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 (CHCl3/CH3OH/Et3N (25/1/1)) was used as eluent. CQ4a was isolated in 64% yield after combining elution fractions. CQ4a was further purified by crystallization (CHCl3 and hexane) to give 120 mg (13% yield) of white solid. 1H NMR (CDCl3) 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(CH3)CH2CH2CH2CH(CH3)CH3), 1.67-1.43 (5H, m, -NHCH(CH3)CH2CH2CH(CH3)CH3), 1.32 (3H, d, -NHCH(CH3)CH2CH2CH2CH(CH3)CH3), 1.23 (2H, d, -NHCH(CH3)CH2CH2CH2CH(CH3)CH3), 0.87 (6H, d, -NHCH(CH3)CH2CH2CH2CH(CH3)CH3).
**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%).

\[ ^1 \text{H NMR (CD}_3\text{OD)} \delta 8.43 (1H, d, \text{1-Ar-H}), 8.33 (1H, d, \text{5-Ar-H}), 7.76 (1H, d, \text{8-Ar-H}), 7.56 (1H, dd, \text{6-Ar-H}), 6.66 (1H, d, \text{2-Ar-H}), 4.26 (1H, m, \text{NHCCH(CH}_3\text{)}_2\text{COOH}), 1.66 (3H, d, \text{NHCH(CH}_3\text{)}_2\text{COOH}) \]

**Synthesis of CQ4c.** CQ4c was synthesized using the same method as CQ4a with corresponding amine (26%).

\[ ^1 \text{H NMR (CDCl}_3\text{)} \delta 8.53 (1H, d, \text{1-Ar-H}), 7.94 (1H, d, \text{5-Ar-H}), 7.70 (1H, d, \text{8-Ar-H}), 7.36 (1H, dd, \text{6-Ar-H}), 6.35 (1H, d, \text{2-Ar-H}), 5.92 (1H, br., \text{Ar-NH}), 3.28 (2H, t, \text{-NHCCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{)}, 2.68 (2H, t, \text{-NHCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{)}, 2.30 (6H, s, \text{-NHCH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\text{)}. \]

**Synthesis of CQ4d.** 4,7-Dichloroquinoline (620 mg, 3.1mmol) and N\text{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\text{2Cl}_2/\text{CH}_3\text{OH/NH}_4\text{OH} (70/28/2), 200 mL) was used as eluent, followed by another eluent (CH\text{2Cl}_2/\text{CH}_3\text{OH/NH}_4\text{OH} (5/20/4), 200 mL). The fractions containing CQ4d (R\text{f} = 0.1 in CH\text{2Cl}_2/\text{NH}_4\text{OH/CH}_3\text{OH} (40/1.5/80)) were collected, combined and subjected to another silica gel column separation (CHCl\text{3}/\text{CH}_3\text{OH /Et}_3\text{N} (10/1/1), 200 mL) to give a pale yellow oil (35%).

\[ ^1 \text{H NMR (CD}_3\text{CD)} \delta 8.52 (1H, d, \text{1-Ar-H}), 7.94 (1H, d, \text{8-Ar-H}), 7.70 (1H, d, \text{5-Ar-H}), 7.35 (1H, dd, \text{6-Ar-H}), 6.32 (1H, d, \text{2-Ar-H}), 5.98 (1H, br., \text{Ar-NH}), 3.36-2.07 (12H, m, \text{-NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHCH(Ch}_3\text{)}_2\text{)}, 1.07 (6H, d, \text{-NHCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHCH(Ch}_3\text{)}_2\text{)}. \]
**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 (CH₂Cl₂/CH₃OH/NH₄OH (70/28/2), 800 mL) was used as eluent, followed by elution in CH₂Cl₂/CH₃OH/NH₄OH ((5/20/4), 200 mL) and CH₃OH/NH₄OH ((1/1), 200 mL). Three different compounds were observed on TLC (CH₂Cl₂/CH₃OH/NH₄OH (5/20/4)). The fraction with Rf = 0.33 was found to be CQ4e. The combined fractions were transferred to another silica-gel column and eluted with (CHCl₃/CH₃OH/Et₃N (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%). ¹H NMR (CD₃CD) 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, -NHCH₂CH₂CH₂N(CH₂CH₂)₂NCH₂CH₂CH₂NH₂), 2.41-2.69 (14H, m, -NHCH₂CH₂CH₂N(CH₂CH₂)₂NCH₂CH₂CH₂NH₂), 1.93 (2H, m, -NHCH₂CH₂CH₂N(CH₂CH₂)₂NCH₂CH₂CH₂NH₂), 1.64 (2H, m, -NHCH₂CH₂CH₂N(CH₂CH₂)₂NCH₂CH₂CH₂NH₂).

