cells were seeded 20 h before treatment of Toc-Cim and Chol-Cim lipids by incubating with p53 therapeutic gene with serum-free RPMI medium. Toc-Cim:p53 and Chol-Cim:p53 were exposed to cells for 4 h at optimized N/P ratios, and medium was changed with complete RPMI medium. Then, the incubation was continued at 37 °C with 5% CO_2 for another 44 h. After the completion of the incubation phase, the medium was removed and washed with 200 μL of 1× PBS. Subsequently, the cells were trypsinized, collected, and resuspended with binding buffer (100 μL) and incubated by adding both Annexin V-FITC (5 μL) and PI dye (1 μL) in the dark place at room temperature for 20 min. Eventually, it was diluted with 400 μL of binding buffer. Then, the p53 transfected CT-26 cells were applied to flow cytometry

analysis (experiment was repeated three times) using a FACS Calibur flow cytometer in combination with the BD FACSAria III Cell Sorter (BD Biosciences, USA).

4.4. Protein Extraction and Western Blot Analysis. Toc-Cim:p53 and Chol-Cim:p53 lipoplexes were transfected to CT-26 cells in a six-well plate with density of 2 × 10^5 per well. After 48 h of incubation, cell lysates were collected from p53 transfected cells, washed twice with 1× PBS, and resuspended with 60 μL of lysis buffer. The resuspension was then collected in Eppendorf vials and centrifuged at 4 °C for 30 min. The superintend cell lysate was obtained and mixed with an equal amount of Laemmli buffer before being heated at 96 °C for 5 min and stored at −20 °C until use. Before loading, the lysate is heated at 53 °C for 10 min and then spinned for 10 s. Samples (15 μL) are loaded and run through the gel with running buffer at 80 V to cross the stacking gel, then the voltage was increased to 100 V and continued the sample running till the end of the gel. After completion of sample run, the gel is transferred to the membrane using the transfer buffer by stirring at 60 V for about 31/2 hours. After transformation of the protein membrane, it was kept for blocking for about 1 h at room temperature with 5% BSA blocking reagent and incubated at 4 °C overnight with primary antibodies. The antibodies are Beta Actin antibody (1:1000) from Cell Signaling Technology, Bax antibody (1:1000) from Protein Research, USA and Bcl-2 antibody (1:1000) from Pierce Protein Research, USA. The membranes are then washed three times for 10 min with PBST (1× PBS + Tween 20). Then, it was incubated with secondary antibodies (Beta Actin-Anti mouse, Bax-Anti Rabbit, Bcl-2-Anti Rabbit) for 1 h at room temperature. Eventually, the membrane was washed three times with PBST 10 min period, after that they were left in PBST. The Chemi-Luminescence (Sigma-Aldrich) western blotting reagent was used to develop the blot and detect chemiluminescent protein signals.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03777.

> Experimental procedure; ^{1}H NMR, ^{13}C NMR, ESI-HRMS spectra; HPLC purity of compounds; cellular uptake qualitative and quantitative analyses in HEK-293 and CAL-27 cells; cytotoxicity of lipids in CT-26 cell visual composition under optical microscopy; and fluorescence spectra of native Toc-Cim and Chol-Cim (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Kumar Pranav Narayan − *Department of Biological Science, Bits Pilani-Hyderabad, Hyderabad, Telangana 500078, India*; Email: pranav@hyderabad.bits-pilani.ac.in

Srilakshmi V. Patri − *Department of Chemistry, National Institute of Technology Warangal, Hanamkonda, Telangana 506004, India*; ⓞ orcid.org/0000-0002-2104-0007; Email: patrisrilaksmi@nitw.ac.in

Authors

Shireesha Manturthi − *Department of Chemistry, National Institute of Technology Warangal, Hanamkonda, Telangana 506004, India*

Dwaipayan Bhattacharya − *Department of Biological Science, Bits Pilani-Hyderabad, Hyderabad, Telangana 500078, India*

Kalyani Rajesh Sakhare − *Department of Biological Science, Bits Pilani-Hyderabad, Hyderabad, Telangana 500078, India*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.2c03777

Notes

The authors declare no competing financial interest.

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