Structure−Function Correlation of Chloroquine and Analogues as Transgene Expression Enhancers in Nonviral Gene Delivery

Jianjun Cheng,[†,‡] Ryan Zeidan,[§] Swaroop Mishra,[§,||] Aijie Liu,[†] Suzie H. Pun,[†,||] Rajan P. Kulkarni,[⊥] Gregory S. Jensen,[†] Nathalie C. Bellocq,[†] and Mark E. Davis*[,§]

Insert Therapeutics, 2585 Nina Street, Pasadena, California 91107, Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, Division of Chemistry and Chemical Engineering, and Division of Biology, California Institute of Technology, Pasadena, California 91125, and Department of Bioengineering, University of Washington, Seattle, Washington 98195

Received June 19, 2006

To understand how chloroquine (**CQ**) enhances transgene expression in polycation-based, nonviral gene delivery systems, a number of **CQ** analogues with variations in the aliphatic amino side chain or in the aromatic ring are synthesized and investigated. Our studies indicate that the aliphatic amino moiety of **CQ** is essential to provide increased gene expression. Further, the enhancements are more dramatically affected by changes to the aromatic ring and are positively correlated to the strength of intercalation between DNA and the **CQ** analogues. Quinacrine (**QC**), a **CQ** analogue with a fused acridinyl structure that can strongly intercalate DNA, enhances transfection similarly to **CQ** at a concentration 10 times lower, while N^4-(4-pyridinyl)-N^1,N^1-diethyl-1,4-pentanediamine (**CP**), a **CQ** analogue that has a weakly intercalating pyridinyl ring, shows no effect on gene expression. Subtle change on the 7-substituent of the chloroquine aromatic structure can also greatly affect the ability of the **CQ** analogues to enhance transgene expression. Transfection in the presence of N^4-(7-trifluoromethyl-4-quinolinyl)-N^1,N^1-diethyl-1,4-pentanediamin e (**CQ7a**) shows expression efficiency 10 times higher than in the presence of **CQ** at same concentration, while transfection in the presence of N^4-(4-quinolinyl)-N^1,N^1-diethyl-1,4-pentanediamine (**CQ7b**) does not reveal any enhancing effects on expression. Through a number of comparative studies with **CQ** and its analogues, we conclude that there are at least three mechanistic features of **CQ** that lead to the enhancement in gene expression: (i) pH buffering in endocytic vesicles, (ii) displacement of polycations from the nucleic acids in polyplexes, and (iii) alteration of the biophysical properties of the released nucleic acid.

Introduction

The intracellular transport of polycation/DNA complexes (polyplexes) involves their cellular uptake, release from the endocytic pathway into the cytoplasm, and delivery of DNA to the cell nucleus.[1] To achieve transgene expression, polyplexes must overcome several extra- and intracellular barriers. Previous studies suggest that entrapment of polyplexes in the endocytic pathway, and their resulting degradation in lysosomes, represents a major impediment to transfection efficiency.[1−3]

Significant increases in gene expression with many nonviral gene delivery systems are observed when chloroquine (**CQ**) is present during the transfection.[4−8] We have also observed increased transfection efficiency in the presence of **CQ** in our studies using cyclodextrin-containing polycations (CDP) for the delivery of nucleic acids.[9−14] Although **CQ** has been widely used as a transfection enhancement reagent, the mechanisms by which **CQ** enhances gene expression remain unclear.

CQ is a weak base with pK_as of 8.1 and 10.2[15] and is known to buffer the luminal pH of endosomes.[16,17] The buffering activity of **CQ** could improve transfection efficiency by facilitating DNA release from the endocytic pathway (as it may generate swelling and destabilization of endosomes)[18] and/or by inhibiting lysosomal enzyme degradation.[19,20] Such hypotheses are supported by the observation that polyethylenimine (PEI), which is known to buffer the pH in endosomes, does not benefit from the presence of **CQ**.[8,21] Noting that **CQ** interacts with DNA,[22,23] others have suggested that **CQ** may increase the transfection efficiency by facilitating dissociation of DNA from polyplexes.[8]

Here, we test postulates of various mechanisms of **CQ**-induced increases in gene expression by designing several **CQ** analogues (Chart 1) and studying gene transfer in the presence of these compounds. By correlating the chemical structures of **CQ** analogues to their contributions in gene transfer, information on the role of **CQ** is obtained. We use the cyclodextrin-containing polycation developed in our laboratories as the main transfection agent in these studies since it does not provide any cellular toxicity at the conditions employed. However, selected experiments are conducted with other transfection reagents in order to confirm that the conclusions obtained are likely applicable to other transfection media. Although similar structure−property approaches have been previously applied to studying the mechanism of **CQ**'s antimalarial activity,[24−27] this is so far the first structure−property investigation of **CQ** in gene transfer.

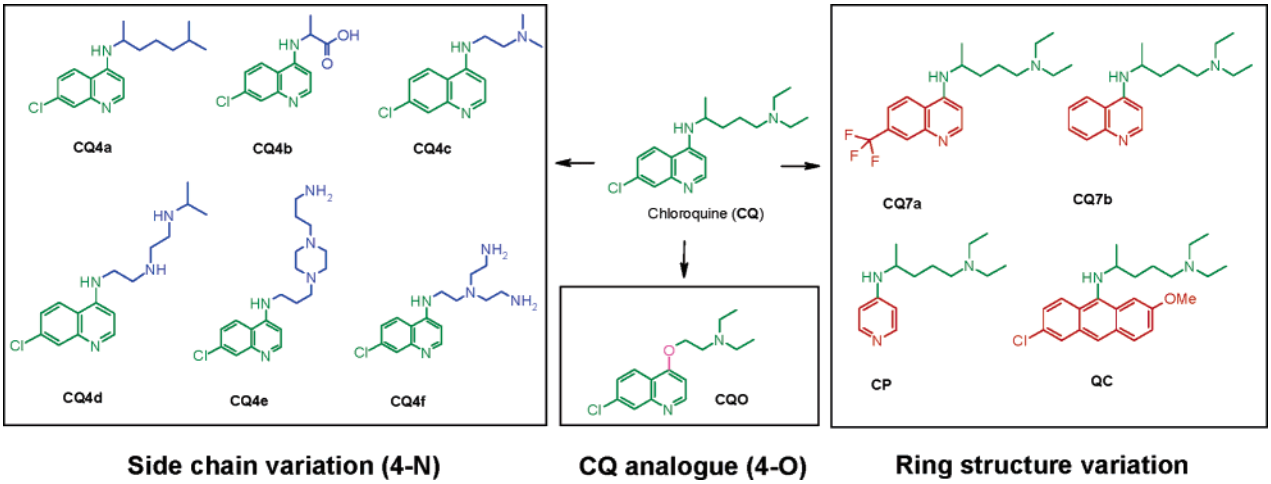
Results

Syntheses of CQ Analogues and Their Evaluation by In Vitro Gene Transfection. Chloroquine (**CQ**) is an analogue of quinoline that has a chloro group at the 7-position and an aliphatic amino side chain at the 4-position (Chart 1). Because of the aromatic ring-structure, **CQ** can intercalate DNA[22,23] and facilitate the unpackaging of DNA from polyplexes.[8] **CQ** is also

* To whom correspondence should be addressed. Phone: (626) 395-4251. Fax: (626) 568-8743. E-mail: mdavis@cheme.caltech.edu.
† Insert Therapeutics.
‡ University of Illinois at Urbana-Champaign.
§ Division of Chemistry and Chemical Engineering, California Institute of Technology.
|| University of Washington.
⊥ Division of Biology, California Institute of Technology.

10.1021/jm060736s CCC: $33.50
Published on Web 10/07/2006

Chart 1. Chemical Structures of Chloroquine and Chloroquine Analogues



widely recognized as an agent buffering endosomal pH[16,17] which may reduce the degradation of polyplexes in acidic endolysosomal environments.[19,20] The pH buffering capability of **CQ** is largely due to its aliphatic amine side chains. To thoroughly investigate the mechanism of action of **CQ** as an enhancing agent in nonviral gene delivery, our strategy was to study a family of **CQ** analogues having either side-chain variations with fixed ring-structure (fixed intercalation capability with variable pH buffering capability) or ring-structure variations with fixed side chain (fixed pH buffering capability with variable intercalation capability). **CQ4a**−**f** and **CQO**, **CQ** analogues with fixed ring structure identical to **CQ**, were readily synthesized in a one-step substitution reaction between 4,7-dichloroquinoline and the corresponding aliphatic amines (see Supporting Information).[28] **CQ** analogues that contain 2-amino-5-diethylaminopentane as the side chain (identical to **CQ**) were prepared to investigate **CQ** analogues with variable 7-substituents on the quinoline ring (**CQ7a**,**b**) or with variable aromatic ring size (**CP**). **CQ7a**,**b** and **CP** were synthesized via the substitution reaction between 2-amino-5-diethylaminopentane and the corresponding 4-chloroquinoline derivatives or 4-bromopyridine, respectively (see Supporting Information).