**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 (CH₂Cl₂/CH₃OH/NH₄OH (70/28/2), 800 mL) was used as eluent, followed by elution with CH₂Cl₂/CH₃OH/NH₄OH ((5/20/4), 200 mL) and CH₃OH/NH₄OH ((1/1), 200 mL). Three different compounds were observed on TLC (CH₂Cl₂/CH₃OH/NH₄OH (5/20/4)). The fraction with Rf = 0.2 was identified to be CQ4f. The combined
fractions with Rf = 0.2 were transferred to another column and eluted with (CHCl3/CH3OH/NH4OH (20/5/1), 200 mL). Clean fractions were collected and combined. Solvent was removed under vacuum to give a colorless oil (15%). 1H NMR (CDCl3) 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, NHCH2CH2N(CH2CH2NH2)2), 2.89-2.68 (6H, m, NHCH2CH2N(CH2CH2NH2)2), 2.63 (4H, t, NHCH2CH2N(CH2CH2NH2)2), 1.59 (5H, br, NHC(H3)CH2CH2N(CH2CH2NH2)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 CHCl3 and purified by preparative TLC using CHCl3/CH3OH/Et3N (25/1/1) as an eluent to yield CQ7a as a colorless oil (32%). 1H NMR (CDCl3) 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), 5.55 (1H, m, Ar-NH), 3.75 (1H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 2.56 (6H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.80 (2H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.67 (2H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.36 (3H, d, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.06 (6H, d, NHCH(CH3)CH2CH2N(CH2CH2NH2)2).

**Synthesis of CQ7b.** CQ7b was synthesized using the same method as CQ4a using 4-chloroquinoline and 2-amino-5-diethylaminopentane (10%). 1H NMR (CDCl3) 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, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 2.57-2.46 (6H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.76 (2H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.62 (2H, m, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.31 (3H, d, NHCH(CH3)CH2CH2N(CH2CH2NH2)2), 1.08 (6H, d, NHCH(CH3)CH2CH2N(CH2CH2NH2)2).
**Synthesis of CP.** 4-Chloropyridine hydrochloride (1.0 g, 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 CHCl₃ and purified by preparative TLC using CHCl₃/CH₃OH/NH₄OH (5/1/0.2) as an eluent to yield a colorless oil (24%) ¹H NMR (CDCl₃) 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(CH₃)CH₂CH₂CH₂N(CH₂CH₃)₂), 2.54-2.41 (6H, m, -NHCH(CH₃)CH₂CH₂CH₂N(CH₂CH₃)₂), 1.18-1.54 (4H, m, -NHCH(CH₃)CH₂CH₂CH₂N(CH₂CH₃)₂), 1.02 (6H, d, -NHCH(CH₃)CH₂CH₂CH₂N(CH₂CH₃)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 N₂. 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 Na₂SO₄, filtered, and concentrated under vacuum to give the title compound in 95% yield (1.29 g). ¹H NMR (CDCl₃) 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, -OCH₂CH₂N(CH₂CH₃)₂), 3.15 (2H, t, -OCH₂CH₂N(CH₂CH₃)₂), 2.71 (4H, q, -OCH₂CH₂N(CH₂CH₃)₂), 1.11 (6H, t, -OCH₂CH₂N(CH₂CH₃)₂).

**Transfection of naked pDNA**

For cell transfection and luciferase assay, cells were plated at 5×10⁴ 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% CO₂), 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.

![Light units](image)

**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 5×10⁴ 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 amount of

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volume of polymer (5 µL of 24.9 mg/mL freshly prepared CDP solution in DNAse-free water) to give charge ratio 5 +/- 0. 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% CO₂), 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 polypeptides 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**

![Graphs](image)

**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 CQ7b (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 ($\lambda_{\text{excitation}}$ 488 nm; $\lambda_{\text{emission}}$ 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 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: