Supporting Information

Structure-Function Correlation of Chloroquine and Analogues as Transgene Expression Enhancer in Non-Viral Gene Delivery

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Synthesis of chloroquine analogues

Chloroquine (**CQ**) analogues with side alkyl amino side chain variation were synthesized via substitution reaction of an appropriate amine and 4,7-dichloroquinoline.[1] **CQ** analogues of ring-size variations and ring-substituent variations were synthesized similarly using an appropriate quinoline or pyridine derivative as starting materials.

Synthesis of CQ4a. A mixture of 4,7-dichloroquinoline (619 mg, 3.1 mmol) and 1,5-dimethylhexylamine (3.1 g, 23.7 mmol) was heated at 100 °C for 1 h and then at 155 °C for 3 h. The reaction was monitored by TLC to ensure the completion of reaction. Unreacted amines were distilled under vacuum and could be reused. The resulting brownish residue was applied to a silica gel flash column and a solvent mixture ($CHCl_3/CH_3OH/Et_3N$ (25/1/1)) was used as eluent. **CQ**4a was isolated in 64% yield after combining elution fractions. **CQ**4a was further purified by crystallization ($CHCl_3$ and hexane) to give 120 mg (13% yield) of white solid. ^{1}H NMR ($CDCl_3$) δ 8.52 (1H, d, *1-Ar-H*), 7.95 (1H, d, *5-Ar-H*), 7.64 (1H, d, *8-Ar-H*), 7.37 (1H, dd, *6-Ar-H*), 6.42 (1H, d, *2-Ar-H*), 4.78 (1H, br, Ar-*NH*), 3.71 (1H, m, $-NHC\mathit{H}(CH_3)CH_2CH_2CH_2CH(CH_3)CH_3$), 1.67-1.43 (5H, m, $-NHCH(CH_3)C\mathit{H}_2C\mathit{H}_2CH_2C\mathit{H}(CH_3)CH_3$), 1.32 (3H, d, $-NHCH(C\mathit{H}_3)CH_2CH_2CH_2CH(CH_3)CH_3$), 1.23 (2H, d, $-NHCH(CH_3)CH_2CH_2C\mathit{H}_2CH(CH_3)CH_3$), 0.87 (6H, d, $-NHCH(CH_3)CH_2CH_2CH_2CH(C\mathit{H}_3)C\mathit{H}_3$).

Synthesis of CQ4b. 4,7-Dichloroquinoline (1.98g, 10 mmol) and L-alanine (1.78 g, 20 mmol) were heated in phenol (8 g) for one hour at 160 °C. The solution was poured into a solution mixture of 10% KI (30 mL) and ethyl ether (30 mL). The aqueous layer was then washed with ether layer (3×30 mL). The combined ether layer was extracted with 10% KI (30 mL). The pH of the combined aqueous phase was adjusted to 7. The aqueous solution was let uncovered for slow evaporation at room temperature. After about 24 h, white crystals were filtered and dried under vacuum (1.25g, 55%). ^{1}H NMR (CD_3OD) δ 8.43 (1H, d, *1-Ar-H*), 8.33 (1H, d, *5-Ar-H*), 7.76 (1H, d, *8-Ar-H*), 7.56 (1H, dd, *6-Ar-H*), 6.66 (1H, d, *2-Ar-H*), 4.26 (1H, m, NHC*H*(CH_3)COOH), 1.66 (3H, d, NHCH(CH_3)COOH)

Synthesis of CQ4c. **CQ4c** was synthesized using the same method as **CQ4a** with corresponding amine (26%). ^{1}H NMR ($CDCl_3$) δ 8.53 (1H, d, *1-Ar-H*), 7.94 d (1H, dd, *5-Ar-H*), 7.70 (1H, d, *8-Ar-H*), 7.36 (1H, dd, *6-Ar-H*), 6.35 (1H, d, *2-Ar-H*), 5.92 (1H, br., Ar-*NH*), 3.28 (2H, t, -NHCH_2CH$_2$N(CH$_3$)$_2$), 2.68 (2H, t, -NHCH$_2$CH_2N(CH$_3$)$_2$), 2.30 (6H, s, -NHCH$_2$CH$_2$N(CH_3)$_2$).