We utilized a linear, cyclodextrin-based polycation (CDP) (Figure 1) to investigate the gene transfer effects of **CQ** and its analogues. CDP is a new class of nontoxic and nonimmunogenic polycation recently developed for use in nonviral gene delivery.[9−14,29−33] The plasmid pGL3-CV (pDNA), encoding the luciferase gene under the control of the SV40 promoter, was complexed with CDP at a charge ratio of 5 ± to give complete pDNA binding.[10] The polyplexes were added in the presence of **CQ** analogues to cultured HepG2 or HeLa cells. Transfection efficiencies were determined by assaying for luciferase protein activity and reported in relative light units (RLU) per milligram of total cellular protein. The concentrations of **CQ** and various

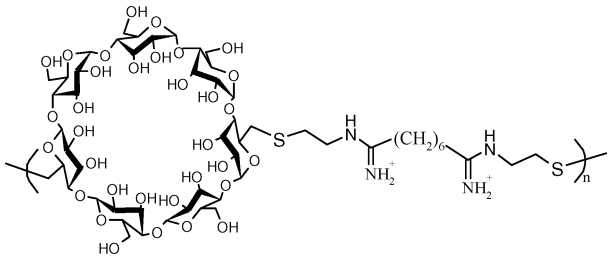


Figure 1. Cyclodextrin polycation (CDP) used as nonviral gene delivery vehicle.

Table 1. Chloroquine and Chloroquine Analogue-Mediated Transfection of CDP/ *p*-DNA in HepG2 and HeLa Cells[a]

CQ analogues (concn, μM)	cell line	RLU/mg protein	cell viability (%)
none	HepG2	4.3×10^6	100
CQ (200)	HepG2	1.6×10^7	60
CQ4a (150)	HepG2	4.7×10^6	89
CQ4b (150)	HepG2	4.3×10^6	84
CQ4c (150)	HepG2	2.5×10^7	64
CQ4d (200)	HepG2	2.2×10^7	74
CQ4e (100)	HepG2	2.0×10^7	74
CQ4f (150)	HepG2	2.5×10^7	83
CQ7a (200)	HepG2	1.7×10^8	61
CQ7b (200)	HepG2	2.7×10^6	100
CP (200)	HepG2	6.4×10^6	85
QC (20)	HepG2	2.5×10^7	57
none	HeLa	1.4×10^5	105
CQ (200)	HeLa	4.3×10^6	82
CQ7a (200)	HeLa	6.7×10^7	83
CP (200)	HeLa	8.2×10^4	101
CQO (200)	HeLa	2.3×10^5	92

[a] HepG2 or HeLa cells were transfected for 4 h with CDP/pGL3-CV polyplexes in the absence or presence of **CQ** or **CQ** analogues. Gene expression was evaluated 48 h later by assaying the luciferase activity (in relative light units, RLU) of cell lysates. The viability of the cells was assessed by measuring the total amount of recovered protein. The **CQ** analogue concentrations represented in the table are those which gave the optimal increase in transfection efficiency. Transfection data with **CQ** or **CQ** analogues at various concentrations using HepG2 cells is provided in Supporting Information.

CQ analogues that resulted in optimal increase in transfection efficiency in HepG2 cells are listed in Table 1. Transfection efficiency increment at other concentrations of **CQ** or **CQ** analogues in HepG2 cells can be found in the Supporting Information.

The observed luciferase activity increases from 4.3×10^6 RLU/mg protein in the absence of **CQ** to 1.6×10^7 RLU/mg protein in the presence of **CQ**. When the tertiary amine side chain of **CQ** was replaced either with a hydrocarbon (**CQ4a**) or with a negatively charged carboxyl group (**CQ4b**), the luciferase activities of transfected cells were roughly the same as that in the absence of **CQ** (Table 1). **CQ4c**, a **CQ** analogue possessing a tertiary amine, enhances transfection to a degree similar to **CQ**. These observations show the importance of the terminal tertiary amine moiety of **CQ** for enhancing polyplex transfection efficiency. However, increasing the basicity on the side chain of **CQ** by increasing the number of amine groups (three amine groups in **CQ4d** and four amine groups in **CQ4e**,**f**) only results in slight increases in maximal luciferase activity

Table 2. Summary of Flow Cytometry Analysis of Polyplex Uptake in the Absence or Presence of **CQ** Analogues

		30 min			2 h		
pDNA or polyplexes	**CQ** or **CQ** analogue	% negativ e	% positiv e	mean fluores cence	% negativ e	% positiv e	mean fluoresce nce
-	-	99.2	0.8	4.1	99.7	0.3	3.45
pDNA	-	99.3	0.7	4.0	98.9	1.1	5.48
polyplexes	-	82.8	17.5	12.9	20.9	79.3	125.7
polyplexes	**CQ**	85.5	11.9	10.0	32.5	67.7	79.3
polyplexes	**CQ7b**	82.2	18.8	15.0	24.7	75.5	88.3
polyplexes	**CQ7a**	85.4	14.8	9.2	35.0	65.2	78.1
polyplexes	**CP**	87.4	12.8	7.6	24.0	76.2	109.0

when compared to **CQ** for HepG2 cells transfected in the presence of those analogues (Table 1).

Dramatic changes in luciferase activity were observed for cells transfected in the presence of **CQ** analogues with variable ring-structures and with side chains identical to **CQ** (Table 1). Replacement of the 7-chloro of **CQ** by a lipophilic trifluoromethyl group (**CQ7a**) results in an order of magnitude increase in luciferase activity (1.6×10^7 RLU/mg protein for **CQ** to 1.7×10^8 RLU/mg protein for **CQ7a**). When the 7-substituent is removed (**CQ7b**), the capacity for enhancing transgene expression is completely lost. Transfection of polyplexes in the presence of **CP**, a **CQ** analogue with a pyridinyl structure, also showed no increase in gene expression. Quinacrine (**QC**), a **CQ** analogue having an acridine ring structure, enhances transfection to a similar degree as **CQ** but at one-tenth the concentration. **QC** is more toxic than **CQ**; a concentration of **QC** higher than 50 μM leads to complete cell death.

To study if there is cell-line dependency in the structure-related gene-expression enhancement capability observed with **CQ** and **CQ** analogues, we also investigated transfections by CDP/pDNA polyplexes in other cells in the presence of **CQ**, **CQ7a**, and **CP**. The variation of expression levels in the presence of these reagents is very similar to that observed in the transfection of HepG2 cells (HeLa cells, Table 1; BHK and A2780 cells, Supporting Information). **CQ7a** gave an order of magnitude increase in luciferase activity (6.7×10^7 RLU/mg protein) compared to **CQ** (4.3×10^6 RLU/mg protein) in HeLa cells (Table 1) as well as in BHK and A2780 cells (see Supporting Information) and gave almost 3 orders of magnitude increase in luciferase activity over **CP** (8.2×10^4 RLU/mg protein) in HeLa cells (Table 1). Interestingly, reducing the overall basicity on **CQ**'s side chain by replacing the aromatic amino group with an oxygen (**CQO**) resulted in an order of magnitude decrease of luciferase activity (2.3×10^5 RLU/mg protein) (Table 1).

To confirm that increases in transfection efficiency were due to interactions among **CQ** analogues, cells, and polyplexes, as opposed to extracellular interactions between **CQ** analogues and unpackaged pDNA, transfection experiments were carried out with pDNA alone (naked pDNA). In the absence of a polycation vector, cells transfected with pDNA in the presence of **CQ** analogues (0.1 mM) did not show significant levels of gene expression. In all cases, luciferase activity was below that observed with polyplexes in the absence of **CQ** analogues (see Supporting Information).

Cellular Uptake Of Polyplexes in the Presence of CQ Analogues. Flow cytometry analysis was used to give a relative measure of pDNA uptake into cells (Table 2). pDNA was labeled with Alexa-fluor-488, condensed into particles with CDP, and exposed to HepG2 cells for 30 min or 2 h in the presence and absence of **CQ** or **CQ** analogues. Cells were then analyzed by fluorescence-activated cell sorting (FACS). Uptake of naked pDNA was minimal, as the mean fluorescence of cells treated with naked pDNA is about the same as that of untreated cells (Table 2). Cells exposed to complexes of labeled pDNA and CDP displayed increases in mean cellular fluorescence, indicating that polyplexes were internalized by cells. Similar observations of internalization have been reported elsewhere.[8,32] At both 30 min and 2 h, cells exposed to polyplexes revealed significant uptake regardless of the presence or absence of **CQ** or **CQ** analogues. 12−19% of cells at 30 min and 65−75% of cells at 2 h contained detectable levels of fluorescently labeled pDNA (% positive, Table 2; FACS plots are provided in Supporting Information). The presence of **CQ** or **CQ** analogues gave slightly reduced uptake relative to the case of polyplexes alone. Also, the fluorescence distribution pattern was very similar at both 30 min and 2 h for cells transfected in the presence and absence of **CQ** or **CQ** analogues. Although cells transfected in the presence of **QC** gave a similar percentage of positive cells and similar mean fluorescence as cells transfected in the presence of other **CQ** analogues, the values may not reflect actual polyplex uptake because **QC** is itself fluorescent at the wavelengths used for the FACS analysis (data not shown).

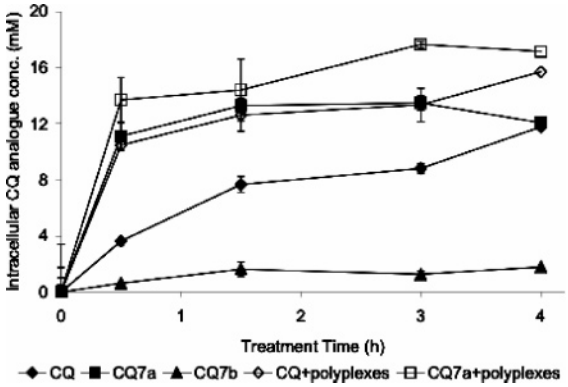


Figure 2. Uptake and intracellular accumulation of **CQ** and **CQ** analogues in HepG2 cells (0.1 mM) in the presence or absence of polyplexes. Fluorescence of cell lysates was used to evaluate the intracellular concentration of **CQ** analogues at various time points after initial exposure.

Uptake and Intracellular Concentration of CQ Analogues. **CQ** can penetrate the cell membrane and accumulate in acidic cellular compartments, especially in lysosomes, in a time-dependent manner.[8] The concentration of **CQ** in HepG2 cells (as measured by its fluorescence in cell lysates) increased as a function of treatment time and reached nearly 12 mM following 4 h of treatment with 100 μM **CQ** (Figure 2). **CQ** accumulated in HepG2 cells with a cell-to-medium concentration ratio of ~100. Relative to **CQ**, **CQ7a** achieved a more rapid intracellular accumulation and reached a concentration of 11 mM in cells only 30 min after exposure to 100 μM **CQ7a** (Figure 2). Beyond this time, the intracellular concentration of **CQ7a** remained steady throughout the 4 h uptake study. The achieved intracellular concentrations of both **CQ** and **CQ7a** noticeably exceeded their concentrations in the surrounding medium.

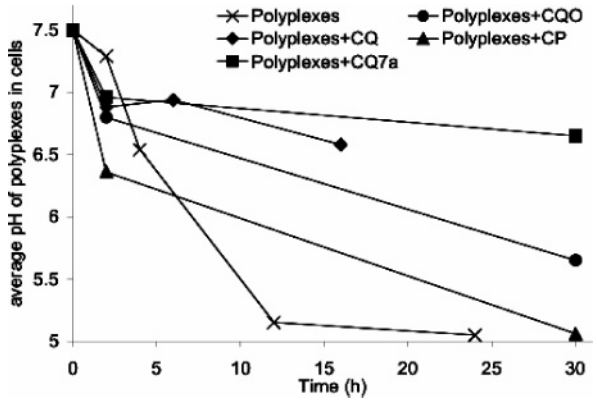


Figure 3. Intracellular buffering activity of **CQ** and **CQ** analogues (0.1 mM) in the transfection of polyplexes containing of CDP and a pH sensitive, SNARF-4F-labeled oligonucleotide in HeLa cells.

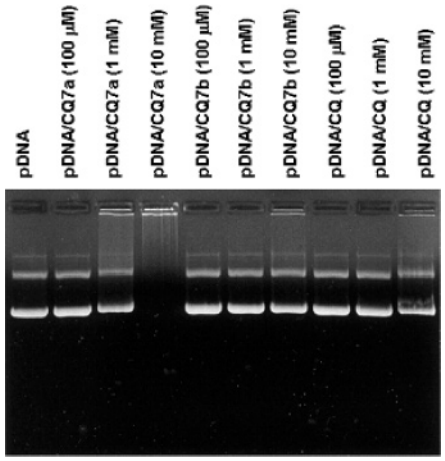


Figure 4. Electrophoretic mobility of pDNA in the presence of **CQ**, **CQ7a**, or **CQ7b** on an agarose gel electrophoresis.

The uptake of **CQ** or **CQ7a** into HepG2 cells was also investigated in the presence of CDP/pDNA polyplexes. Both **CQ** and **CQ7a** exhibited strong uptake; after 4 h, intracellular concentrations were 13−15 mM, slightly higher than their concentrations in the absence of polyplexes (Figure 2). When the substituent at the 7-position of **CQ** was removed, the resulting **CQ** analogue (**CQ7b**) showed a lower tendency to accumulate in cells. The maximum intracellular concentration of **CQ7b** was around 2 mM after 4 h treatment, about five times lower than that of **CQ** or **CQ7a**. These uptake analyses demonstrate that the transmembrane capability of **CQ** and its analogues is closely related to the substituent at the 7-position of the quinoleic ring. The trifluoromethyl group is a strong electron-withdrawing, lipophilic group, and **CQ7a** that contained this group showed fast cell-membrane penetration and intracellular accumulation relative to **CQ**.

The observed concentrations are averages for the entire cell volume. When taken into cells, **CQ** analogues that accept protons at the relevant pH are likely found at much greater concentrations in vesicles of the endocytic pathway. **CQ** is believed to accumulate in such vesicles as a result of its intravesicular protonation[17] and its interactions with vesicle membranes.[34]

One of the motivations of our investigation on the uptake of **CQ** and **CQ** analogues was to elucidate the role of endocytic vesicle buffering by **CQ** in gene transfer. To assist in this study, we synthesized an additional **CQ** analogue, **CQO** (where an oxygen replaces the aniline amino group at the 4 position of chloroquine), in order to examine a compound that has less ability to buffer the pH of endocytic compartments.

To evaluate the actual intracellular buffering activity of **CQO** and other **CQ** analogues, we employed the method of Kulkarni et al.,[35] where polyplexes labeled with the pH-sensitive fluorophore SNARF-4F were administered to cells and used to evaluate the local pH environment of polyplexes within individual cells. The data collected provide a direct measurement of the buffering activity of **CQ** analogues within live cells (Figure 3). Using this technique, **CQ** and **CQ7a** (0.1 mM) displayed strong buffering activity relative to the absence of any **CQ** analogues. **CQO** displayed mild buffering activity, while **CP** exhibited none.

Interaction of CQ Analogues with pDNA. Electrophoretic Mobility of pDNA in the Presence of CQ Analogues. It is known that **CQ** can bind DNA.[22,37] Previous work has indicated that **CQ** exists as a doubly protonated cation in dilute aqueous solutions at physiological pH,[15] and its binding to DNA is related to insertion of its quinoleic moiety into DNA base pairs and to electrostatic interaction between the cationic side chain and the phosphate anions along the DNA backbone.[22] For the examinations of **CQ** analogue interaction with nucleic acids (and/or polyplexes), we focused on a smaller set of molecules for further study: **CQ**, **CQ7a** (shows strong effects on transfection and strong interactions with DNA), and **CP** (not effective in increasing transfection; a single-ring structure that should limit interaction with DNA).

The interaction of pDNA and **CQ** or **CQ** analogues was analyzed by studying the retardation of pDNA movement on a gel in the presence of these small molecules. The pDNA was incubated in the presence of **CQ** analogues at various concentrations and then subjected to gel electrophoresis (Figure 4). The band from pDNA treated with **CQ7a** at 100 μM showed essentially the same pattern as the untreated control, indicating that **CQ7a** had little effect on the mobility of pDNA at this concentration. The pDNA treated with **CQ7a** at a one-order-of-magnitude higher concentration (1 mM) showed slightly retarded movement while the migration of the pDNA was completely prohibited when treated with **CQ7a** at 10 mM (Figure 4). In contrast, **CQ7b** had little effect on pDNA retardation across the same concentration range (0.1−10 mM). **CQ** at 100 μM and 1 mM did not retard pDNA, but did so slightly at 10 mM.

DNA Displacement from Polyplexes by CQ, CQ7a, or CQ7b. Interactions between **CQ** and pDNA could destabilize the association between the plasmid and cationic delivery vectors.[8] To assess the stability of CDP/pDNA polyplexes against **CQ** analogues, solutions of polyplexes were allowed to mix with **CQ** analogues for 30 min and then were filtered to remove intact polyplexes. The unpackaged pDNA in the filtrate was measured by a fluorescence method (Figure 5). **CQ7a** was able to displace 40% of total condensed pDNA at a concentration as low as 500 μM. 40−50% of the pDNA was found in the filtrate for **CQ7a** concentrations of 500 μM to 10mM, and over 70% of pDNA was displaced with a **CQ7a** concentration of 20 mM. **CQ** displayed weaker ability to displace pDNA than **CQ7a**. The pDNA displaced from the polyplexes by **CQ** gradually increased from 10% with 500 μM **CQ** to roughly 50% with 20 mM **CQ**. With **CQ7b** concentrations of 2.5 mM or lower, less than 3% of the pDNA was detected in the filtrate. The displaced pDNA gradually increased from 10% to about 30% when polyplexes were treated with 5 mM to 20 mM of **CQ7b**, respectively (Figure 5).

The comet assay is a standard method of evaluating DNA damage.[36] In this assay, cells are gently lysed, embedded in

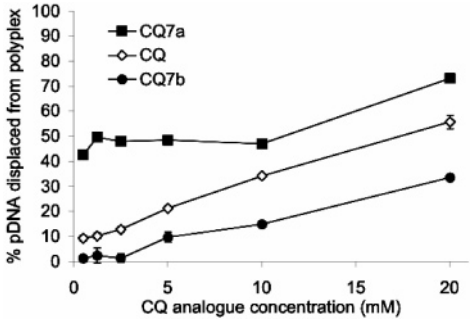


Figure 5. DNA displacement from polyplexes by **CQ**, **CQ7a**, or **CQ7b** after incubating CDP/pDNA polyplexes with various concentrations of **CQ**, **CQ7a**, or **CQ7b** followed by the filtration to remove intact polyplexes and the quantification of the displaced DNA in the filtrate.

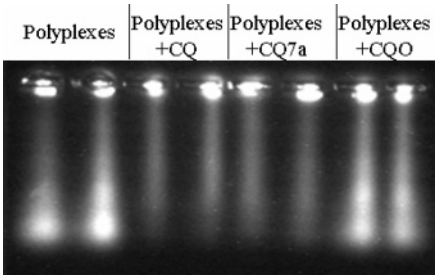


Figure 6. Agarose gel electrophoresis of the FITC-labeled nucleic acid collected from HeLa cells transfected with CDP/FITC−oligonucleotide polyplexes in the presence of **CQ**, **CQ7a**, and **CQO**.

an agarose gel on a glass slide or coverslip, and exposed to electrophoresis. Only damaged cellular DNA is reduced to a size that permits migration within the gel, and this damaged DNA will generate a characteristic "comet" pattern upon subsequent intragel labeling of nucleic acids by a fluorescent marker. The size and fluorescence intensity of the comet reflects the extent of DNA damage. By this method, individual cells may be visually evaluated for DNA damage.

We modified this assay to produce an aggregate separation and visualization of the delivered and unpackaged nucleic acids. Rather than evaluating samples on an individual-cell basis, cells are collected by centrifugation, lysed, embedded in an agarose gel, and subjected to electrophoresis in order to generate a relative measure of DNA unpackaging for a particular sample. We transfected cells using nucleic acids conjugated with a fluorescent marker rather than adding a fluorescent label after electrophoresis. Thus, we are able to distinguish exogenous (delivered) nucleic acids from cellular DNA. The unpacked oligonucleotide measured by this assay is that which migrates through the agarose gel to the same extent as in the case of direct gel loading of the oligonucleotide.

HeLa cells were transfected in the presence of **CQ** analogues and examined by the modified comet assay (Figure 6). Unlike the behavior of **CQ** and **CQ7a** in displacing pDNA from polyplexes (Figure 4), samples transfected with these **CQ** analogues generate less unpackaged (migrating) DNA than samples transfected without **CQ** analogues or samples transfected in the presence of **CQO**.

PicoGreen experiences significant fluorescence enhancement upon intercalation in nucleic acids, such that its exclusion indicates inaccessibility of the nucleic acids to intercalation.[37] This characteristic was used to evaluate the relative accessibility of pDNA in the presence of **CQ** analogues. Polyplexes were incubated with various concentrations of **CQ** analogues, combined with solutions of PicoGreen, and evaluated for fluorescence intensity (Figure 7). **CQ7a**, **CQ**, and **CQO** demonstrated

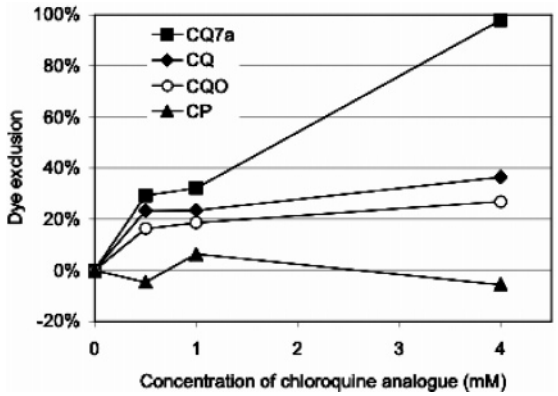


Figure 7. PicoGreen dye exclusion from pDNA in the presence of **CQ** analogues.

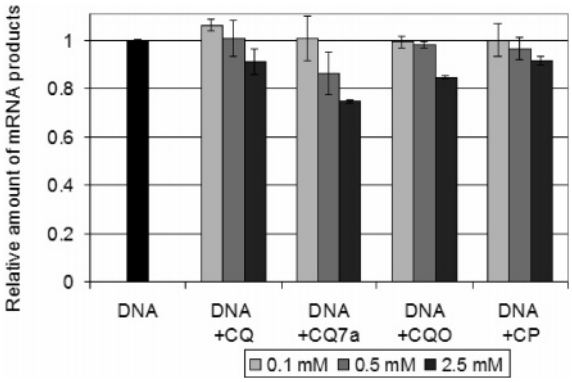


Figure 8. In vitro transcription in the presence of **CQ** analogues. Solutions of pDNA and **CQ** analogues were subjected to in vitro transcription, and mRNA transcripts were quantified by absorption at 260 nm and normalized against the transcription in the absence of **CQ**.

enhancements in dye exclusion with increasing concentration. Dye exclusion was ∼30% for 0.5 or 1 mM **CQ7a** and rose to almost 100% at 4 mM. Dye exclusion was ∼23% for **CQ** (0.5 or 1 mM) and ∼17% for **CQO** (0.5 or 1 mM), with slightly greater exclusion for **CQ**/**CQO** at 4 mM. PicoGreen was not excluded from DNA by **CP**.

Interactions between pDNA and **CQ** analogues may render pDNA less accessible to enzymes such as polymerases that initiate gene expression. To evaluate this hypothesis, solutions of pDNA and **CQ** analogues were subjected to in vitro transcription. Relative to in vitro transcription in the absence of **CQ** analogues, pDNA retained at least 75% of its transcript production with **CQ** analogue concentrations of up to 2.5 mM (Figure 8). However, small decreases in transcription were observed as **CQ** analogue concentrations were increased. The most pronounced reduction was observed with **CQ7a**, less of an effect was shown with **CQ** and **CQO**, and the weakest effect was recorded with **CP**. The correlation between dye exclusion (Figure 7) and reduced mRNA production (Figure 8) suggests that in vitro transcription is inhibited by interactions between pDNA and **CQ** analogues.

Discussion

Chloroquine (**CQ**) can enhance the transfection efficiency in many nonviral, polycation-based gene delivery vectors, but the mechanisms by which this occurs are not well established. In this study, we contribute to the understanding of **CQ**-related enhancements in gene expression by synthesizing and investigating a number of **CQ** analogues whose structures are designed specifically to probe hypothesized mechanisms, i.e., pH buffer-

ing of endocytic vesicles, displacement of polycations from DNA, etc. This is so far the first systematic study to correlate the **CQ** structure with its function during gene transfer.

As shown by the data in Table 1, a side chain with at least one basic amino moiety is essential for the capability of these small molecules to enhance transfection (**CQ** and **CQ4c**−**f** vs **CQ4a** and **CQ4b**). **CQ4a** and **CQ4b**, the **CQ** analogues that lack side-chain amino groups, show no effects on gene transfer. However, the effects of the side-chain amino group alone are not entirely responsible for **CQ**'s activity because transfection is not enhanced by some of the **CQ** analogues containing this group (**CQ7b** and **CP**) or by an aliphatic amine alone.[8]

CQ analogues bearing more than two amino moieties (**CQ4d**−**f**) are more basic than **CQ**. Expression levels in the presence of those **CQ** analogues are essentially the same as with **CQ** or in the presence of a **CQ** analogue with only two amino moieties (**CQ4c**). These results indicate that the level of basicity exceeding that of **CQ** does not enhance the gene transfection.

The aromatic ring structure of **CQ** contributes to its function in nonviral gene transfer (Table 1). **CP**, a pyridinyl-based **CQ** analogue, does not increase gene expression at all, while **QC**, an acridine-based **CQ** analogue with three fused aromatic rings, enhances the transfection at an optimal concentration of 20 μM; 10 times lower than the optimal concentration of **CQ**.

Dramatic effects on gene expression occur with changes in the 7-substituent of the **CQ** aromatic ring (while maintaining the side chain of **CQ**). When the 7-Cl group of **CQ** is replaced with a proton (**CQ7b**), the **CQ** analogue is not able to enhance gene expression. If a trifluoromethyl group (**CQ7a**) is used instead of the 7-Cl group, the transfection enhancement is markedly improved relative to the enhancement by **CQ** (Table 1).

The presence of the **CQ** analogues does not significantly affect the uptake of polyplexes by cells (Table 2), and the intracellular concentrations of **CQ** and **CQ7a** are similar at 4 h (Figure 2). Apparently, the differences in transfection enhancement between **CQ** and **CQ7a** are not due to differences in uptake of either the DNA or the **CQ** and **CQ7a**. However, **CQ7b** demonstrates that the lipophilic or the electron-withdrawing character of the 7-substituent can contribute to the uptake of a **CQ** analogue, providing an explanation for the lack of effect on transfection efficiency by **CQ7b** and possibly **CP**. Direct uptake measurements of **CP** would be helpful, but the absorbance spectra of **CP** (and **CQO**) do not allow their cellular uptake to be measured using the method employed for other **CQ** analogues.

