Anisamide-targeted cyclodextrin nanoparticles for siRNA delivery to prostate tumours in mice

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Abstract

A hepta-guanidino-β-cyclodextrin (G-CD), its hepta-PEG conjugate (G-CD-PEG), and the corresponding anisamide-terminated PEG conjugate (G-CD-PEG-AA) have been synthesised and compared as delivery vectors for siRNA to prostate cancer cells and tumours in vivo. The G-CD-PEG-AA.siRNA formulations (in which anisamide targets the sigma receptor), but not the non-targeted formulations, induced prostate cell-specific internalisation of siRNA resulting in approximately 80% knockdown in vitro of the reporter gene, luciferase. Following intravenous administration of the anisamide-targeted formulation in a mouse prostate tumour model significant tumour inactivation with corresponding reductions in the level of vascular endothelial growth factor (VEGF) mRNA were achieved, without demonstrating enhanced toxicity. This data imply significant potential for anisamide-conjugated cyclodextrin vectors for targeted delivery of therapeutic siRNAs in the treatment of prostate cancer.

1. Introduction

As a leading cause of cancer-related deaths (over 32,000 fatalities annually in the male population of the United States [1]), prostate cancer is an obvious disease state demanding safe and effective treatment. The expanding knowledge of the genetic basis of cancer has recently promoted the application of RNA interference (RNAi) as a new generation of cancer therapeutics [2,3]. However, the clinical potential of siRNA is greatly retarded by inept delivery [4-7].

We have developed a strategy which uses cyclodextrins (CDs) as core molecules for gene delivery [8-13]. There are particular advantages associated with this approach: cyclodextrins have low immunogenicity and are unique among oligosaccharides in providing chemically equivalent multiple sites for attachment of functional groups such as ions, lipophiles, polyethylene glycol and targeting ligands [14-17].

The sigma receptor, a membrane bound protein, is known to be overexpressed on many human malignancies including prostate cancer [18]. This receptor binds haloperidol and various other neuroleptics showing a high affinity with the anisamide moiety. Anisamide-conjugated nanoparticles have achieved site-specific delivery of siRNAs in certain cancers [19-21]. The aim of this study was to synthesise an anisamide-targeted pegylated cyclodextrin and to evaluate this as a delivery vector for siRNA in prostate tumours. To achieve this, a hepta-guanidino-β-cyclodextrin (G-CD) was grafted to polyethylene glycol chains (giving G-CD-PEG), which acted as a spacer for the attachement of the anisamide-targeting moiety (in G-CD-PEG-AA). The presence of the cationic guanidino group enables binding and charge neutralization of the siRNA and the presence of the PEG is designed to stabilise the nanoparticles in physiological environments thereby prolonging their systemic circulation.

In most tumours new blood vessels are formed via a process termed angiogenesis, from pre-existing vasculature [3]. Consequently, VEGF (one of the most important angiogenesis oncogenes) has been investigated as an anti-angiogenesis target [22-25]. Accordingly, complexes of the anisamide-targeted cyclodextrin with anti-VEGF siRNA

were investigated here for their efficacy in a mouse prostate carcinoma model.

2. Materials and methods

2.1 Synthesis of the modified cyclodextrins

Monomethyl-PEG_{500} and 1-amino-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontyl 32-azide were obtained from Aldrich. To simplify nomenclature, 3,6,9,12,15,18,21,24,27,30-decaoxadotriacontyl is referred to as 1-deoxy-PEG_{440}-yl.

tert-Butyl-N-(2-mercaptoethyl) carbamate [26] and anisic acid N-hydroxysuccinimide [27] were synthesised by literature methods. Heptakis(6-bromo)-β-cyclodextrin and heptakis(6-O-*tert*-butyldimethylsilyl)-β-cyclodextrin [28,29] were synthesised by literature methods.

Heptakis(6-O-tert-butyldimethylsilyl-2-O-propargyl)-β-cyclodextrin **(7)** and heptakis(6-bromo-6-deoxy-2-O-propargyl)-β-cyclodextrin **(8)** were synthesised as previously described by us [13].

Three new CD-based compounds were synthesised as described below. In compound **5** the CD was modified on the primary face with guanidino to give a cationic cyclodextrin (G-CD) (Figure1a). A separate synthetic process, as outlined in Figure 1b, resulted in the production of two further CDs. Both of these CDs retain the cationic guanidino on the primary side but are further modified on the secondary side initially with a PEG chain resulting in a PEGylated cationic CD (G-CD-PEG) **(11a).** This PEG chain was then used as a linker for attachement of the targeting ligand anisamide to produce the third compound (G-CD-PEG-AA) **(11b)** (Figure 1b).

2.1.1 Heptakis(6-Boc-aminoethylthio)-β-cyclodextrin **(2)**

tert-Butyl-N-(2-mercaptoethyl)carbamate (2.8313 g, 16 mmol, 19.5 eq.) was dissolved in 50 ml of anhydrous DMF. Sodium hydride (38 mg, 16 mmol, 19.5 eq.) was added and the mixture was stirred at 60 °C for 2 hours under nitrogen. Heptakis(6-bromo)-β-cyclodextrin (1.3 g, 0.8 mmol, 1 eq.) (dried under vacuum

overnight) was added to the solution and the mixture stirred at 60 °C overnight. The solvent was evaporated and product precipitated in cyclohexane. The precipitate was washed several times with water and cyclohexane to give product **(2)** in quantitative yield.

^{1}H NMR (400 MHz, DMSO-d_6) δ 6.65-6.85 (m, 1H), 5.65-6.05 (m, 3H), 4.75-4.90 (m, 1H), 3.65-4.1 (m, 2H), 3.50-3.65 (m, 1H), 3.20-3.50 (m, 3H), 3.00-3.15 (m, 2H), 2.50-2.70 (m, 2H), 1.25-1.45 (m, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 155.93, 102.71, 78.15, 72.85, 40.45, 39.89, 38.10, 34.44, 33.00, 30.50, 28.66.

2.1.2 Heptakis(6-Boc-aminoethylthio-2-O-propargyl)-β-cyclodextrin **(3)**

Heptakis(6-Boc-aminoethylthio)-β-cyclodextrin **(2)** (309 mg, 0.14 mmol, 1 eq), dissolved in 40 ml of anhydrous DMF, was cooled to 0 °C before addition of 33 mg (1.37 mmol, 10 eq) of sodium hydride (95% in oil). After stirring for 12 hours at 0 °C, then at room temperature (RT), the solution was cooled to 0 °C and 150 µl (1.37 mmol, 10 eq) of propargyl bromide (80% in toluene) was added before stirring at RT for 12 hours. The solution was concentrated under reduced pressure and the residue purified by column chromatography over SiO2 (ethyl acetate-cyclohexane 1:1) to give product **(3)** (66 mg, 20%).