Synthesis of CQ4d. 4,7-Dichloroquinoline (620 mg, 3.1mmol) and N^1-isopropyl-diethylenetriamine (1.76g, 12.3 mmol) was heated for 1 h at 100 °C and then for additional 3 h at 155 °C. TLC analysis indicated that 4,7-Dichloroquinoline was completely consumed after 4 h. Unreacted amine was distilled at 85-115 °C using a Kugelrohr apparatus under a vacuum of approximately 50 millitorr. The remaining brown oil was transferred to a silica gel flash column. A solvent mixture ($CH_2Cl_2/CH_3OH/NH_4OH$ (70/28/2), 200 mL) was used as eluent, followed by another eluent ($CH_2Cl_2/CH_3OH/NH_4OH$ (5/20/4), 200 mL). The fractions containing **CQ4d** (R*f* = 0.1 in $CH_2Cl_2/NH_4OH/CH_3OH$ (40/1.5/80)) were collected, combined and subjected to another silica gel column separation ($CHCl_3/CH_3OH$ /Et_3N (10/1/1), 200 mL) to give a pale yellow oil (35%). ^{1}H NMR (CD_3CD) δ 8.52 (1H, d, *1-Ar-H*), 7.94 (1H, d, *8-Ar-H*), 7.70 (1H, d, *5-Ar-H*), 7.35 (1H, dd, *6-Ar-H*), 6.32 (1H, d, *2-Ar-H*), 5.98 (1H, br., Ar-*NH*), 3.36-2.07 (12H, m, -N*H*CH_2CH_2N*H*CH_2CH_2N*H*C*H*(CH$_3$)$_2$), 1.07 (6H, d, -NHCH$_2$CH$_2$NHCH$_2$CH$_2$NHCH(CH_3)$_2$).

Synthesis of CQ4e. 4,7-Dichloroquinoline (500mg, 2.5 mmol) and excess 1,4-piperazine-dipropanamine (5 g, 25 mmol) were heated for 12 h at 135 °C. TLC analysis indicated that 4,7-Dichloroquinoline was completely consumed after 12 h reaction. Unreacted amine was distilled at 85-110 °C using a Kugelrohr apparatus under a vacuum of approximately 50 millitorr. The remaining brown oil was transferred to a silica gel flash column. A solvent mixture ($CH_2Cl_2/CH_3OH/NH_4OH$ (70/28/2), 800 mL) was used as eluent, followed by elution in $CH_2Cl_2/CH_3OH/NH_4OH$ ((5/20/4), 200 mL) and CH_3OH/NH_4OH ((1/1), 200 mL). Three different compounds were observed on TLC ($CH_2Cl_2/CH_3OH/NH_4OH$ (5/20/4)). The fraction with R*f* = 0.33 was found to be **CQ4e**. The combined fractions were transferred to another silica-gel column and eluted with ($CHCl_3/CH_3OH/Et_3N$ (10/1/1), 200 mL) for another time. Clean fractions were obtained and combined. Solvent was removed under vacuum to give a colorless oil (31%). ^{1}H NMR (CD_3CD) δ 8.35 (1H, d, *1-Ar-H*), 8.07 (1H, d, *5-Ar-H*), 7.77 (1H, d, *8-Ar-H*), 7.39 (1H, dd, *6-Ar-H*), 6.53 (1H, d, *2-Ar-H*), 3.39 (2H, t, -$NHCH_2CH_2CH_2N(CH_2CH_2)_2NCH_2CH_2CH_2NH_2$), 2.41-2.69 (14H, m, -$NHCH_2CH_2CH_2N(CH_2CH_2)_2NCH_2CH_2CH_2NH_2$), 1.93 (2H, m, -$NHCH_2CH_2CH_2N(CH_2CH_2)_2NCH_2CH_2CH_2NH_2$), 1.64 (2H, m, -$NHCH_2CH_2CH_2N(CH_2CH_2)_2NCH_2CH_2CH_2NH_2$).

