Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Synthesis of AZT-Based Cationic Lipids and In Vitro Evaluation of siRNA Delivery

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Experimental Part

General Information

All chemicals were obtained from Aldrich Chemical Company or Novabiochem and were used without further purification. Each reaction was executed under an inert atmosphere of dry argon and using glassware that was flame-dried under vacuum. Flash chromatography was performed using silica gel 60 (230–400 mesh; ASTM). Melting points are uncorrected and were obtained using an Electrothermal 1A 9000 series apparatus. FTIR spectra were recorded on a Brucker model FT-IR PS55+ spectrometer. High-resolution FAB^+ mass spectra were obtained using a JEOL JMS-AX505WA (FAB) spectrometer.

^1H and ^{13}C NMR spectra were recorded using a Bruker Aspect 300 NMR spectrometer. Chemical shifts these spectra are reported in parts per million (ppm) downfield relative to the internal standard, tetramethylsilane (TMS). Coupling constants are reported in hertz (Hz). Spectral splitting patterns are designed as s, singlet; d, doublet; dd, double doublet; dt, distorted triplet; t, triplet; m, multiplet; and br, broad.

3'-[4-(Methoxycarbonyl)-1*H*-1,2,3-triazol-1-yl]-thymidine (2)

In H_2O/*t*-BuOH (1:1) 50 ml, AZT (**1**) (1.00 g, 3.74 mmole) was dissolved, then $[Cu(CH_3CN)_4]PF_6$ (462 uL, 1.5 eq), and propiolic methyl ester (70 mg, 5 mol%) was added. The reaction mixture was stirred for 9 hours at 50-60 °C. The reaction mixture was concentrated in vacuo. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (80:0→30:1, v/v) as eluent to give **2** as a yellow solid. yield; 1.18 g (91%)

^1H NMR (300 MHz, $CDCl_3$): δ 8.34 (s, 1H), 7.63(s, 1H), 6.32(t, *J*=6.50 Hz, 1H), 5.47(m, 1H), 4.41(m, 1H), 3.77(m, 2H), 3.40(s, 3H), 2.91(m, 2H), 1.94(s, 3H); ^{13}C NMR(75 MHz, $CDCl_3$): δ 166.5, 162.4, 152.5, 140.8, 138.4, 129.7, 111.9, 86.9, 86.4, 62.3, 61.7, 52.8, 39.2, 12.6 ; HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{14}H_{17}N_5O_6$: 352.1257; found: 352.1257 $(M+H)^+$.

3′-[1*H*-1,2,3-triazole-4-didodecanopeptidyl]-thymidine (3a)

In CH_2Cl_2/DMF (1:1) 50 ml, compound **2** (416 mg, 1.19 mmole) was dissolved, and then NaOH (20 mg, 1.5 eq) was added. The reaction mixture was stirred for 9 hours at room temperature. The reaction mixture was neutralized by DOWEX-50 for 1 hr. The residue was filtered and concentrated in vacuo. The residue was dried in vacuo overnight and then dissolved in DMF. DCC (318 mg, 1.30 eq), HOBt (192 mg, 1.20 eq) was added to the solution. In the end, didodecylamine (510 mg, 1.2 eq) was applied. The reaction mixture was stirred for overnight at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (100:0→70:1, v/v) as eluent to give **3a** as a yellow solid. yield; 645 mg (81%).

^1H NMR (300 MHz, $CDCl_3$): δ 8.46 (s, 1H), 7.62 (s, 1H), 6.25 (t, *J*=6.60 Hz, 1H), 5.52 (m, 1H; C4), 4.44-4.46 (br, 1H), 3.45-3.49 (m, 2H), 2.96-3.03 (m, 2H), 1.96 (s, 3H), 1.31 (br, 40H), 0.89 (m, 10H); ^{13}C NMR(75 MHz, $CDCl_3$):

δ 163.8, 160.7, 150.7, 145.4, 137.9, 128.9, 111.6, 89.0, 85.5, 61.7, 59.6, 48.9, 47.4, 37.7, 32.1, 29.9, 29.6, 27.8, 27.3, 26.9, 22.9, 14.3, 12.7; HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{37}H_{64}N_6O_5$: 673.5016; found: 673.5016 $(M+H)^+$.

3′-[1*H*-1,2,3-triazole-4-dioctadecanopeptidyl]-thymidine (3b)

In CH_2Cl_2/DMF (1:1) 50 ml, compound **2** (260 mg, 0.74 mmole) was dissolved, and then NaOH (20 mg, 1.5 eq) was added. The reaction mixture was stirred for 9 hours at room temperature. The reaction mixture was neutralized by DOWEX-50 for 1 hr. The residue was filtered and concentrated in vacuo. The residue was dried in vacuo overnight and then dissolved in DMF. DDC (203 mg, 1.3 eq), HOBt (124 mg, 1.2 eq) was added to the solution. In the end, dioctadecylamine (480 mg, 1.2 eq) was applied. The reaction mixture was stirred for overnight at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (100:0→70:1, v/v) as eluent to give **3b** as a yellow solid. yield; 574 mg (86%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.41 (s, 1H), 7.45 (s, 1H), 6.22 (t, *J*=6.42Hz, 1H), 5.31-5.56 (m, 1H), 4.43-4.45 (m, 1H), 3.78-4.03 (m, 4H), 3.45-3.50 (m, 2H), 2.98-3.04 (m, 2H), 1.92 (s, 3H), 1.64-1.67 (br, 4H), 1.25-1.39 (br, 60H), 0.86-0.90 (m, 6H); ^{13}C NMR(75 MHz, $CDCl_3$): δ 164.1, 160.8, 150.8, 145.3, 137.9, 129.1, 111.5, 88.9, 85.4, 61.5, 59.5, 49.0, 47.5, 37.7, 32.1, 29.9, 29.7, 27.8, 27.3, 26.9, 22.9, 14.3, 12.6; HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{49}H_{88}N_6O_5$: 841.6894; found: 841.6894 $(M+H)^+$.

5′-*O*-{Boc-L-Orn (Boc)}-3′-[1*H*-1,2,3-triazole-4-didodecanopeptidyl]-thymidine (4a)

In CH_2Cl_2 50ml, compound **3a** (1.04 g, 1.55 mmole) was dissolved, and then Boc-L-Orn(Boc)-OH (534 mg, 1.61 mmole) and DMAP (19 mg, 0.15 mmole) was added. After that DCC (105 mg, 0.51 mmole) was added dropwise to the solution at 0 °C. The reaction mixture was stirred for 1 hour at 0 °C and then stirred for 4hours at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (100:0→70:1, v/v) as eluent to give **4a** as a pale yellow solid. yield; 1.12 g (73%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.31 (s, 1H), 7.18 (s, 1H), 6.08 (br s, 1H), 5.44 (br, 2H), 4.97 (br, 1H), 4.52 (br, 2H), 3.93-3.82 (m, 2H), 3.49-3.39 (m, 2H), 3.13 (br, 4H), 1.93 (s, 5H), 1.65-1.60 (m, 6H), 1.39 (br, 18H), 1.24 (br, 36H), 0.88-0.84 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.7, 163.7, 160.7, 156.4, 155.8, 150.2, 145.5, 137.6, 129.1, 111.7, 88.6, 82.1, 80.3, 79.6, 63.1, 59.9, 53.7, 48.9, 47.3, 40.2, 37.3, 32.1, 29.9, 29.8, 29.7, 29.6, 29.6, 28.6, 27.8, 27.4, 26.9, 26.5. 22.9, 14.3, 12.7; HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{52}H_{91}N_8O_{10}$: 987.68; found: 987.68 $(M+H)^+$.

5′-*O*-{Boc-L-Orn(Boc)}-3′-[1*H*-1,2,3-triazole-4-dioctadecanopeptidyl]-thymidine (4b)

In CH_2Cl_2 50ml, compound **3b** (282 mg, 0.33 mmole) was dissolved, and then Boc-L-Orn(Boc)-OH (123 mg, 0.37 mmole) and DMAP (4 mg, 0.03 mmole) was added. After that DCC (76 mg, 0.37 mmole) was added dropwise to the solution at 0 °C. The reaction mixture was stirred for 1 hour at 0 °C and then stirred for 4hours at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (100:0→70:1, v/v) as eluent to give **4b** as a pale yellow solid. yield; 342 mg (90%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.80 (br, 1H), 8.22 (s, 1H), 7.16 (s, 1H), 6.05 (br, 1H), 5.43-5.30 (br, 2H), 4.92 (br, 1H), 4.54-4.41 (br, 2H), 3.97-3.81, 3.51-3.40 (br, 6H), 3.16 (m, 2H), 1.95 (s, 5H), 1.68-1.61 (m, 6H), 1.41 (br, 18H), 1.25 (br, 36H), 0.90-0.85(m, 6H); ^{13}C NMR(75 MHz, $CDCl_3$): δ 172.6, 163.6, 160.6, 156.3, 155.7, 150.1, 145.4, 137.6, 129.1, 111.6, 88.6, 81.9, 80.3, 79.6, 77.4, 63.0, 59.8, 53.6, 48.9, 47.2, 40.1, 37.2, 32.1, 29.9, 29.8, 29.6, 29.6, 29.5, 28.8, 28.6 28.4, 27.7, 27.3, 26.9, 26.4, 22.8, 14.3, 12.6; HRMS-FAB (m/z): $(M+Na)^+$ calcd for $C_{64}H_{114}N_8O_{10}Na$: 1177.86; found: 1177.86 $(M+Na)^+$.

5′-*O*-{Boc-L-Lys(Boc)}-3′-[1*H*-1,2,3-triazole-4-didodecanopeptidyl]-thymidine (4c)

In CH_2Cl_2 50ml, compound **3a** (120 mg, 0.19 mmole) was dissolved, and then Boc-L-Lys(Boc)-OH (87 mg, 0.19 mmole) and DMAP (2.4 mg, 0.02 mmole) was added. After that DCC (56 mg, 0.27 mmole) was added dropwise to the solution at 0 °C. The reaction mixture was stirred for 1 hour at 0 °C and then stirred for 4hours at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (100:0→70:1, v/v) as eluent to give **4c** as a pale yellow solid. yield; 114 mg (64%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 9.13 (s, 1H), 8.30 (s, 1H), 7.20(s, 1H), 6.12(br, 1H, C1), 5.41-5.43 (m, 2H), 4.77 (br, 1H), 4.25 (br, 1H), 3.84-3.94 (m, 2H), 3.12 (br, 4H), 1.95 (s, 3H), 1.80 (s, 2H), 1.64-1.69 (m, 4H), 1.25-1.49 (m, 54H), 0.88 (m, 6H); HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{53}H_{92}N_8O_{10}$: 1001.7015; found: 1001.7015 $(M+H)^+$.

5′-*O*-{Boc-L-Lys(Boc)}-3′-[1*H*-1,2,3-triazole-4-dioctadecanopeptidyl]-thymidine (4d)

In CH_2Cl_2 50 ml, compound **3b** (230 mg, 0.26 mmole) was dissolved, and then Boc-L-Lys(Boc)-OH (119 mg, 0.26 mmole) and DMAP (4 mg, 0.03 mmole) was added. After that DCC (76 mg, 0.37 mmole) was added dropwise to the solution at 0 °C. The reaction mixture was stirred for 1 hour at 0 °C and then stirred for 4 hours at room temperature. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (100:0→70:1, v/v) as eluent to give **4d** as a pale yellow solid. yield; 222 mg (73%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 9.44 (s, 1H), 8.34 (s, 1H), 7.21 (s, 1H), 6.14(br, 1H, C1), 5.44-5.46 (m, 2H), 4.81 (br, 1H), 4.50 - 4.55 (br, 3H), 4.34 - 4.38 (br, 2H), 4.24 (br, 1H), 3.86 - 3.92 (m, 2H), 3.45 - 3.48 (m, 4H), 2.72 (br, 2H), 1.95 (s, 3H), 1.67 (m, 4H), 1.41 (br, 18H), 1.25 (br, 60H), 0.86-0.89 (m, 6H); ^{13}C NMR(75 MHz, $CDCl_3$): δ 171.1, 160.7, 149.8, 145.3, 128.9, 111.4, 98.4, 86.4, 81.9, 62.2, 53.8, 48.7, 47.1, 39.5, 37.7, 31.9, 29.7, 28.4, 27.2, 22.7, 19.4, 14.1, 12.5; HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{65}H_{116}N_8O_{10}$: 1169.8393; found: 1169.8393 $(M+H)^+$.

5′-*O*-L-Ornithinyl-3′-[1*H*-1,2,3-triazole-4-didodecanopeptidyl]-thymidine (5a)

In $CHCl_3$ 20 ml, compound **4a** (96 mg, 0.10 mmole) was dissolved, and then TFA (20%, v/v) was added to the solution. The reaction mixture was stirred for 5 hours at room temperature. The residue was evaporated and dried in vacuo. yield; 113 mg (Quant.).

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.59 (s, 1H), 8.41 (s, 1H), 7.88 (br, 6H), 7.21 (s, 1H), 5.92 (br, 1H), 5.61 (br, 1H), 4.63 (br, 1H), 4.56 (br, 1H), 4.29 (br, 1H), 4.19 (br, 1H), 3.76 (br, 2H), 3.43 (br, 2H), 3.05 (br, 4H), 2.10 (br, 2H), 1.90

(br, 2H), 1.79 (s, 3H), 1.69-1.60 (br, 4H), 1.24 (br, 36H), 0.88-0.84 (m, 6H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 168.9, 165.3, 161.6, 161.4, 161.3, 150.9, 143.9, 129.3, 117.3, 115.0, 112.7, 111.5, 81.7, 63.9, 59.2, 52.9, 49.5, 47.6, 39.4, 36.7, 32.1, 29.9, 29.9, 29.8, 29.6, 29.6, 27.6, 27.3, 26.9, 22.9, 14.3, 11.9; MS (LRFAB) (*m/z*) : $(M+H)^+$ calcd. for $C_{42}H_{75}N_8O_6$: 787.6 ; found: 787.7 $(M+H)^+$.

5′-*O*-L-Ornithinyl-3′-[1*H*-1,2,3-triazole-4-dioctadecanopeptidyl]-thymidine (5b)

In $CHCl_3$ 20 ml, compound **4b** (75.2 mg, 0.07 mmole) was dissolved, and then TFA (20%, v/v) was added to the solution. The reaction mixture was stirred for 5hours at room temperature. The residue was evaporated and dried in vacuo yield; 82 mg (Quant.).

^{1}H NMR (300 MHz, $CDCl_3$) δ 10.59 (s, 1H), 8.63 (br, 2H), 8.44 (s, 1H), 7.25 (s, 1H), 6.00 (br, 1H), 5.65 (br, 1H), 4.59 (br, 1H), 4.35 (br, 1H), 4.19 (br, 1H), 3.81 (br, 2H), 3.44 (br, 2H), 3.05-3.13 (br, 4H), 2.07 (br, 2H), 1.94 (br, 2H), 1.81 (s, 3H), 1.71-1.62 (br, 4H), 1.29-1.31 (br, 60H), 0.89-0.91 (m, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.6, 164.9, 161.7, 160.9, 150.7, 143.9, 139.4, 128.8, 119.5, 112.54, 111.3, 81.6, 63.6, 59.1, 52.7, 49.2, 47.4, 39.1, 36.4, 31.9, 29.7, 29.7, 29.4, 29.4, 29.2, 28.8, 27.4, 27.1, 26.7, 22.8, 22.7, 14.1, 11.7. HRMS-FAB (m/z): $(M+H)^+$ calcd. for $C_{54}H_{99}N_8O_6$: 955.76 ; found: 955.77 $(M+H)^+$.

5′-*O*-L-Lysinyl-3′-[1*H*-1,2,3-triazole-4-didodecanopeptidyl]-thymidine (5c)

In $CHCl_3$ 20 ml, compound **4c** (114 mg, 0.11 mmole) was dissolved, and then TFA (20%, v/v) was added to the solution. The reaction mixture was stirred for 5hours at room temperature. The residue was evaporated and dried in vacuo. yield; 120 mg (Quant.).

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.65 (s, 1H), 8.66 (s, 1H), 7.93 (br, 1H), 6.03 (br, 1H), 5.67-5.70 (m, 2H), 4.37-4.60 (m, 6H), 4.13 (br, 1H), 3.84 (br, 4H), 3.43 (br, 4H), 1.95 (s, 3H), 1.69 (s, 4H), 1.64-1.69 (m, 4H), 1.25-1.44 (m, 40H), 0.85-0.88 (m, 6H); HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{43}H_{76}N_8O_6$: 801.5966; found: 801.5966 $(M+H)^+$.

5′-*O*-L-Lysinyl-3′-[1*H*-1,2,3-triazole-4-dioctadecanopeptidyl]-thymidine (5d)

In $CHCl_3$ 20 ml, compound **4d** (80 mg, 0.07 mmole) was dissolved, and then TFA (20%, v/v) was added to the solution. The reaction mixture was stirred for 5hours at room temperature. The residue was evaporated and dried in vacuo. yield; 85 mg (Quant.).

^{1}H NMR (300 MHz, $CDCl_3$): δ 10.70 (s, 1H), 8.68 (s, 1H), 7.95 (br, 1H), 6.06 (br, 1H), 5.68 (br, 1H), 4.59 (br, 2H), 3.80-4.32 (m, 9H), 3.40 (br, 4H), 2.97 (br, 2H), 1.78 (s, 3H), 1.60-1.68 (s, 8H), 1.25-1.34 (m, 60H), 0.87-0.89 (m, 6H); ^{13}C NMR(75 MHz, $CDCl_3$): δ 169.0, 165.0, 162.4, 150.9, 144.5, 139.3, 114.8, 81.8, 68.4, 53.2, 49.3, 47.5, 45.9, 39.4, 36.7, 32.1, 29.9, 27.4, 23.2, 14.3, 8.7; HRMS-FAB (m/z): $(M+H)^+$ calcd for $C_{43}H_{76}N_8O_6$: 801.5966; found: 801.5966 $(M+H)^+$.

Lipoplex preparation

Cationic lipids stock solutions were prepared by solubilizing the compounds in H_2O/MeOH (10:1). The mixtures were vortexed for 30 s and incubated for 30 min at room temperature before adding the siRNAs. Lipoplexes were then prepared by dilution the stock solution with DMEM and mixing the corresponding siRNA quantities of each formulation with N/P ratio. Finally, the formulations were mixed and incubated for 30 min at room temperature before use.

MTT assay

Lipids (1.25-125 μM with N/P ratio of 6.25) were complexed with VEGF siRNA (certain concentration as for lipid) and added to the cells. Cells were incubated with complexes at 37 °C and 5% CO_2 for 24 h. After incubation, media were removed, the cells washed with 100 mL of DPBS buffer (2 times) and then a 100 μL of MTT solution (1 mg/mL of MTT dissolved in phenol red free medium, Sigma) was added to each well and incubated at 37 °C for 4 h. After incubation, 100 μL DMSO was added to each well and absorbances were measured at a wavelength of 570 nm using a microplate reader (Asys) and converted to percentage of cell viability (relative to control cells).

Transfection experiment

HeLa cells were cultured in Dulbecco's modified Eagle's medium (HyClone), supplemented with 10% fetal bovine serum (HyClone), 100 μg/mL of streptomycin, and 100 U/mL of penicillin at 37 °C in 5% CO_2 incubator. Cells were split, using trypsin/EDTA medium when almost confluent. HeLa cells were seeded at a density of 2.5 x 10^5 cells per well, each well of which contained 2 mL of 10% FBS supplemented DMEM, and incubated for 4 hours. HeLa cells were transfected in the absence of serum with VEGF siRNAs using lipid (**5a** and **5d**), Lipofectamine™ 2000 (Invitrogen), or without any transfection reagent. The cells were allowed to incubate at 37°C for 6 hours in CO_2 incubator followed by replacement of 2 mL of DMEM containing 10% FBS. After more 18hrs incubation, cell medium were collected and analyzed the VEGF expression level by using VEGF ELISA kit (QIA51 VEGF ELISA Kit, Human, Calbiochem).

RT-PCR experiment

VEGF siRNA-treated cells were washed by PBS and lyzed in 1.0 ml of TRIzol®-reagent (Invitrogen) and total RNA was isolated. A 1 μg sample of RNA was used in reverse transcription with Improm-II™ Reverse Transcription System (Promega) and the procedures were performed by the manufacturer's protocols. The reverse transcription reaction was carried out at 25 °C (5 min), 42 °C (60 min) and 70 °C for 15 min followed by PCR: 1 cycle, 95 °C, 5 min; 30 cycles, 95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 1 cycle, 72 °C, 5 min. For VEGF mRNA amplification forward and reverse primers were 5'-GGGCAGAATCATCACGAAGT-3' and 5'-TGGTGATGTTGGACTCCTCA-3', respectively. The PCR products were analyzed by electrophoresis on a 1.5 % agarose gel (80V, 50 min) stained with ethidium bromide.

Confocal microscope experiment

At first, cover glasses (1.13 cm^2, Deckglaser) were put into 6-well plate, and the plate was coated with 0.2% gelatin. HeLa cells were seeded at a density of 2.5 x 10^5 cells per well, each well of which contained 2 mL of 10% FBS supplemented DMEM, and incubated for 12 hours. HeLa cells were transfected in the absence of serum with 100 nM of FITC-tagged VEGF siRNA using Lipofectamine™ 2000 (Invitrogen), lipid **5d** (N/P ratio: 10) or without any reagent. The cells were allowed to incubate at 37°C in the presence of oligonucleotides for 4 hours in CO_2 incubator followed by replacement of 2 mL of DMEM containing 10% FBS. For preparation, 4 hours after transfection, cells were washed with DPBS, and then cover glasses were detached from bottom of plate. The cells on cover glass were fixed with 3.5% paraformaldehyde solution. After additional washing with DPBS, the cover glasses were transferred onto slide glass. Confocal images were obtained from the fixed cell by using Olympus FluoView™ FV1000 confocal microscope (*Olympus Optical Co., Ltd.,* Tokyo).

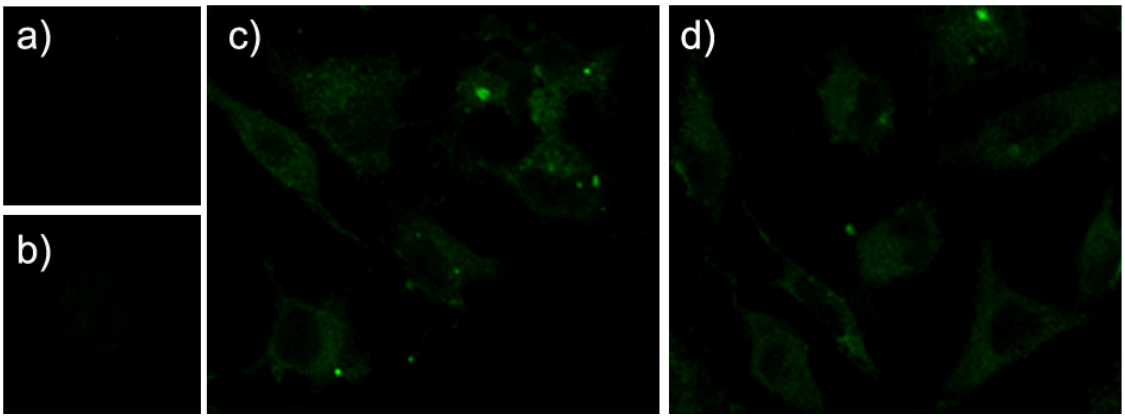

Fig. S1 Confocal images of a) HeLa cell; b) HeLa cell incubated with 100 nM FITC-tagged VEGF siRNA; c) HeLa cell incubated with 100 nM FITC-tagged VEGF siRNA and lipofectamine; d) HeLa cell incubated with 100 nM FITC-tagged VEGF siRNA and lipid **5d**.