1H NMR (400 MHz, CDCl3) δ 5.32 (m, 1H), 5.01 (m, 1H), 4.80 (m, 1H), 4.55 (m, 2H), 3.90 (m, 2H), 3.66 (m, 2H), 3.48 (m, 1H), 3.32 (m, 2H), 3.09 (m, 1H), 2.75 (m, 2H), 2.51 (m, 1H), 1.44 (m, 9H).

^{13}C NMR (101 MHz, CDCl3) δ 155.88, 101.43, 85.77, 79.19, 78.56, 75.07, 73.18, 70.84, 59.51, 40.03, 33.42, 31.61, 29.62, 28.49. MALDI-MS: 2507.11 $[M+H]^+$, 2529.53 $[M+Na]^+$.

2.1.3 Heptakis(6-aminoethylthio-2-O-propargyl-)-β-cyclodextrin trifluoroacetate **(4)**

Heptakis(6-Boc-aminoethylthio-2-O-propargyl)-β-cyclodextrin **(3)** (66 mg) was dissolved in 50 ml of dichloromethane and 500 µl of trifluoroacetic acid was added.

The solution was stirred at 0 °C for 5 hours. The solution was concentrated under reduced pressure and purified by size exclusion chromatography over SEPHADEX LH20 (methanol, 100) (Rf = 0.05 CH_2Cl_2/MeOH 8/2) to give product **(4)** in quantitative yield. MALDI-MS: 1817.06 $[M+H]^+$, 1839.31 $[M+Na]^+$.

2.1.4 Heptakis(6-guanidinoethylthio-2-O-propargyl-)-β-cyclodextrin trifluoroacetate **(5)**

2-Propargyl-6-thioethylamine-CD (55 mg, 0.02 mmol, 1 eq) was dissolved in 5mL of dichloromethane. 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine (52 mg, 0.133 mmol, 6.3 eq) (Aldrich) was added with 24 µl of triethylamine.

The solution was stirred under N_2 at room temperature for 12 hours. The solution was diluted with 10 ml of dichloromethane and extracted twice with potassium bisulfide and brine. The solution was concentrated under reduced pressure and purified by column chromatography over SiO_2 (CH_2Cl_2/MEOH 95/5 – Rf = 0.40 90/10 CH_2Cl_2/MEOH).

^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (s, 1H), 8.60 (s, 1H), 5.00 (s, 1H), 4.74 (s, 1H), 4.53 (m, 2H), 3.80-4.01 (m, 2H), 3.54-3.76 (m, 4H), 2.72-3.07 (m, 2H), 2.51 (s, 1H), 1.44-1.57 (m, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.58, 156.03, 153.01, 101.43, 82.98, 79.23, 79.07, 75.51, 73.18, 71.16, 59.47, 53.42, 33.09, 29.70, 27.86.

The previous product was dissolved in 5 ml of dichloromethane and 500 µl of TFA. The solution was stirred at 0 °C for 5 hours. The solution was concentrated under reduced pressure and purified by size exclusion chromatography over SEPHADEX LH20 (methanol, 100) to yield 19.1 mg of 2-propargyl-6-thioethylguanidine-CD **(5).**

^{1}H NMR (400 MHz, CD_3OD) δ 7.42 (s, 2H), 5.02 (m,1H), 4.53 (m, 2H), 4.16 (m, 2H), 3.78-3.98 (m, 2H), 3.61-3.71 (m, 1H), 3.50-3.59 (m, 1H), 3.38-3.46 (m, 1H), 3.00-3.14 (m, 1H), 2.71-2.96 (m, 2H), 2.59-2.68 (m,1H). MALDI-MS: 2109 $[M+H]^+$.

2.1.5 ω-Methoxy-O-methylsulfonyl-PEG_{500}

Monomethyl-PEG_{500} 1.42 g (2.57 mmol, 1 eq.) was dissolved in 80 ml of DCM and 3.2 ml (18.02 mmol, 7 eq.) of di-isopropylethylamine. The solution was cooled to 0 °C and 1 ml (12.87 mmol, 5 eq.) of methylsulfonyl chloride was added. The solution was stirred for 12 hours at RT under nitrogen, then concentrated under reduced pressure and the residue purified by column chromatography over SiO_2 (CH_2Cl_2/methanol, 95:5) to give product (1.52 g, 94%).

^{1}H NMR (500 MHz, cdcl$_3$) δ 4.27 – 4.22 (m, 2H), 3.66 – 3.62 (m, 2H), 3.62 – 3.45 (m, 40H), 3.43 – 3.40 (m, 2H), 3.24 (s, 3H), 2.96 (s, 3H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 71.70, 70.53 – 69.85 (m), 69.19, 68.77, 58.72, 37.45. MS (ESI$^-$/TOF) m/z: 506.73 (16%) $[M-H]^-$; 551.16 (57%) $[M-H]^-$; 595.28 (73%) $[M-H]^-$; 639.13 (81%) $[M-H]^-$; 683.30 (100%) $[M-H]^-$; 727.29 (96%) $[M-H]^-$; 771.21 (65%) $[M-H]^-$; 859.40 (23%) $[M-H]^-$; 903.41 (12%) $[M-H]^-$.

2.1.6 ω- Methoxy-PEG_{500}-yl azide

ω-Methoxy-O-methylsulfonyl-PEG_{500} (1.52 g, 2.38 mmol, 1 eq.) was dissolved in 30 ml of DMF. 620 mg (9.55 mmol, 4 eq.) of sodium azide was added. The solution was heated at 80 °C overnight. The solution was concentrated under reduced pressure and the residue was purified by column chromatography over SiO_2 (CH_2Cl_2/methanol, 95:5) to give product (1.21 g, 87%).

^{1}H NMR (400 MHz, cdcl$_3$) δ 3.70 – 3.50 (m, 48H), 3.50 – 3.44 (m, 2H), 3.34 – 3.28 (m, 5H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 71.85, 70.91 – 70.18 (m), 69.95, 58.93, 50.60. MS (ESI$^+$/TOF) m/z: 476.15 (15%) $[M+Na]^+$; 520.14 (51%) $[M+Na]^+$; 564.14 (100%) $[M+Na]^+$; 608.19 (93%) $[M+Na]^+$; 652.18 (62%) $[M+Na]^+$; 696.24 (26%) $[M+Na]^+$; 740.29 (15%) $[M+Na]^+$.

2.1.7 Heptakis[6-bromo-6-deoxy-2-O-(N-(ω-methoxy--PEG_{500}-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin **(9a)**

Heptakis(6-bromo-6-deoxy-2-O-propargyl)-β-cyclodextrin **(8)** (100 mg, 0.054 mmol, 1 eq.) and 317 mg (0.54 mmol, 10 eq.) of ω-methoxy-O-methoxy-PEG_{500} -yl- azide were dissolved in 6 ml of DMF and 6 ml of water. Copper (II) sulphate pentahydrate (13.5

mg, 0.054 mmol, 1 eq.) was added, then 32 mg (0.16 mmol, 3 eq.) of sodium ascorbate, and the solution was stirred at 80 °C for 12 hours. The solution was concentrated under reduced pressure and the residue purified by size exclusion chromatography over Sephadex LH20 (methanol 100%) to give product **(9a)** (282 mg, 88%).

^{1}H NMR (400 MHz, cdcl$_3$) δ 7.90 (s, 1H), 5.07 – 4.89 (m, 3H), 4.84 (s, 1H), 4.69 – 4.47 (m, 1H), 4.01 – 3.23 (m, 52H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 143.62, 124.66, 101.35, 85.54, 78.41, 73.15 – 69.03 (m, 23), 64.90, 58.97, 50.25, 33.01.

2.1.8 Heptakis[6-Boc-aminoethylthio-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin

tert-Butyl-N-(2-mercaptoethyl)carbamate (242 mg, 1.36 mmol, 28 eq.) and 33 mg (1.36 mmol, 28 eq.) of sodium hydride were dissolved in 25 ml of anhydrous DMF. The solution was stirred at 80 °C under nitrogen for 2 hours. The solution was then cooled to room temperature and 282 mg (0.05 mmol, 1 eq.) of **8a** in 5 ml of anhydrous DMF was added. The solution was stirred overnight at 80 °C under nitrogen, then concentrated under reduced pressure and the residue purified by size exclusion column chromatography over Sephadex LH20 (methanol 100%) to give product (285 mg, 90%).

^{1}H NMR (500 MHz, cdcl$_3$) δ 7.87 (s, 1H), 5.54 – 5.25 (m, 1H), 5.08 – 4.84 (m, 3H), 4.83 – 4.74 (m, 1H), 4.64 – 4.53 (m, 2H), 3.97 – 3.90 (m, 2H), 3.90 – 3.44 (m, 46H), 3.38 (s, 3H), 3.32 – 3.22 (m, 2H), 3.13-2.95 (m, 1H), 2.76 – 2.61 (m, 2H), 1.42 (s, 9H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 155.87, 143.93, 124.49, 101.34, 85.70, 79.14, 73.02, 71.95, 70.58, 69.49, 65.21, 59.00, 50.20, 40.08, 33.43, 28.54.

2.1.9 Heptakis[6-aminoethylthio-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin trifluoroacetate **(10a)**

Heptakis[6-(Boc-aminoethylthio)-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin (285 mg, 0.044 mmol) was dissolved in 50 ml of DCM and 20 ml of trifluoroacetic acid. The solution was stirred for 6 hours at RT. The solution was concentrated under reduced pressure and purified by purified by size exclusion column

chromatography over Sephadex LH20 (methanol 100%) to give product **(10a)** (250 mg, 86%).

^{1}H NMR (400 MHz, cdcl$_3$) δ 8.72 – 7.91 (m, 3H), 7.91 – 7.38 (m, 1H), 5.19 – 4.25 (m, 6H), 3.96 – 2.59 (m, 54H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 162.48 – 160.35 (m), 143.85, 124.60, 119.78 – 113.18 (m), 101.12, 84.97, 78.62, 73.01, 71.84, 70.98 – 69.86 (m, 23C), 69.35, 64.94, 58.95, 50.19, 38.58, 30.11. MALDI–MS: M_{avg}=5808.45 g/mol.

2.1.10 Heptakis[6-diBoc-guanidinoethylthio-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin

Heptakis[6-(2''-aminoethylthio)-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin trifluoroacetate (250 mg, 0.038 mmol, 1eq.) was dissolved in 50 ml of DCM and 1 ml of di-isopropylethylamine. N,N′-diBoc-1H-pyrazole-1-carboxamidine (580 mg, 1.87 mmol, 50 eq.) (Aldrich) was added. The solution was stirred at RT under N_2 for 12 hours. The solution was concentrated under reduced pressure and the residue was purified by size exclusion chromatography over Sephadex LH20 (methanol, 100) to give product (208 mg, 73%).

^{1}H NMR (400 MHz, cdcl$_3$) δ 11.40 (s, 1H), 8.75 – 8.22 (m, 1H), 7.76 (s, 1H), 5.09 – 4.62 (m, 4H), 4.56 – 4.42 (m, 2H), 3.97 – 3.68 (m, 4H), 3.63 – 3.33 (m, 46H), 3.29 (s, 3H), 3.04 – 2.40 (m, 4H), 1.65 – 1.08 (m, 18H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 163.59, 156.04, 152.98, 144.00, 124.36, 101.35, 85.28, 82.92, 79.40, 78.98, 73.11, 71.97, 71.29, 70.88 – 70.11 (m, 22C), 69.53, 65.47, 59.06, 50.58, 50.18, 39.86, 32.93, 28.40, 28.14.

2.1.11 Heptakis[6-guanidinoethylthio-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin trifluoroacetate **(11a)**

Heptakis[6-(diBoc-guanidinoethylthio)-2-O-(N-(ω-methoxy--PEG$_{500}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin (208 mg) was dissolved in 10 ml of DCM and 10 ml of trifluoroacetic acid. The solution was stirred for 6 hours at RT. The solution was concentrated under reduced pressure and dried under high vacuum for 12 hours to give product (215 mg, quant.).

^{1}H NMR (500 MHz, cdcl$_3$) δ 11.07 – 9.55 (m, 2H), 7.95 (s, 1H), 7.50 (s, 1H), 6.89 (s, 3H), 5.23 – 4.40 (m, 6H), 3.93 – 3.22 (m, 53H), 2.99 – 2.33 (m, 4H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 160.45 (q, *J* = 37.4 Hz), 157.38, 143.48, 125.46, 115.88 (q, *J* = 289.3 Hz), 101.19, 85.11, 79.12, 72.99, 71.89, 71.46, 71.25 – 69.83 (m, 22C), 69.20, 64.34, 58.96, 50.73, 41.01, 32.32. MALDI-MS: M_{avg}=6086.13 g/mol.

2.1.12 1-Deoxy-ω-(*p*-methoxybenzamido)-PEG$_{440}$-yl azide

1-Amino-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontyl 32-azide (133 mg, 0.25 mmol, 1 eq.), 284 mg (1.14 mmol, 4.5 eq.) of anisic acid N-hydroxysuccinimide and 330 µl (1.90 mmol, 7.6 eq.) of di-isopropylethylamine were dissolved in 25 ml of DCM. The solution was stirred in the dark at RT under nitrogen for 12 hours, concentrated under reduced pressure and purified by column chromatography over silica (CH_2Cl_2-methanol, 95:5 to 90:10) to give product (167 mg, quant.).

^{1}H NMR (600 MHz, cdcl$_3$) δ 7.79 – 7.74 (m, 2H), 6.93 – 6.87 (m, 2H), 6.73 (s, 1H), 3.83 (s, 3H), 3.70 – 3.55 (m, 42H), 3.37 (t, *J* = 5.1 Hz, 2H). ^{13}C NMR (151 MHz, cdcl$_3$) δ 167.15, 129.04, 127.17, 113.84, 71.98 – 69.33 (m), 55.57, 50.91, 39.93. MS (ESI$^+$/TOF) m/z: 661.34 (100) $[M+H]^+$; 683.33 (41) $[M+Na]^+$; 699.29 (34) $[M+K]^+$.

2.1.13 Heptakis[6-bromo-6-deoxy-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin **(9b)**

Heptakis(6-bromo-6-deoxy-2-O-propargyl)-β-cyclodextrin (54 mg, 0.029 mmol, 1 eq.) and 192.9 mg (0.29 mmol, 10 eq.) of 1-deoxy-ω-(*p*-methoxybenzamido)-PEG$_{440}$-yl azide were dissolved in 5 ml of DMF and 5 ml of water. Copper (II) sulfate pentahydrate (7.25 mg, 0.029 mmol, 1 eq.) was added, then 17.2 mg (0.087 mmol, 3 eq.) of sodium ascorbate was added and the solution was stirred at 80 °C for 12 hours. The solution was concentrated under reduced pressure and purified by size exclusion column chromatography over Sephadex LH20 (methanol 100%) to give product **(9b)** (153 mg, 82%).

^{1}H NMR (400 MHz, cdcl$_3$) δ 7.85 (s, 1H), 7.73 (d, *J* = 8.3 Hz, 2H), 6.87 (d, *J* = 8.3 Hz, 2H), 6.75 (s, 1H), 5.21 – 4.69 (m, 5H), 4.60 – 4.46 (m, 2H), 4.00 – 3.12 (m, 50H). ^{13}C

NMR (101 MHz, cdcl$_3$) δ 166.97, 162.04, 143.60, 128.84, 126.85, 124.69, 113.60, 101.36, 85.56, 78.42, 72.76, 71.20 – 69.65 (m, 18C), 69.34, 64.88, 55.36, 50.30, 39.70, 33.03. MALDI-MS: 6492.21 $[M+H]^+$; 6533.64 $[M+K]^+$.

2.1.14 Heptakis[6-Boc-aminoethylthio-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl) -1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin

tert-Butyl-N-(2-mercaptoethyl)carbamate (117 mg, 0.66 mmol, 28 eq.) and 15.8 mg (0.66 mmol, 28 eq.) of sodium hydride were dissolved in 25 ml of anhydrous DMF. The solution was stirred at 80 °C under nitrogen for 2 hours. The solution was then cooled to room temperature and 153 mg (0.024 mmol, 1 eq.) of heptakis[6-bromo-6-deoxy-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-triazol e-4'-yl-methyl)]-β-cyclodextrin in 5 ml of anhydrous DMF was added. The solution was stirred overnight at 80 °C under nitrogen. The solution was concentrated under reduced pressure and purified by purified by size exclusion column chromatography over SEPHADEX LH20 (methanol 100%) to give product (146 mg, 86%).

^{1}H NMR (500 MHz, cdcl$_3$) δ 7.83 (s, 1H), 7.75 (d, *J* = 8.7 Hz, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.73 (s, 1H), 5.35 (s, 1H), 5.05 – 4.84 (m, 3H), 4.76 (s, 1H), 4.60 – 4.51 (m, 2H), 3.95 – 3.84 (m, 3H), 3.82 (s, 3H), 3.70 – 3.42 (m, 40H), 3.39 – 2.47 (m, 9H), 1.40 (s, 9H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 167.06, 162.17, 155.99, 144.03, 128.96, 127.03, 124.61, 113.74, 101.40, 85.80, 79.24 (2C), 73.11, 71.11 – 69.82 (m, 18C), 69.57, 65.27, 55.50, 50.29, 40.17, 39.83, 33.52 (2C), 28.63.

2.1.15 Heptakis[6-aminoethylthio-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H* -triazole-4'-yl-methyl)]-β-cyclodextrin trifluoroacetate **(10b)**

Heptakis[6-(Boc-aminoethylthio)-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-t riazole-4'-yl-methyl)]-β-cyclodextrin (146 mg) was dissolved in 10 ml of DCM and 10 ml of trifluoroacetic acid. The solution was stirred for 6 hours at room temperature. The solution was concentrated under reduced pressure and dried under high vacuum for 12 hours to give product **(10b)** (150 mg, quant.).

^{1}H NMR (500 MHz, cdcl$_3$) δ 8.12 (s, 1H), 7.76 (d, *J* = 8.5 Hz, 2H), 7.64 – 7.17 (m, 4H), 6.92 (d, *J* = 8.6 Hz, 2H), 5.25 – 4.74 (m, 3H), 4.62 (s, 2H), 4.08 – 2.54 (m, 56H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 169.25, 162.93, 161.08 – 158.21 (m), 142.49, 129.35, 126.47, 125.49, 118.47 – 114.30 (m, *J* = 276.0 Hz), 114.07, 101.03, 84.84, 79.15, 73.04, 71.70 – 68.34 (m, 19C), 63.24, 55.55, 51.47, 40.27, 39.41, 32.52, 30.44. MALDI-MS: 6470.93 $[M+H]^+$; 6511.42 $[M+K]^+$.