Synthesis of CQ4f. 4,7-Dichloroquinoline (680 mg, 3.4 mmol) and excess of Tris-(2-aminoethyl)-amine (5 g, 34 mmol) were heated for 12 h at 135 °C. TLC analysis indicated that 4,7-Dichloroquinoline was completely consumed after 12 h reaction. Unreacted amine was distilled at 85-110 °C using a Kugelrohr apparatus under a vacuum of approximately 50 millitorr. The remaining brown oil was transferred to a silica gel flash column. A solvent mixture ($CH_2Cl_2/CH_3OH/NH_4OH$ (70/28/2), 800 mL) was used as eluent, followed by elution with $CH_2Cl_2/CH_3OH/NH_4OH$ ((5/20/4), 200 mL) and CH_3OH/NH_4OH ((1/1), 200 mL). Three different compounds were observed on TLC ($CH_2Cl_2/CH_3OH/NH_4OH$ (5/20/4)). The fraction with R*f* = 0.2 was identified to be **CQ4f**. The combined

fractions with R*f* = 0.2 were transferred to another column and eluted with ($CHCl_3/CH_3OH/NH_4OH$ (20/5/1), 200 mL). Clean fractions were collected and combined. Solvent was removed under vacuum to give a colorless oil (15%). ^{1}H NMR ($CDCl_3$) δ 8.50 (1H, d, *1-Ar-H*), 7.98 (1H, d, *5-Ar-H*), 7.92 (1H, d, *8-Ar-H*), 7.32 (1H, dd, *6-Ar-H*), 6.69 (1H, br, Ar-*NH*), 6.35 (1H, d, *2-Ar-H*), 3.36 (2H, m, -$NHC\mathit{H}_2CH_2N(CH_2CH_2NH_2)_2$), 2.89-2.68 (6H, m, -$NHCH_2C\mathit{H}_2N(C\mathit{H}_2CH_2NH_2)_2$), 2.63 (4H, t, -$NHCH_2CH_2N(CH_2C\mathit{H}_2NH_2)_2$), 1.59 (5H, br, -$N\mathit{H}CH_2CH_2N(CH_2CH_2N\mathit{H}_2)_2$).

Synthesis of CQ7a. 4-chloro-7-trifluoromethyl quinoline (0.5g, 2.16 mmol) and 2-amino-5-diethylaminopentane (2.61 g, 15.7 mmol) were heated together at 100 °C for 1 hour and then 155 °C for 4.5 hours. Reaction was then cooled to room temperature. Unreacted amine was removed by Kugelrohr distillation at 120 °C under vacuum. The remaining brown crude oil was then dissolved in $CHCl_3$ and purified by preparative TLC using $CHCl_3/CH_3OH/Et_3N$ (25/1/1) as an eluent to yield **CQ7a** as a colorless oil (32%). ^{1}H NMR ($CDCl_3$) δ 8.62 (1H, d, *1-Ar-H*), 8.23 (1H, d, *8-Ar-H*), 7.91 (1H, d, *5-Ar-H*), 7.58 (1H, dd, *6-Ar-H*), 6.52 (1H, d, *2-Ar-H*), 5.55 (1H, m, Ar-*NH*), 3.75 (1H, m, -$NHC\mathit{H}(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 2.56 (6H, m, -$NHCH(CH_3)CH_2CH_2C\mathit{H}_2N(C\mathit{H}_2CH_3)_2$), 1.80 (2H, m, -$NHCH(CH_3)C\mathit{H}_2CH_2CH_2N(CH_2CH_3)_2$), 1.67 (2H, m, -$NHCH(CH_3)CH_2C\mathit{H}_2CH_2N(CH_2CH_3)_2$), 1.36 (3H, d, -$NHCH(C\mathit{H}_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 1.06 (6H, d, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2C\mathit{H}_3)_2$).

Synthesis of CQ7b. **CQ7b** was synthesized using the same method as **CQ4a** using 4-chloroquinoline and 2-amino-5-diethylaminopentane (10%). ^{1}H NMR ($CDCl_3$) δ 8.53 (1H, d, *1-Ar-H*), 7.97 (1H, d, *8-Ar-H*), 7.76 (1H, d, *5-Ar-H*), 7.61 (1H, dd, *7-Ar-H*), 7.40 (1H, d, *6-Ar-H*), 6.42 (1H, d, *2-Ar-H*), 5.21 (1H, br., Ar-*NH*), 3.75 (1H, m, -$NHC\mathit{H}(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 2.57-2.46 (6H, m, -$NHCH(CH_3)CH_2CH_2C\mathit{H}_2N(C\mathit{H}_2CH_3)_2$), 1.76 (2H, m, -$NHCH(CH_3)C\mathit{H}_2CH_2CH_2N(CH_2CH_3)_2$), 1.62 (2H, m, -$NHCH(CH_3)CH_2C\mathit{H}_2CH_2N(CH_2CH_3)_2$), 1.31 (3H, d, -$NHCH(C\mathit{H}_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 1.08 (6H, d, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2C\mathit{H}_3)_2$).

Synthesis of CP. 4-Chloropyridine hydrochloride (1.0g, 6.66 mmol) and 2-amino-5-diethylaminopentane (8.06 g, 50.88 mmol) were heated at 100°C for 1 hour, 155°C for 3 hours, and 135°C for 16 hours. Reaction was then cooled to room temperature and the unreacted excess amine was removed by Kugelrohr distillation of the crude reaction mixture at 120°C under vacuum. The remaining crude brown oil was then dissolved in $CHCl_3$ and purified by preparative TLC using $CHCl_3/CH_3OH/NH_4OH$ (5/1/0.2) as an eluent to yield a colorless oil (24%) ^1H NMR ($CDCl_3$) δ 8.14 (1H, dd, *2,6-Py-H*), 6.37 (1H, dd, *3,5-Py-H*), 4.44 (1H, br., Py-*NH*), 3.51 (1H, m, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 2.54-2.41 (6H, m, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 1.18-1.54 (4H, m, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 1.19 (3H, d, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$), 1.02 (6H, d, -$NHCH(CH_3)CH_2CH_2CH_2N(CH_2CH_3)_2$).

Synthesis of CQO. NaH (60% dispersion in mineral oil, 0.234 g, 5.84 mmol) was added to a flame-dried round bottom. Anhydrous DMF (16.1 mL) was added to the flask under N_2. To this suspension was added dropwise a solution of N,N-diethyl-2-aminoethanol (0.71 mL, 5.35 mmol) in DMF (16.1 mL) and the resulting mixture was stirred 30 minutes (gas evolution had stopped). A solution of 4,7-dichloroquinoline (0.96 g, 4.87 mmol) in DMF (14.6 mL) was then added to the reaction. Reaction was complete after 30 minutes as monitored by TLC. The reaction mixture was then poured into 200 mL water. The product was extracted with EtOAc (3 × 100mL). The organic layer was then dried over Na_2SO_4, filtered, and concentrated under vacuum to give the title compound in 95% yield (1.29 g). ^1H NMR ($CDCl_3$) δ 8.72 (1H, d, *1-Ar-H*), 8.12 (1H, d, *5-Ar-H*), 8.0 (1H, d, *8-Ar-H*), 7.44 (1H, dd, *6-Ar-H*), 6.74 (1H, d, *2-Ar-H*), 4.28 (2H, t, -$OCH_2CH_2N(CH_2CH_3)_2$), 3.15 (2H, t, -$OCH_2CH_2N(CH_2CH_3)_2$), 2.71 (4H, q, -$OCH_2CH_2N(CH_2CH_3)_2$), 1.11 (6H, t, -$OCH_2CH_2N(CH_2CH_3)_2$)

Transfection of naked pDNA

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 appropriate amount of **CQ** (or **CQ** analogues) solution, and 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 1x cell culture lysis buffer (Promega). Cell lysates were analyzed for luciferase activity with luciferase assay reagent (Promega). Light units were integrated over 10 s in duplicate with a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA).