The results obtained with **CQ**, **CQ7a**, and **CQO** show a correlation between enhancements in transfection efficiency and buffering of the pH experienced by polyplexes within cells (Figure 3). **CQO** interacts with nucleic acids in a manner similar to **CQ** (Figure 7) but displays reduced buffering activity (Figure 3) and does not enhance transfection efficiency (Table 1). **CQ** analogues without a side chain amino group (**CQ4a,b**) should have reduced pH-buffering activity, and they do not reveal the enhancement in gene expression that is seen with variants containing one or more side chain amino groups (**CQ**, **CQ4c**−**f**) (Table 1). Together, these results support the hypothesis that **CQ**'s pH-buffering of endocytic vesicles is a necessary but not sufficient condition for enhancing transfection efficiency.

While buffering of the endocytic vesicles appears to be necessary for transfection enhancements, it does not provide a complete explanation of how **CQ** increases transfection efficiency. The results from varying both the side chain and the aromatic ring structures (Table 1) suggest that **CQ** achieves its overall effect through multiple mechanisms. Also, the intracellular buffering by **CQ** and **CQ7a** is functionally similar, so the buffering hypothesis does not explain the significant increase in transfection efficiency in all the four cell lines tested when **CQ** is replaced with **CQ7a** (HepG2 and HeLa, Table 1; BHK and A2780, see Supporting Information) When poly-lysine (PLL)/pDNA complexes were transfected with **CQ** or **CQ7a** in HepG2 cells, results similar to those of CDP were obtained (see Supporting Information). The significantly enhanced gene expression observed with **CQ7a** compared to **CQ** cannot be simply ascribed to the buffering effect. Compounds such as monensin, methylamine, spermine, and ammonium chloride are known to buffer endocytic vesicles but do not contribute to transfection efficiency,[8] and these data provide additional evidence that buffering activity is not a sufficient condition for improved transfection.

CQ, **CQ7a**, and **QC** all confer significant gains in transfection efficiency. However, the dramatic variations in their performance suggest that their effects on transfection efficiency could be related to their interactions with DNA. Parker et al. have demonstrated that **CQ** can bind to both DNA and RNA in vitro, an interaction which can alter the biological and physical properties of the DNA.[38] Interactions between DNA and **CQ** analogues are strongly associated with the aromatic structures of those ligands. Because of its acridinyl fused aromatic structure, **QC** exhibits strong interactions with DNA,[39] and this molecule is a potent enhancer of gene transfer at relatively low concentration (Table 1). The single aromatic ring of **CP** renders this molecule unable to bind DNA as effectively as its multiring analogues (Figure 7), consistent with the fact that **CP** does not enhance gene transfer (Table 1).

In addition to ring structure, the electron-withdrawing character of the substituent at the 7-position can affect the strength of binding between **CQ** analogues and DNA.[23] This effect is demonstrated by gel retardation of pDNA at various concentrations of **CQ**, **CQ7a**, and **CQ7b** (Figure 4). **CQ7a** shows the strongest interaction with pDNA, displaying some retardation of pDNA electrophoresis at 1 mM and essentially complete retardation at 10 mM. **CQ7b** shows a weaker interaction with pDNA, as pDNA mobility did not change in the presence of **CQ7b** over a wide range of concentrations. The strength of interaction between **CQ** and pDNA is likely intermediate to those of **CQ7a** and **CQ7b** since **CQ** slightly retarded pDNA electrophoresis at 10 mM.

pDNA must be dissociated from polycations in order to achieve gene expression. We have shown that CDP/pDNA polyplexes do not reach the nucleus of transfected cells but deliver unpackaged pDNA to both the cytoplasm and cell nucleus.[32] It is likely that gene transfer is improved with enhanced intracellular unpackaging.[40] The presence of **CQ** analogues can destabilize polyplexes, generating unpackaged pDNA (Figure 5). The ability to displace pDNA from CDP decreases in the order of **CQ7a**, **CQ**, and **CQ7b**, corresponding to the order of pDNA binding strengths displayed in the gel retardation assay.

Cells exposed to solutions of pDNA and **CQ** analogues do not show gene expression (see Supporting Information), suggesting that extracellular interactions between pDNA and **CQ** analogues do not play a significant role in transfection. Although cells may be exposed to relatively low concentrations of **CQ** analogues during transfection, the concentrations within cells are found to be much higher (Figure 2). For **CQ** and **CQ7a**, these concentrations are sufficient to disrupt polyplexes (Figure 5). The average cellular concentrations of **CQ** analogues are likely less than the concentrations found in vesicles of the

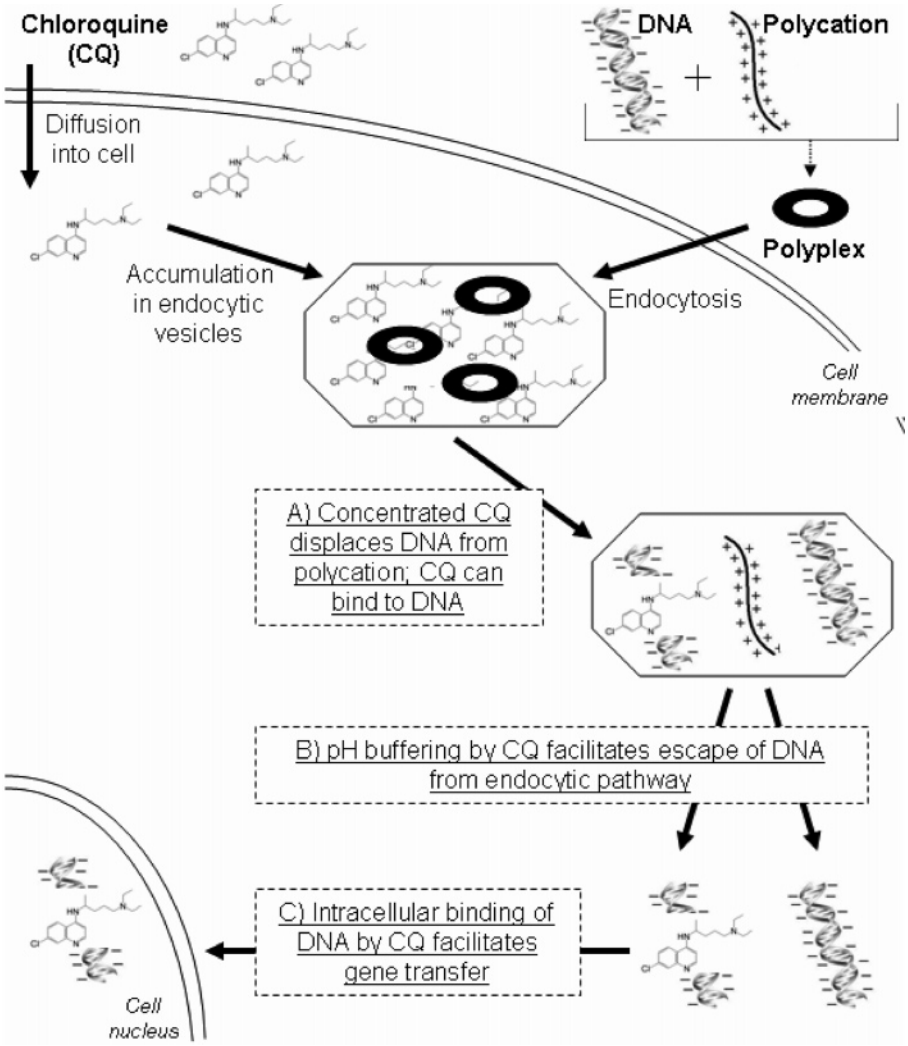


Figure 9. Proposed mechanism for the enhancement of transgene expression by **CQ** in nonviral gene delivery: accumulated intracellular high concentrations of **CQ** in the endocytic pathway (1) leading to enhanced unpackaging of delivered DNA; (2) buffering the pH of endocytic vesicles; (3) altering the trafficking, processing, or degradation of the DNA within cells.

endocytic pathway, as acidic intracellular compartments tend to accumulate weakly basic compounds such as **CQ**.[17,41−44] The pH-partitioning may contribute to this effect,[41,45] but data suggest that such accumulation occurs to a greater extent than would be predicted by pH-partitioning alone.[46] Colombo and Bertini have proposed that **CQ** accumulates in lysosomes due to pH-partitioning as well as its interactions with the lysosomal membrane.[34] Thus, it is likely that **CQ** analogues accumulated in the endocytic pathway at or above 10 mM can destabilize polyplexes and unpack pDNA from polyplexes.

The biological and physical properties of DNA can be altered when it is bound by **CQ**.[38] **CQ7a**, which displays stronger binding affinity for DNA than **CQ**, can be expected to have similar or greater effects on altering the properties of DNA. Beyond their activity in displacing DNA from polyplexes, **CQ** and **CQ7a** appear to interact directly with nucleic acids, even when those nucleic acids have been delivered to cells (Figures 4, 6−8). Results from the modified comet assay (Figure 6) suggest that **CQ** and **CQ7a** may be associated with the DNA that has been released from the polyplexes (lack of mobility in the gel suggests that a cationic entity is bound to the DNA that has been released from the polyplexes when **CQ** and **CQ7a** are utilized in the transfection). Strong binding by numerous **CQ7a** molecules could alter the physical properties of pDNA and reduce the overall negative charge of its backbone (via electrostatic interaction between anionic phosphate groups of pDNA and cationic amino side chains of **CQ7a**) (Figure 4). When bound by **CQ**, pDNA could achieve improved gene transfer due to changes in its intracellular processing or its rate of intracellular degradation.[47,48] The pDNA can be transcribed in the presence of **CQ** analogues, although increasing concentrations of **CQ** analogues lead to declining levels of mRNA transcripts (Figure 8). Reduced accessibility of pDNA to transcription-initiating polymerases may imply reduced pDNA accessibility to degradative enzymes, but efforts to evaluate DNAse protection by **CQ** analogues were inconclusive due to difficulty in purifying pDNA from **CQ** analogues after DNAse exposure (data not shown).

CQ7a greatly enhances gene expression over **CQ** in all the four cell lines we studied (Table 1 and Supporting Information), an effect that is not explained by its intracellular buffering effect alone. The observed higher luciferase activity from transfection in the presence of **CQ7a** is likely due to additional features such as greater displacement of polycations (Figure 5) and stronger interactions with intracellular nucleic acids that may protect and traffic more of the intact nucleic acid into the nucleus (Figures 4, 6−8).

In summary, a structure−function study among CQ and its analogues in nonviral, polycation-based gene delivery is carried out for the first time. It is demonstrated that the tertiary amino

moiety of CQ is important to enhancing gene expression. Further, changes in the aromatic ring of CQ significantly affect its ability to increase gene expression. A CQ analogue with a three fused aromatic ring structure (**QC**) shows much higher cytotoxicity than CQ and can enhance transfection similarly to CQ at a concentration 10 times lower. A CQ analogue with one pyridinyl aromatic ring (**CP**) gives lower cytotoxicity than CQ but has no effect on gene transfer. Variation at the 7-position of CQ dramatically changes its ability to enhance transfection. Transfection with a 7-trifluoromethyl CQ analogue (**CQ7a**) shows luciferase activity an order of magnitude higher than transfection with the same concentration (0.2 mM) of CQ, while transfection is not affected in the presence of a 7-hydro CQ analogue (**CQ7b**). The fused aromatic ring structure (acridinyl ring) in **QC** and the strong electron-withdrawing group (trifluoromethyl) in **CQ7a** endow these molecules with higher binding affinity to DNA. The stronger intercalator, **CQ7a**, has a higher tendency than CQ to competitively displace the polycation from CDP/pDNA polyplexes at a concentration that is achievable within cells after several hours' treatment. Finally, CQ and **CQ7a** appear to interact directly with nucleic acids in cells.

Our study suggests that the mechanisms of CQ action are at least 3-fold (Figure 9). One, intracellular accumulation of CQ drives its concentration to a point where it is able to facilitate unpackaging of nucleic acids from the polyplexes. CQ accumulates in cells to concentrations greatly exceeding that in the extracellular medium; and it reaches still higher concentrations in the vesicles of the endocytic pathway where internalized polyplexes are also delivered. Second, consistent with numerous literature reports, CQ buffers the vesicles of the endocytic pathway, helping delivered nucleic acids escape from these vesicles and/or avoid lysosomal degradation. Third, intracellular interactions between CQ and unpackaged nucleic acids contribute to enhanced gene expression. CQ can bind to the delivered DNA via interaction with DNA bases and through electrostatic interaction with anionic phosphate groups. These interactions lead to changes in the biological and physical properties of the delivered nucleic acids, possibly altering their intracellular processing or slowing their degradation.

Experimental Section

Chloroquine (**CQ**), quinocrine (**QC**), and other chemicals reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification unless otherwise indicated. NMR spectra were collected on Varian 300 MHz and Inova 500 MHz spectrometers. β-Cyclodextrin polymer (CDP) containing two positively charged amidine groups in each repeat with weight average molecular weight (M_w) of 6000 Da was synthesized and used for condensing DNA or oligonucleotide in this study.[10,11] Each repeat unit contains two positively charged amidine groups that can efficiently bind and condense DNA in plasmid (pDNA) or oligonucleotide form.[10]

General Procedure for the Synthesis of CQ Analogues. CQ analogues with alkylamino side chains and 7-chloroquinoline structure (**CQ4a**−**f** and **CQO**) were synthesized via substitution reaction of an appropriate amine and 4,7-dichloroquinoline (see Supporting Information). **CQ** analogues with aromatic ring-size and ring-substituent variations were synthesized similarly using an appropriate quinoline or pyridine derivative as starting materials (see Supporting Information).

Cell Culture and Transfection Experiments. The DNA plasmid pGL3-CV (Promega, Madison, WI) containing the firefly luciferase gene under the control of the SV40 promoter was amplified by *E. coli* strain DH5α and was then purified using the Ultramobius 1000 plasmid kit (Novagen, San Diego, CA). HepG2 and HeLa cells were purchased from the ATCC (Rockville, MD). Cells were cultured according to recommended procedures using Minimum Essential Medium (HepG2) or Dulbecco's Modified Eagle's Medium (HeLa), with 2 mM l-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Media and supplements were purchased from Gibco BRL (Gaithersburg, MD). Cells were transfected in serum-reduced medium with CDP/pDNA polyplexes as described previously, in the presence of **CQ** or **CQ** analogues at various concentrations.

For cell transfection and luciferase assay, cells were plated at 5×10^4 cells/well in 24-well plates 24 h in advance. Immediately prior to transfection, cells were rinsed once with PBS (pH 7.4), and 200 μL of Opti-MEM (Gibco) was added to each well. pGL3-CV (1 μg, 5 μL of a 0.2 μg/μL solution in DNAse-free water) was mixed with an equal volume of polymer solution (5 μL of 24.9 mg/mL freshly prepared CDP solution in DNAse-free water) to give a charge ratio 5 +/−. An Opti-MEM solution (185 μL) and an appropriate amount of **CQ** (or **CQ** analogues) in 5 μL of Opti-MEM solution were mixed with CDP/pDNA complexes (10 μL) to give a final volume of 200 μL. These solutions were immediately transferred to each well. After 4 h of incubation (37 °C, 5% CO_2), the media in each well was replaced with 1 mL of culture media. After another 44 h, the media was removed by aspiration. Cells were washed twice with PBS (pH 7.4) before addition of 100 μL of 1× cell culture lysis buffer (Promega). Cell lysates were analyzed for luciferase activity with luciferase assay reagent (Promega). Light units were measured in duplicate with a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA).

Determination of cell viability and total protein concentration was conducted by a modified Lowry protein assay. The amount of protein in cell lysates obtained 48 h after transfection was used as a measure of cell viability. Protein levels of transfected cells were determined by the DC Protein Assay (Bio-Rad, Hercules, CA) and normalized with protein levels of cells transfected with naked DNA. A protein standard curve was run with various concentrations of bovine IgG (Bio-Rad) in cell culture lysis buffer.

Uptake of Polyplexes in the Absence or Presence of CQ Analogues. pDNA was prepared for fluorophore binding by reacting with Label-IT Amine (Mirus, Madison, WI). After 1 h at 37 °C, the amine-terminated DNA was recovered by precipitation with ethanol and then incubated overnight with Alexa-fluor-488 NHS ester (Molecular Probes, Eugene, OR). The labeled DNA was purified by precipitation with ethanol. The percentage of pDNA labeling was determined to be 3.3 wt % by measuring the fluorescence intensity of labeled DNA ($\lambda_{excitation}$ 488 nm; $\lambda_{emission}$ 535 nm) using a Spectrafluor Plus plate reader (Tecan, Durham, NC) and comparing with a standard curve.

Labeled pDNA was condensed with CDP prior to being administered to HepG2 cells at a concentration 5 μg DNA/mL culture medium (same concentration as in gene transfection study). Uptake was terminated at 30 min and at 2 h. Cells were treated with 0.25% trypsin, washed with phosphate-buffered saline and Hanks balanced salt solution, and analyzed by fluorescence-activated cell sorting (FACS).

Uptake and Intracellular Accumulation of CQ, CQ7a, and CQ7b. HepG2 cells (2×10^6 cells) were incubated at 37 °C in 2 mL of Opti-MEM (pH 7.4) with or without CDP/pDNA (20 nmol/3 pmol) in the presence of 100 μM of **CQ**, **CQ7a**, or **CQ7b**. After incubation in a humidified atmosphere (37 °C, 5% CO_2) for scheduled time, the medium was removed; cells were washed three times with cold PBS containing 100 μM of corresponding **CQ** analogue, washed three more times with cold PBS, and then lysed by 20 min incubation in 1 mL of 1× cell culture lysis buffer. The lysates was diluted with 1 mL of NaOH (0.1 N), and 80 μL of such solution was analyzed on a Spectrafluor Plus plate reader (Tecan) at excitation wavelength of 360 nm and emission wavelength of 465 nm. The resulting fluorescence results were converted to concentrations of **CQ** or **CQ** analogues using a standard curve. The cellular concentration of **CQ** or **CQ** analogues was calculated assuming a cell volume of 3×10^{-6} μL.[8]

Intracellular Buffering Activity of CQ Analogues. The activity of CDP in buffering the pH environment of intracellular polyplexes

was evaluated using the method of Kulkarni et al.[35] Polyplexes were prepared by mixing CDP and a 21-base RNA phosphorothioate oligonucleotide (5′-GACGUAAACGGCCACAAGUUC-3′) labeled at the 5′ position with the pH-sensitive fluorophore SNARF-4F (Molecular Probes, Carlsbad, CA).[35] HeLa cells were treated with the polyplexes and imaged using an inverted Zeiss LSM 510 META confocal microscope with a 63× oil objective (NA 1.4). Excitation of the SNARF fluorophore was achieved with the 543 nm line of the He−Ne laser line supplied with the microscope. After passing through a variable confocal pinhole, the SNARF-4F emission was collected by the META detector in distinct wavelength bins of 569−601 nm and 633−687 nm. Using a standard curve, the relative intensity of individual signals across these bins was used to evaluate the pH environment of the corresponding intracellular polyplexes.

Electrophoretic Mobility of pDNA in the Presence of CQ Analogues. Each polycation was examined for its ability to bind pDNA through gel electrophoresis experiments as previously described.[9,10] pGL3-CV (5 μL of a 0.2 μg/μL solution in DNAse-free water) was mixed with an appropriate amount of **CQ**, **CQ7a**, or **CQ7b**. Each solution was incubated for approximately 10 min. Loading buffer (5 μL) was added to each sample, followed by an appropriate volume of Borax buffer (0.025 M, pH 8.4) to give a final volume of 25 μL for each sample. 10 μL of such mixture was transferred into each well of a 0.5% agarose gel (6 μg of ethidium bromide/100 mL TAE buffer (40 mM Tris-acetate, 1 mM EDTA)) and electrophoresed.

Displacement of DNA from Polyplexes by CQ Analogues. pGL3-CV (2 μg, 0.6 pmol) in 10 μL of DI water was complexed with CDP (24 μg, 4 nmol) in 10 μL. The solution was diluted by adding 80 μL of PBS and kept for 30 min at 25 °C. After a further 30 min at 25 °C, each solution was passed through a Pall Life Science Supor Membrane (100 nm) presoaked and rinsed with a solution containing the equivalent concentration of the **CQ** analogues. An amount of 40 μL of the filtrate was mixed with 40 μL of 5.6 μM 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI). The amount of unpackaged DNA was determined from the fluorescence intensities ($\lambda_{excitation}$ 350 nm; $\lambda_{emission}$ 465 nm) of the DAPI-containing filtrate solution.[8] The percentage of pDNA dissociated from polyplexes was calculated according to $(I_f - I_0)/(I_t - I_0) \times 100$, where I_t is the fluorescence intensity of DAPI in the presence of the total full of pDNA, I_0 is the fluorescence intensity of DAPI in the absence of pDNA, and I_f is the fluorescence intensity of the filtrate containing dissociated pDNA.

Measure of Unpackaged Intracellular Nucleic Acids. HeLa cells in 24-well plates were transfected with polyplexes of CDP and a 25-base DNA oligonucleotide (5′-ACTGCTTACCAGG-GATTTCAGTGCA-3) labeled at the 5′ position with FITC (FITC-oligo). Fourteen hours after transfection, cells were collected by trypsinization and pelleted by centrifugation (6 min at 2400 rpm). Each sample was resuspended in 10 μL of 1× cell culture lysis buffer (Promega) and stored at 4 °C. Controls consisted of known amounts of FITC-oligo in 1× cell culture lysis buffer. After 30 min incubation, each sample was mixed with 25 μL of 37 °C low-melting-point agarose (1% in TAE buffer) and transferred immediately to a well of an agarose gel (0.5% in 60 mL TAE buffer). The agarose gel was subjected to gel electrophoresis and imaged under UV illumination. Detection of FITC-oligo was achieved through its fluorescent signal. Migration of FITC-oligo from control samples was used to indicate the expected migration of FITC-oligo unpackaged from cells.

Dye Exclusion Assay To Evaluate Accessibility of Condensed pDNA. The accessibility of polyplexes in the presence of **CQ** analogues was evaluated by dye exclusion through adaptation of a published procedure.[37] After formulation, polyplexes were diluted 2.5× in dH_2O. For each sample, 25 μL of polyplex solution was transferred to a well of an opaque, black 96-well plate and combined with 25 μL of **CQ** analogue solution at twice the concentration of interest. Separately, a 200× dilution of the supplied PicoGreen stock solution (Molecular Probes) was prepared in 10 mM HEPES buffer. After polyplex−**CQ** analogue solutions were incubated for 5 min at room temperature, each 50 μL sample was combined with 50 μL of PicoGreen solution. The fluorescence ($\lambda_{excitation}$ 488 nm, $\lambda_{emission}$ 535 nm) of the resulting solutions was evaluated with a Spectrafluor Plus plate reader (Tecan, Durham, NC). The percentage dye exclusion was calculated from the ratio $((F_{DNA} - F_{sample})/(F_{DNA} - F_{H2O}))$, where F_{DNA} is the fluorescence of a sample of DNA alone (no polycation), F_{sample} is the sample fluorescence, and F_{H2O} is the fluorescence of a blank (control) sample.

In Vitro Transcription in the Presence of Chloroquine Analogues. An in vitro transcription assay was used to evaluate the transcription accessibility pDNA in the presence of **CQ** analogues. The plasmid pT7-luc contains the firefly luciferase gene under the control of the T7 promoter. This plasmid was amplified by *E. coli* strain DH5α and purified using the Ultramobius 1000 plasmid kit. The MAXIscript T7 kit (Ambion, Austin, TX) was used to produce firefly luciferase mRNA from mixtures of pT7-luc and **CQ** analogues. Following the reaction, pT7-luc was digested using the TURBO DNase supplied with the kit. The RNA reaction product was purified by ammonium acetate/ethanol precipitation, resuspended in dH_2O, and quantified by absorbance at 260 nm. Yields were normalized using reactions conducted in the absence of **CQ** analogues.

Acknowledgment. We thank Insert Therapeutics for financial support.

Supporting Information Available: Detailed synthesis and characterization of chloroquine analogues, in vitro transfection of CDP/pDNA in the presence of chloroquine and chloroquine analogues in HepG2, HeLa, and A2780 and BHK cells, transfection of naked pDNA in the presence of CQ and CQ analogues, transfection of polylysine/pDNA in the presence of chloroquine and chloroquine analogue, fluorescence-activated cell sorting (FACS) histogram of the time-dependent uptake of polyplexes in the absence or presence of CQ analogues, and UV analysis of the interaction between DNA and CQ or CQ analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hwang, S. J.; Davis, M. E. Cationic polymers for gene delivery: Designs for overcoming barriers to systemic administration. *Curr. Opin. Mol. Ther.* **2001**, *3*, 183−191.
(2) Davis, M. E. Nonviral gene delivery systems. *Curr. Opin. Biotechnol.* **2002**, *13*, 128−131.
(3) Lechardeur, D.; Verkman, A. S.; Lukacs, G. L. Intracellular routing of plasmid DNA during non-viral gene transfer. *Adv. Drug Delivery Rev.* **2005**, *57*, 755−767.
(4) Wagner, E.; Zenke, M.; Cotten, M.; Beug, H.; Birnstiel, M. L. Transferrin-Polycation Conjugates as Carriers for DNA Uptake into Cells. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3410−3414.
(5) Zenke, M.; Steinlein, P.; Wagner, E.; Cotten, M.; Beug, H.; Birnstiel, M. L. Receptor-Mediated Endocytosis of Transferrin Polycation Conjugates− an Efficient Way to Introduce DNA into Hematopoietic-Cells. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3655−3659.
(6) Midoux, P.; Mendes, C.; Legrand, A.; Raimond, J.; Mayer, R.; Monsigny, M.; Roche, A. C. Specific Gene-Transfer Mediated by Lactosylated Poly-l-Lysine into Hepatoma-Cells. *Nucleic Acids Res.* **1993**, *21*, 871−878.
(7) Monsigny, M.; Roche, A. C.; Midoux, P.; Mayer, R. Glycoconjugates as Carriers for Specific Delivery of Therapeutic Drugs and Genes. *Adv. Drug Delivery Rev.* **1994**, *14*, 1−24.
(8) Erbacher, P.; Roche, A. C.; Monsigny, M.; Midoux, P. Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA lactosylated polylysine complexes. *Exp. Cell Res.* **1996**, *225*, 186−194.
(9) Gonzalez, H.; Hwang, S. J.; Davis, M. E. New class of polymers for the delivery of macromolecular therapeutics. *Bioconjugate Chem.* **1999**, *10*, 1068−1074.
(10) Hwang, S. J.; Bellocq, N. C.; Davis, M. E. Effects of structure of beta-cyclodextrin-containing polymers on gene delivery. *Bioconjugate Chem.* **2001**, *12*, 280−290.
(11) Pun, S. H.; Davis, M. E. Development of a nonviral gene delivery vehicle for systemic application. *Bioconjugate Chem.* **2002**, *13*, 630−639.
(12) Reineke, T. M.; Davis, M. E. Structural effects of carbohydrate-containing polycations on gene delivery. 1. Carbohydrate size and its distance from charge centers. *Bioconjugate Chem.* **2003**, *14*, 247−254.

(13) Reineke, T. M.; Davis, M. E. Structural effects of carbohydrate-containing polycations on gene delivery. 2. Charge center type. *Bioconjugate Chem.* **2003**, *14*, 255−261.
(14) Popielarski, S. R.; Mishra, S.; Davis, M. E. Structural effects of carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization. *Bioconjugate Chem.* **2003**, *14*, 672−678.
(15) Irvin, J. L.; Irvin, E. M. Spectrophotometric and Potentiometric Evaluation of Apparent Acid Dissociation Exponents of Various 4-Aminoquinolines. *J. Am. Chem. Soc.* **1947**, *69*, 1091−1099.
(16) Maxfield, F. R. Weak Bases and Ionophores Rapidly and Reversibly Raise the Ph of Endocytic Vesicles in Cultured Mouse Fibroblasts. *J. Cell Biol.* **1982**, *95*, 676−681.
(17) Poole, B.; Ohkuma, S. Effect of Weak Bases on the Intralysosomal Ph in Mouse Peritoneal-Macrophages. *J. Cell Biol.* **1981**, *90*, 665−669.
(18) Sonawane, N. D.; Szoka, F. C.; Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* **2003**, *278*, 44826−44831.
(19) Wibo, M.; Poole, B. Protein Degradation in Cultured-Cells .2. Uptake of Chloroquine by Rat Fibroblasts and Inhibition of Cellular Protein Degradation and Cathepsin-B1. *J. Cell Biol.* **1974**, *63*, 430−440.
(20) Poole, B.; Ohkuma, S.; Warburton, M. J. Accumulation of Weakly Basic Substances in Lysosomes and Inhibition of Intracellular Protein Degradation. *Acta Biol. Med. Ger.* **1977**, *36*, 1777−1788.
(21) Behr, J. P. The proton sponge: A trick to enter cells the viruses did not exploit. *Chimia* **1997**, *51*, 34−36.
(22) Allison, J. L.; Obrien, R. L.; Hahn, F. E. DNA−Reaction with Chloroquine. *Science* **1965**, *149*, 1111−1113.
(23) Cohen, S. N.; Yielding, K. L. Spectrophotometric studies of the interaction of chloroquine with deoxyribonucleic acid. *J. Biol. Chem.* **1965**, *240*, 3123−3131.
(24) Egan, T. Structure−Function Relationships in Chloroquine and Related 4-Aminoquinoline Antimalarials. *Mini-Rev. Med. Chem.* **2001**, *1*, 113−123.
(25) Egan, T. J.; Hunter, R.; Kaschula, C. H.; Marques, H. M.; Misplon, A.; Walden, J. Structure−function relationships in aminoquinolines: Effect of amino and chloro groups on quinoline-hematin complex formation, inhibition of beta-hematin formation, and antiplasmodial activity. *J. Med. Chem.* **2000**, *43*, 283−291.
(26) Kaschula, C. H.; Egan, T. J.; Hunter, R.; Basilico, N.; Parapini, S.; Taramelli, D.; Pasini, E.; Monti, D. Structure−activity relationships in 4-aminoquinoline antiplasmodials. The role of the group at the 7-position. *J. Med. Chem.* **2002**, *45*, 3531−3539.
(27) De, D. Y. D.; Krogstad, F. M.; Byers, L. D.; Krogstad, D. J. Structure−activity relationships for antiplasmodial activity among 7-substituted 4-aminoquinolines. *J. Med. Chem.* **1998**, *41*, 4918−4926.
(28) De, D. Y.; Byers, L. D.; Krogstad, D. J. Antimalarials: Synthesis of 4-aminoquinolines that circumvent drug resistance in malaria parasites. *J. Heterocycl. Chem.* **1997**, *34*, 315−320.
(29) Bellocq, N. C.; Pun, S. H.; Jensen, G. S.; Davis, M. E. Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. *Bioconjugate Chem.* **2003**, *14*, 1122−1132.
(30) Bellocq, N. C.; Kang, D. W.; Wang, X. H.; Jensen, G. S.; Pun, S. H.; Schluep, T.; Zepeda, M. L.; Davis, M. E. Synthetic biocompatible cyclodextrin-based constructs for local gene delivery to improve cutaneous wound healing. *Bioconjugate Chem.* **2004**, *15*, 1201−1211.
(31) Pun, S. H.; Tack, F.; Bellocq, N. C.; Cheng, J. J.; Grubbs, B. H.; Jensen, G. S.; Davis, M. E.; Brewster, M.; Janicot, M.; Janssens, B.; Floren, W.; Bakker, A. Targeted delivery of RNA-cleaving DNA enzyme (DNAzyme) to tumor tissue by transferrin-modified, cyclodextrin-based particles. *Cancer Biol. Ther.* **2004**, *3*, 641−650.
(32) Mishra, S.; Webster, P.; Davis, M. E. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur. J. Cell Biol.* **2004**, *83*, 97−111.
(33) Pun, S. H.; Bellocq, N. C.; Liu, A. J.; Jensen, G.; Machemer, T.; Quijano, E.; Schluep, T.; Wen, S. F.; Engler, H.; Heidel, J.; Davis, M. E. Cyclodextrin-modified polyethylenimine polymers for gene delivery. *Bioconjugate Chem.* **2004**, *15*, 831−840.
(34) Colombo, M. I.; Bertini, F. Properties of Binding-Sites for Chloroquine in Liver Lysosomal Membranes. *J. Cell. Physiol.* **1988**, *137*, 598−602.
(35) Kulkarni, R. P.; Mishra, S.; Fraser, S. E.; Davis, M. E. Single cell kinetics of intracellular, nonviral, nucleic acid delivery vehicle acidification and trafficking. *Bioconjugate Chem.* **2005**, *16*, 986−994.
(36) Singh, N. P.; McCoy, M. T.; Tice, R. R.; Schneider, E. L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **1988**, *175*, 184−191.
(37) O'Brien, R.; Allison, J.; Hahn, F. Evidence for intercalation of chloroquine into DNA. *Biochim. Biophys. Acta* **1966**, *129*, 622−624.
(38) Parker, F. S.; Irvin, J. L. The Interaction of Chloroquine with Nucleic Acids and Nucleoproteins. *J. Biol. Chem.* **1952**, *199*, 897−909.
(39) Doglia, S. M.; Albinsson, B.; Hiort, C.; Norden, B.; Graslund, A. Quinacrine−Spectroscopic Properties and Interactions with Polynucleotides. *Biopolymers* **1993**, *33*, 1431−1442.
(40) Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol. Bioeng.* **2000**, *67*, 598−606.
(41) Lloyd, J. B. Lysosome membrane permeability: implications for drug delivery. *Adv. Drug Delivery Rev.* **2000**, *41*, 189−200.
(42) Rashid, F.; Horobin, R. W.; Williams, M. A. Predicting the behaviour and selectivity of fluorescent probes for lysosomes and related structures by means of structure−activity models. *Histochem. J.* **1991**, *23*, 450−459.
(43) Diwu, Z.; Chen, C. S.; Zhang, C.; Klaubert, D. H.; Haugland, R. P. A novel acidotropic pH indicator and its potential application in labeling acidic organelles of live cells. *Chem. Biol.* **1999**, *6*, 411−418.
(44) Hurwitz, S. J.; Terashima, M.; Mizunuma, N.; Slapak, C. A. Vesicular anthracycline accumulation in doxorubicin-selected U-937 cells: participation of lysosomes. *Blood* **1997**, *89*, 3745−3754.
(45) de Duve, C.; de Barsy, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F. Commentary. Lysosomotropic agents. *Biochem. Pharmacol.* **1974**, *23*, 2495−2531.
(46) Duvvuri, M.; Krise, J. P. A novel assay reveals that weakly basic model compounds concentrate in lysosomes to an extent greater than pH-partitioning theory would predict. *Mol. Pharmacol.* **2005**, *2*, 440−448.
(47) Lukacs, G. L.; Haggie, P.; Seksek, O.; Lechardeur, D.; Freedman, N.; Verkman, A. S. Size-dependent DNA mobility in cytoplasm and nucleus. *J. Biol. Chem.* **2000**, *275*, 1625−1629.
(48) Lechardeur, D.; Sohn, K. J.; Haardt, M.; Joshi, P. B.; Monck, M.; Graham, R. W.; Beatty, B.; Squire, J.; O'Brodovich, H.; Lukacs, G. L. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther.* **1999**, *6*, 482−497.

JM060736S