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.

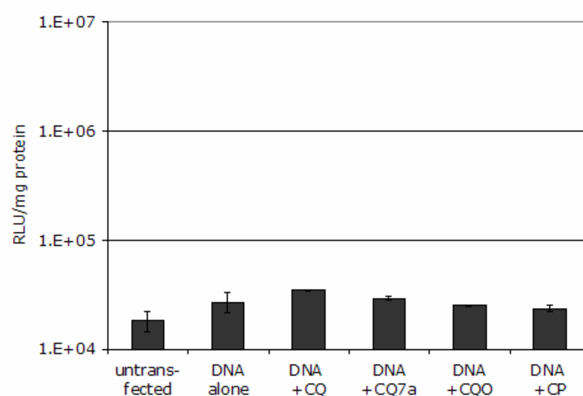

Figure S1. Effect of **CQ** analogues on transfection of HeLa cells with pDNA. HeLa cells were transfected with pDNA in the presence of various **CQ** analogues (0.1 mM). Luciferase activity was used as a measure of transfection efficiency.

Transfection of CDP/pDNA in HepG2 cells in the presence of CQ and CQ analogues

HepG2 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 (5 μL of 24.9 mg/mL freshly prepared CDP solution in DNAse-free water) to give charge ratio 5 +/-. Opti-MEM (185 μL) and an appropriate amount of **CQ** (or **CQ** analogues) in 5 μL 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) (Figure S2)

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 (Figure S2).

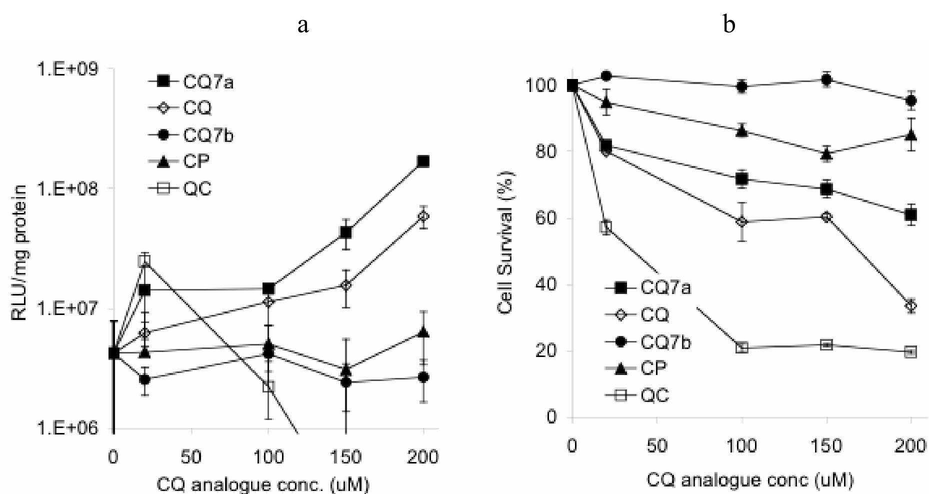

Figure S2. Effect of **CQ** analogues on transfection of HepG2 cells. HepG2 cells were transfected with CDP/pDNA polyplexes in the presence of various **CQ** analogues. Luciferase activity was used as a measure of transfection efficiency (a) and total protein was used as a measure of cell viability (b).

Transfection of CDP/pDNA in BHK and A2780 cells in the presence of CQ and CQ analogues

Transfection and expression of CDP/pDNA in BHK and A2780 cells in the presence or absence of **CQ** or **CQ7a** was similarly carried out as in HepG2 cells (see procedure in previous section) (Figure S3).

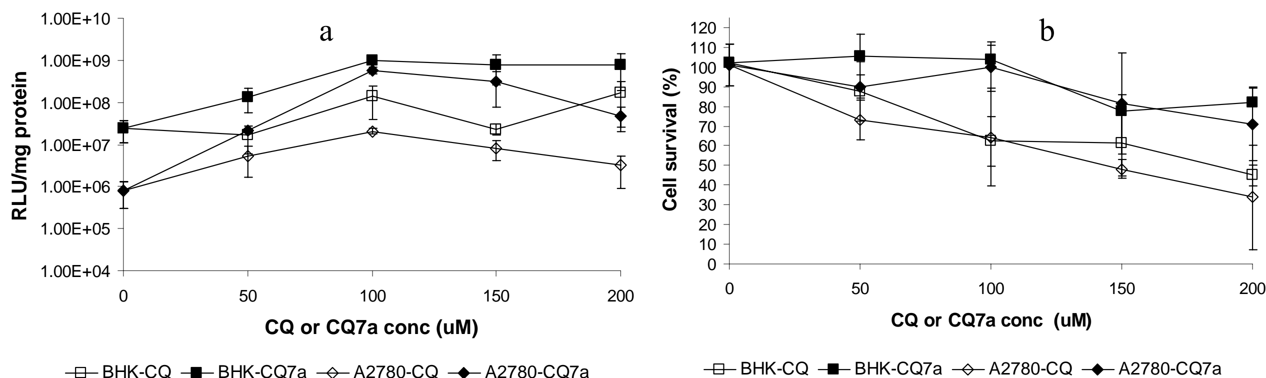

Figure S3. Effect of **CQ** analogues on transfection of BHK and A2780 cells. BHK or A2780 cells were transfected with CDP/pDNA polyplexes in the presence of **CQ** or **CQ7a** at variable concentrations. Luciferase activity was used as a measure of transfection efficiency (a) and total protein was used as a measure of cell viability (b).

Transfection of Poly-lysine/pDNA in the presence of CQ and CQ7a

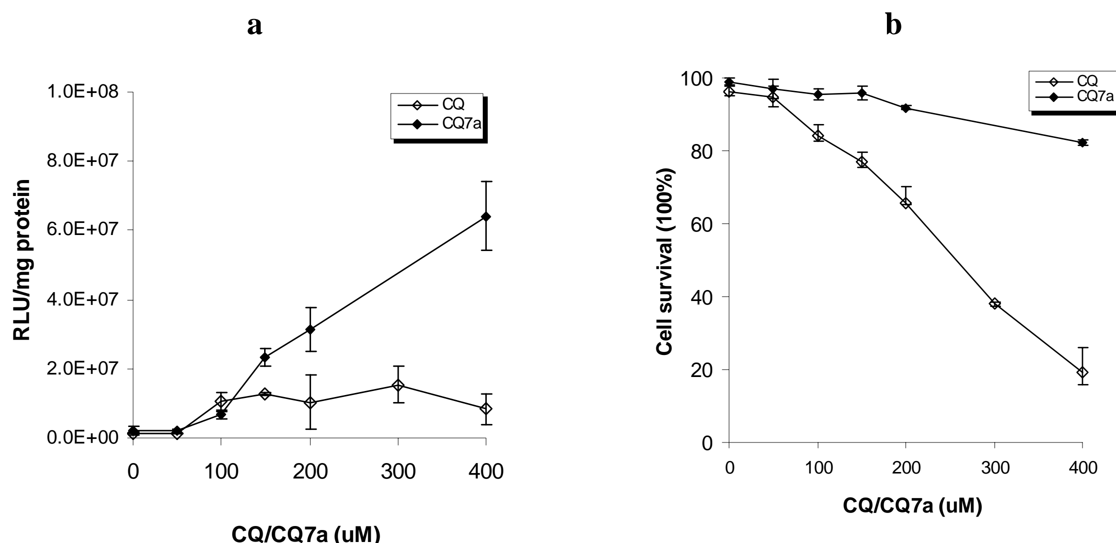

Figure S4. Effect of **CQ** and **CQ7a** on transfection of Poly-L-Lysine/pDNA in HepG2 cells. HepG2 cells were transfected in the presence of **CQ** or **CQ7a** at variable concentrations. Luciferase activity was used as a measure of transfection efficiency (a) and total protein was used as a measure of cell viability (b).

Transfection of Poly-lysine (MW 15-30 kDa, Sigma)/pDNA (N/P = 15/1) in HepG2 cells (Figure S4) in the presence of **CQ** and **CQ7a** is similarly carried out as CDP/pDNA in HepG2 cells (see previous description).

Uptake of polyplexes in the absence or presence of CQ analogues

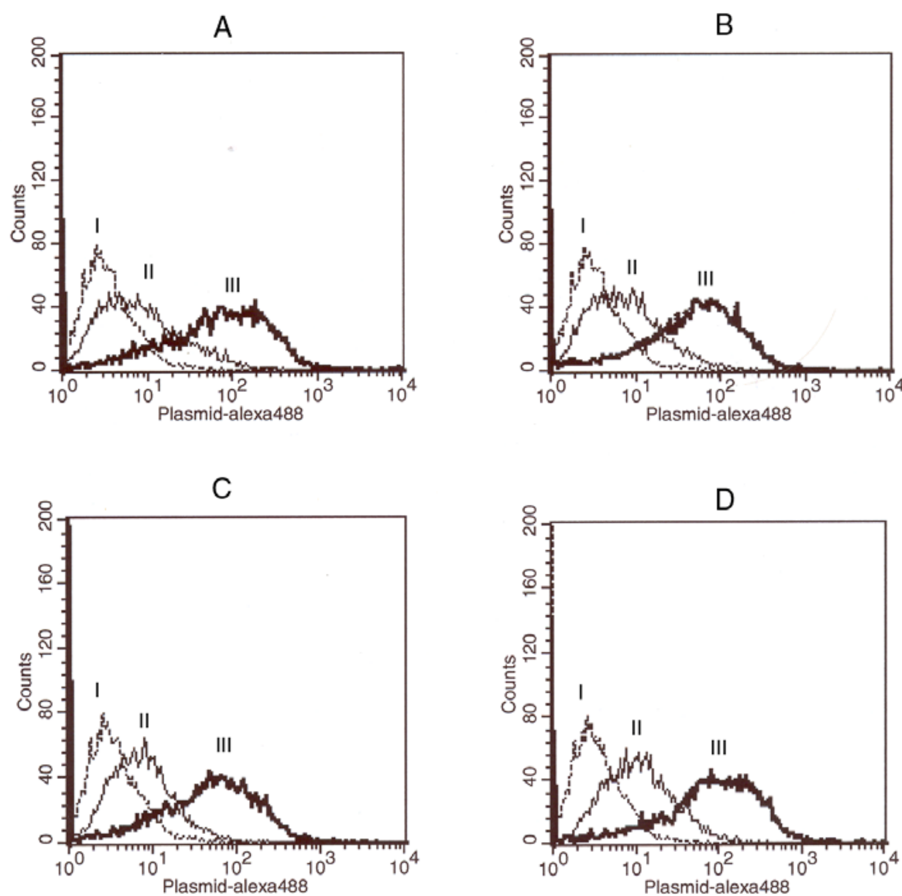

Figure S5 Flow cytometry analysis of polyplex uptake in the presence of **CQ** analogues**.** pDNA labeled with Alexa-fluor-488 was condensed with CDP and administered to HepG2 cells. Uptake was terminated at 30 min and 2 h and cells were analyzed by flow cytometry. Uptake of polyplexes was similar in the absence of any **CQ** analogue (a) or in the presence of 200 μM **CQ** (b), 200 μM **CQ**7b (c), or 200 μM **CQ7a** (d). The cellular fluorescence was analyzed at 0 min (I), 30 min (II), or 2 h (III).

pDNA was prepared for fluorophore binding by reacting with Label-IT Amine (Mirus, Madison, WI). After 1 hour at 37 °C, the amine-terminated DNA was recovered by precipitation with ethanol, 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 (λexcitation 488 nm; λemission 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 hours. 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) (Figure S5).

References:

(1) Surrey, A.; Hammer, H. F. The Preparation of 7-Chloro-4-(4-(N-ethyl-N-ß-hydroxyethylamino)-1-methylbutylamino)-quinoline and Related Compounds. *J. Am. Chem. Soc.* **1950**, *72*, 1814-1815.