2.1.16 Heptakis[6-diBoc-guanidinoethylthio-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin

Heptakis[6-aminoethylthio-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin trifluoroacetate (150 mg, 0.020 mmol, 1eq.) was dissolved in 50 ml of DCM and 1 ml of di-isopropylethylamine. N,N′-diBoc-1H-pyrazole-1-carboxamidine (311 mg, 1.00 mmol, 50 eq.) was added. The solution was stirred at RT under nitrogen for 12 hours. The solution was then concentrated under reduced pressure and the residue was purified by size exclusion chromatography over Sephadex LH20 (methanol, 100) to give product (133 mg, 81%).

^{1}H NMR (500 MHz, cdcl$_3$) δ 11.42 (s, 1H), 8.47 (s, 1H), 7.78 (s, 1H), 7.72 (d, *J* = 8.8 Hz, 2H), 6.85 (d, *J* = 8.8 Hz, 2H), 6.80 – 6.73 (m, 1H), 5.03 – 4.67 (m, 4H), 4.56 – 4.45 (m, 2H), 3.97 – 3.18 (m, 51H), 3.07 – 2.32 (m, 4H), 1.59 – 1.29 (m, 18H). ^{13}C NMR (126 MHz, cdcl$_3$) δ 167.04, 163.63, 162.14, 156.09, 153.03, 144.04, 128.95, 127.00, 124.40, 113.70, 101.39, 85.34, 82.96, 80.05 – 78.59 (m, 2C), 73.14, 71.97 – 68.61 (m, 19C), 65.50, 55.47, 50.23, 39.80 (s, 2C), 32.97, 29.03 – 27.47 (m, 7C).

2.1.17 Heptakis[6-guanidinoethylthio-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin trifluoroacetate **(11b)**

Heptakis[6-diBoc-guanidinoethylthio-2-O-(N-(ω–(*p*-methoxybenzamido)-PEG$_{440}$-yl)-1'*H*-triazole-4'-yl-methyl)]-β-cyclodextrin (100 mg) was dissolved in 10 ml of DCM and 10 ml of trifluoroacetic acid. The solution was stirred for 6 hours at room temperature, then concentrated under reduced pressure and the residue dried under high vacuum for 12 hours to give product **(11b)** (105 mg, quant.).

^{1}H NMR (500 MHz, cdcl$_3$) δ 8.19 (s, 1H), 7.78 (d, J=10 Hz, 2H), 7.31 (s, 2H), 6.95 (d, *J* = 10 Hz, 2H), 6.70-6.80 (m, 2H), 5.10 – 4.80 (m, 3H), 4.70 – 4.4.60 (m, 2H), 4.05 – 3.30 (m, 51H), 3.10 – 2.35 (m, 4H).

^{13}C NMR (126 MHz, cdcl$_3$) δ 169.16, 162.76, 160.12-159.18 (1C), 157.05, 141.97, 129.14, 126.42, 125.19, 118.75, 116.44, 114.14, 113.89, 111.86, 100.63, 84.36, 79.30, 72.81, 71.16, 70.71-69.58 (19C), 69.27, 68.45, 62.90, 55.35, 51.57, 40.81, 40.15, 32.51, 32.12. MALDI-MS: 6745.46; 6797.07.

2.2 Preparation and characterisation of cyclodextrin complexes with siRNA

The cyclodextrins (G-CD, G-CD-PEG and G-CD-PEG-AA) were dissolved in aqueous solutions (deionised water and phosphate buffer saline (PBS)), before addition of siRNA solution (prepared in RNase-free water following QIAGEN and SIGMA recommendations) at different mass ratios (MRs) of cyclodextrins to siRNA, followed by 1-hour incubation at RT.

The ability of cyclodextrins to complex siRNA was analysed by gel retardation [25]. Complexes of cyclodextrins and siRNA (containing 0.5 μg siRNA) at different mass ratios were prepared as described above and loaded onto 1% (w/v) agarose gels in Tris/Borate/EDTA (TBE) buffer containing ethidium bromide. Electrophoresis was performed at 120 V for 30 minutes and the resulting gels were photographed under UV.

The morphology of cyclodextrins with siRNA was examined using transmission electron microscopy (TEM) [30]. Cyclodextrins or complexes (MR = 75) containing 0.5 μg siRNA in deionised water (deionised water was filtered through a 0.2 μm membrane) were left on 400-mesh carbon-filmed copper grids (Agar Scientific) for several minutes, then grids were blotted with filter paper. The grids were then stained with 2% (w/w) uranyl acetate, blotted again and left at RT overnight. Samples were analysed using a JEOL 2000 FXII transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Particle sizes and zeta potentials were measured with a Malvern Nano-ZS (Malvern

Instruments, UK). PBS (0.2 µm membrane-filtered, used for particle size analysis) and deionised water (0.2 µm membrane-filtered, applied for zeta potential evaluation) were added to the cyclodextrin or the complexes and made up to 1 ml, 4 hours before measurement. The concentration of cyclodextrins (cyclodextrins alone and cyclodextrin.siRNA complexes) was fixed at 0.2 mg/ml.

In order to compare the stability in serum of siRNA alone and CD complexes, siRNA (0.5 µg) or cyclodextrin.siRNA complexes (MR75) were incubated for different time intervals in 50% (v/v) fetal bovine serum (FBS) at 37 ºC [25]. Samples were then treated for 1 hour with excess heparin (1,000 I.U./ml) at room temperature. After loading onto 1.5% (w/v) agarose gels in TBE buffer containing ethidium bromide, electrophoresis was performed at 120 V for 30 minutes, and the resulting gels were photographed under UV light.

2.3 Molecular biology, cell culture and cytotoxicity

PC-3 cells (human prostate cancer cell line, European Collection of Cell Cultures (ECACC), UK) were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS. TRAMP C1 cells (a transgenic adenocarcinoma of the mouse prostate cell line, was kindly donated by Cork Cancer Research Centre (CCRC), Ireland) were maintained in Dulbecco's modified Eagle's medium high glucose with L-glutamine and without sodium pyruvate (Gibco, UK) supplemented with 5% FBS, 5% Nu Serum IV (BD Biosciences, UK), 5 µg/ml insulin from bovine pancreas, 10^{-8} mol/l 5a-Androstan-17b-ol-3-one. All cells were grown in an incubator (ThermoForma) at 37 ºC with 5% CO_2 and 95% relative humidity.

The pGL3-luc plasmid contains the firefly luciferase gene under the control of the simian vacuolating virus 40 (SV40) promoter/enhancer. The pGL3-luc plasmid was maintained and propagated in competent *E. coli*. It was isolated by alkaline lysis, purified by anion exchange chromatography using Plasmid Mega kit (QIAGEN) in accordance with the manufacturer's instructions and stored in deionised water at -20 °C. Plasmid concentration was determined from the absorbance at 260 nm using

GeneRay UV-photometer Biometra®. Plasmid with A260/A280=1.8-2.0 was used for experiments.

Cytotoxicity of β-CD, G-CD, G-CD-PEG and G-CD-PEG-AA was estimated using the MTT assay with 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. PC-3 cells (5000 per well) were seeded in 96-well plates one day before transfection. The cyclodextrins were incubated with cells for 48 hours under the normal growth conditions. After incubation, the cyclodextrin solution was replaced with 200 µl fresh growth medium, and 20 µl MTT stock (5 mg/ml in PBS) was added and incubated with cells for 4 hours at 37 ºC. The contents were removed and 100 µl DMSO was added to dissolve the purple formazan products. The results were measured at 570 nm using a microplate reader. The concentration of CDs leading to 50% cell growth inhibition (IC50) was estimated from the plot of the percentage of viable cells versus the concentration of cyclodextrins for each treatment.

2.4 Internalisation of cyclodextrin.fluorescein-siRNA complexes

PC-3 cells (100,000 per well) were seeded in 12-well plates and incubated for 24 hours under normal growth conditions. Following incubation, cells were treated (or left untreated) with 40 µM haloperidol for 3 hours for the competitive binding study [31]. Cells were then washed with growth medium and transfected with fluorescein-siRNA (sense sequence 5' – UUC UCC GAA CGU GUC ACG U – 3', modified by 3'-fluorescein on sense sequence, QIAGEN) (50 nM) or its cyclodextrin complex followed by 4-hour incubation in 10% FBS containing growth medium. After this, cells were treated with CellScrub™ (Genlatins) to remove uninteranlised complexes according to manufacturer's instructions. The medium was then removed by aspiration, and cells were washed twice with pre-warmed PBS and trypsinised. After centrifugation (1000 rpm for 5 minutes) the supernatant was discarded and pellets were re-suspended in 1000 µl cold PBS in Polystyrene Round-Bottom Tubes (Becton Dickinson). For each sample 10,000 cells were measured out according to the Becton Dickinson FACScalibur manual. Fluorescein-positive cells were analysed by Dot Plot and by Histogram Plot (data not shown).

2.5 Intracellular fate of G-CD-PEG-AA.fluorescein-siRNA complexes

TRAMP C1 cells (50,000 per well) were seeded in 12-well culture plates with glass bottoms (MatTek™). Anisamide-targeted formulations containing fluorescein-siRNA were applied to cells with a final siRNA concentration of 100 nM and incubated under the normal growth conditions for 30 minutes, 4 hours and 24 hours. In order to label late endosomes and lysosomes, 75 nM LysoTracker Red (Molecular Probes, Invitrogen) was added to cells for 30 minutes at 37 °C. The media was then replaced with fresh growth medium and observed using an Olympus FV 1000 microscope. Fluorescein-labeled siRNA was detected using excitation at 488 nm and emission of 505-530 nm (green). LysoTracker Red was detected using excitation at 543 nm and an emission band pass filter 560 nm (red).

2.6 *In vitro* reporter gene silencing by cyclodextrin.anti-luciferase siRNA complexes

PC-3 cells were seeded at a density of 50,000 cells per well in 24-well plates. Following 24-hour incubation, the media was replaced with 10% FBS containing growth medium and transfected with 0.4 μg of pGL3-luc plasmid DNA complexed with FuGENE6® DNA transfection reagent (Roche). Following a 8-hour incubation, the media was replaced with 10% FBS containing growth medium, and luciferase GL3 siRNAs (sense sequence 5' – CUU ACG CUG AGU ACU UCG A – 3', QIAGEN) (100 nM) formulated with G-CD-PEG or G-CD-PEG-AA (MR75) was applied to the cells for a further 24-hour transfection at 37 °C. Luciferase GL3 siRNA alone (100 nM) and negative control siRNA (sense sequence 5' – UUC UCC GAA CGU GUC ACG U – 3', no modification, QIAGEN) (100 nM) formulated with G-CD-PEG-AA were used as negative controls. At the endpoint of transfection, cells were washed with PBS, treated with 250 μl of cell lysis buffer (Promega) and a freeze-thaw cycle [25]. Cellular debris was removed by centrifugation at 13,000 rpm for 5 minutes. The luciferase activity in cell lysate (20 μl) was measured with 100 μl of luciferin substrate (Promega) in a luminometer (BERTHOLD Technologies) after 10 seconds. The protein content was determined with Pierce BCA® Assay according to manufacturer's instructions (Thermo Scientific). Luciferase gene expression was expressed as relative

light units (RLU) per microgram of protein.

2.7 *In vivo* anti-tumour effect of cyclodextrin.VEGF siRNA complexes

Male C57 BL/6 mice (Harlan Laboratories, UK), aged 6-8 weeks, were used. The animal ethics committee of University College Cork approved all experiments. The mice were kept at 22 ºC with a natural day/night light cycle in a conventional animal colony. All mice were maintained in a pathogen free animal facility for at least 2 weeks before the experiments. The tumour-bearing animal model was established by subcutaneous injection of 5 x 10^6 TRAMP C1 cells into the right flank [32]. Tumour growth and body weight were recorded regularly, and tumour volume was calculated using the formula $a^2b(\pi/6)$, where a is the minor diameter of the tumour and b is the major diameter perpendicular to diameter a.

When tumour volume attained 100 mm³ (day 0), tumour-bearing mice (5 mice/group) were given intravenous injections through the tail vein on day 1, 4, 8, 11, 15 and 18, using mouse VEGF siRNAs (sense sequence 5'-AGG UUU CAA UAU ACA UUU ATT-3', SIGMA) at a dose of 0.5 mg/kg body weight formulated in G-CD-PEG and G-CD-PEG-AA in a 0.1 ml injection volume. PBS, VEGF siRNA alone and G-CD-PEG-AA alone were used as negative controls. The mice were sacrificed on day 22, and blood samples were collected for monitoring of Alanine aminotranferease (ALAT) and aspartate aminotransferase (ASAT) levels, the analysis was performed by the Institut Clinique de la Souris (ICS, France).

Determination of VEGF mRNA expression was as follows. Tumor homogenates (obtained using a MagNA Lyser Instrument, Roche) were centrifuged at 15,000 rpm for 2 minutes at 4 °C to remove insoluble tissue debris and the supernatant was collected for reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the supernatant using an Absolutely RNA Miniprep Kit (Stratagene), according instructions supplied. First-strand cDNA was generated from total RNA samples using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Quantitative real-time RT-PCR was performed using a Light Cycler System (Roche).

RT-PCR was carried out at the following thermal conditions: an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of 15 seconds at 95 °C, annealing for 5 seconds at 55 °C, and 15 seconds at 72 °C. The primers used were: mouse 18S ribosomal RNA (rRNA) (forward 5'-gcaattattccccatgaacg-3' and reverse 5'-gggacttaatcaacgcaagc-3', Eurofins), and mouse VEGF-a (forward 5'-gcagcttgagttaaacgaacg-3' and reverse 5'-ggttcccgaaaccctgag-3', Eurofins). The quantitative level of each VEGF mRNA was measured as a fluorescent signal corrected according to the signal for 18S rRNA.

2.8 Statistical analysis

An unpaired Student's t-test (two-tailed) was used to test the significance of differences between two mean values. One-way ANOVA was used to test the significance of differences in three or more groups. Two-way ANOVA with repeated measures was used to test the significance of differences in measured tumour growth and body weight. In all cases, $p<0.05$ was considered statistically significant.

3. Results and discussion

3.1 Synthesis of modified cyclodextrins

Beta CD was modified, as described above, on the primary side with a cationic group, guanidino **(5),** which is reported to enhance cell penetration [3,6] (Figure 1a). This cationic CD (G-CD), in addition to the potential to enhance cellular uptake, is designed to complex the siRNA. A click chemistry approach, previously described by us [13], was used to modify the CD on the secondary side with a PEG chain to produce G-CD-PEG **(11a).** The PEG chain is designed to mask any residual charge, to reduce toxicity and to enhance stability. In addition, the PEG chain was used as a spacer to attach the targeting ligand anisamide **(11b)** resulting in the synthesis of a targeted PEGylated CD, G-CD-PEG-AA (Figure 1b). To our knowledge there is no previous example of a CD-based vector which incorporates these three distinct design features, a cationic group (guanadino) in tandem with a PEG chain linked to a targeting moiety

(anisamide), all covalently attached in one CD compound.

3.2 Formation of cyclodextrin.siRNA complexes

The formation of cyclodextrin and siRNA complexes by interaction between positively charged guanidino groups of the cyclodextrins and negatively charged siRNA was examined by gel retardation (Figure 2a). Complete condensation of siRNA with G-CD took place from mass ratio MR5 onwards (data not shown), yet in the case of the PEGylated derivatives complete complexation of siRNA occurred only from MR50 (G-CD-PEG) (data not shown) and MR75 (G-CD-PEG-AA) onwards, probably due to steric interference from the PEG-AA groups.

Using dynamic light scattering (DLS) at MR75 (Figure 2c and d), the diameters of the PEGylated CDs, G-CD-PEG and G-CD-PEG-AA, and their complexes with siRNA after 4-hour incubation in PBS were in the range of ~210-260 nm, a similar size for the G-CD-PEG-AA was observed by TEM (Figure 2b). In contrast the particle sizes of the non-PEGylated CDs, G-CD and G-CD.siRNA complex, were significantly greater in the presence of PBS (Figure 2c). Zeta potentials of the PEGylated CDs (~ 14-18 mV) were much lower than that of the guanidino cyclodextrin (~ 63 mV), and their surface charges were further reduced to nearly neutral (~ 7-11 mV) by condensation of siRNA (Figure 2d).

To examine the ability of the cyclodextrins to protect siRNA from serum nucleases, the G-CD-PEG-AA.siRNA complex (MR75) was incubated in 50% FBS at 37 ºC for 24 and 48 hours. Naked siRNA was completely degraded after 24-hour incubation, in contrast, up to 48 hours only slight degradation of siRNA was noted on formulation with G-CD-PEG-AA (Figure 2e).

The *in vivo* stability of siRNA-vector particles depends on the vector structure, as well as on the surface charge. Although positively charged vectors can bind and protect siRNA against serum nuclease they tend to be toxic in vivo as they may adsorb to serum albumin and other anionic proteins resulting in clot-like accumulations in the blood [3]. These are either entrapped in the lung endothelial capillary bed or taken up

by the reticuloendothelial system [33]. PEGylation has been widely reported as an effective method to mask the surface charge, forming so called ‘stealth’ particles [6]. In addition, the PEG can be used as a linker to attach a targeting ligand to enhance cell specific uptake and compensate for the negative effects of PEG on cellular internalisation [6]. In this work a PEGylated CD has been synthesised and this molecule was further modified to attach, via the PEG chain, anisamide as a targeting ligand. Physicochemical charasterisation of the PEGylated CD particles, in contrast to the cationic nonPEGylated control, indicates the ability of the PEG to efficiently shield the surface charge of complexes and avoid aggregation in saline solutions. Such properties are a prerequisite for prospective extended blood circulation. In addition, the G-CD-PEG-AA.siRNA construct protected against serum nucleases and produced particle sizes capable of exploiting enhanced permeation and retention via the leaky vasculature in tumours [33].

3.3 Cytotoxicity of cyclodextrins

It has been reported that significantly higher cytotoxicity is caused by polycationic complexes compared with PEGylated complexes. In this study, the cytotoxicity of cyclodextrins was studied in prostate cancer cell lines using an MTT assay (Figure 3a). In the PC-3 cell line (a human prostate cancer cell line), G-CD, G-CD-PEG and G-CD-PEG-AA displayed IC50 values of 65, 850 and 790 µg/ml respectively (Figure 3a). In the TRAMP C1 cells (a mouse prostate carcinoma cell line), G-CD, G-CD-PEG and G-CD-PEG-AA IC50 values were 45, 1050 and 890 µg/ml (data not shown). The data confirmed that PEGylated cyclodextrins are considerably less toxic, although the anisamide moiety slightly increased toxicity. Due to higher cytotoxicity, the non-PEGylated CD was not used in the following *in vitro* and in *vivo* investigations. In the case of the PEGylated CDs the concentrations used for subsequent in vitro studies ensured cell viability of > 90%.

3.4 Internalisation, intracellular trafficking and *in vitro* reporter gene silencing with cyclodextrin.fluorescein-siRNA complexes

The sigma receptor is overexpressed on many human malignancies including prostate cancer [18]. It has been demonstrated that anisamide-conjugated lipidic nanoparticles achieved successfull site-specific delivery of siRNAs to such cancers [19-21]. Here, fluorescein-siRNA was used to examine the internalisation of anisamide-conjugated cyclodextrin.siRNA in PC-3 cells which overexpress sigma receptors [34]). Uptake of siRNA formulated with G-CD-PEG was similar to that observed for siRNA on its own (Figure 3b), whereas formulation with the anisamide-targeted cyclodextrin resulted in 65-70% fluorescein-positive cells. Cellular uptake was significantly ($P<0.05$) reduced by pre-treatment with haloperidol, a known sigma antagonist [31] (Figure 3b). The cellular uptake of the G-CD-PEG-AA complex in TRAMP C1 cells (also overexpress with sigma receptors [35]) was similar to that observed in PC3 cells (data not shown). In addition, we have also demonstrated that G-CD-PEG-AA did not achieve cellular internalisation in CT26 cells (a sigma-receptor-negative cell line [19]) (data not shown). These results indicate that the targeted G-CD-PEG-AA.siRNA construct has an increased affinity for prostate cancer cells and that cellular uptake is mediated via the sigma receptor.

The intracellular trafficking of the G-CD-PEG-AA.siRNA complex was studied in the mouse TRAMP C1 cells using fluorescently labelled siRNA (Figure 3c). After 30 minutes incubation, no fluorescence signal was detected inside the cytoplasm. At 4 hours, the fluorescein-siRNA was detected inside the cells, mainly in the endolysosomes, where the yellow colour observed is consistent with co-localisation with the endosomal/lysomomal marker, LysoTracker Red (Figure 3c). However, after 24 hours, the green fluorescence became evenly dispersed and more pronounced than the red fluorescence, implying release of siRNA from the endolysosomes [36]. In contrast, other amphiphilic cationic cyclodextrins used by us, with lipid chains at the 6-positions and amino groups on the oligo(ethylene oxide) chains at the 2-positions, have shown cell entry within 30 minutes after incubation [10], suggesting that endocytosis is highly influenced by the surface characteristics of nanoparticles. In addition, the intracellular trafficking of the G-CD-PEG-AA complex in PC-3 cells was

similar to that observed in TRAMP C1 cells (data not shown).

The ability of G-CD-PEG-AA to deliver siRNA was further confirmed using luciferase GL3 siRNA to silence pGL3-luc plasmid expression in PC-3 cells. Luciferase siRNA formulated with anisamide-targeted cyclodextrin induced significant gene inactivation compared to naked luciferase GL3 siRNA and siRNA formulated with G-CD-PEG (Figure 3d). Relative to the scrambled siRNA formulation the siRNA formulated with the targeted G-PEG-CD-AA caused approximately 80% gene knockdown, indicating that the effect is due to RNA interference. The *in vitro* luciferase gene silencing of the anisamide-conjugated complex in TRAMP C1 cells was similar to that recorded in PC-3 cells (data not shown).

3.5 Anti-tumour effect following intravenous administration of cyclodextrin.anti-VEGF siRNA complexes

The effect of in vivo administration of anti-VEGF siRNA formulated with PEGylated cyclodextrins was assessed by intravenous injections through the tail vein of TRAMP C1 tumour-bearing mice (n = 5 mice/group). Tumour growth (Figure 4a) showed that neither naked VEGF siRNA, G-CD-PEG-AA alone, nor VEGF siRNA formulated with G-CD-PEG, had a significant effect on tumour growth compared to the negative control (PBS). In contrast, G-CD-PEG-AA.VEGF siRNA achieved a significant ($P<0.05$) inhibitory effect relative to the vehicle alone and approximately a 3-fold reduction in tumour volume relative to PBS. RT-PCR was used to measure the expression of VEGF mRNA in tumour samples. The targeted G-CD-PEG-AA complex significantly improved VEGF knockdown compared to any of the other groups (i.e. ~ 3.5-fold and ~ 5.5-fold reductions in VEGF mRNA relative to G-CD-PEG complex and G-CD-PEG-AA alone, respectively) (Figure 4b). This data indicates that the improved down-regulation of VEGF expression is due to site-specific delivery of siRNA by G-CD-PEG-AA. In addition, monitoring of mouse body weight demonstrated no significant loss compared to PBS controls over the treatment period (Figure 4c), indicating the absence of formulation-induced toxicity.

Alanine aminotranferease (ALAT) and aspartate aminotransferase (ASAT) are enzymes in liver cells that leak into general circulation when the cells are injured. Following treatment with the G-CD-PEG-AA.siRNA complex no significant increase in ALAT or ASAT was observed (Figures 4d and 4e) this taken together with the unaltered body weight (Figure 4c), indicates further the absence of any critical toxicity at the dose used.

4 Conclusions

A click chemistry approach was used to attach a PEG chain to a cationic CD. This PEGylated chain masked the cationic charge, and in addition, facilitated the attachment of the targeting group anisamide. The resulting siRNA.CD construct produced a near neutral particle with acceptable size. The in vitro studies with the anisamide targeted vector show cell specific uptake on competition with haloperidol indicating the integrity of the targeting ligand. Following intravenous administration a reduction in tumour growth was reflected in a corresponding decrease in target mRNA levels. Preliminary toxicity indicators, body weight and liver enzymes, indicated that the treatment was well tolerated. These results indicate that the anisamide conjugated cyclodextrin vector has potential for siRNA delivery for prostate cancer therapy.

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Figure captions

Figure 1. (a) Synthesis of the cationic cyclodextrin, G-CD **(5)** (i), PPh_3, Br_2, DMF; (ii), *tert*-Butyl-N-(2-mercaptoethyl)-carbamate, NaH, DMF, 60 °C; (iii), propargyl bromide, NaH, DMF, r.t. (iv), TFA, CH_2Cl_2; (v), (a) 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine, triethylamine, DCM r.t.; b) TFA, CH_2Cl_2, r.t. **(b)** Synthesis of the PEGylated CD, G-CD-PEG **(11a),** and, the anisamide targeted CD, G-CD-PEG-AA **(11b)** (i), TBDMSCl, pyridine; (ii), propargyl bromide, NaH, DMF; (iii), PPh_3, Br_2, CH_2Cl_2, (iv), □□-methoxy-O-methoxy-PEG_{500} -yl- azide (For **9a**) or 1-deoxy-□-(*p*-methoxybenzamido)-PEG_{440}-yl azide (For **9b**) , $CuSO_4$.5 H_2O, sodium ascorbate, DMF-H_2O, 80 °C; (v) a) *tert*-Butyl-N-(2-mercaptoethyl)- carbamate, NaH, 80 °C; b) TFA, CH_2Cl_2 (vi), a) N,N′-diBoc-1H-pyrazole-1-carboxamidine, DCM, DIPEA, r.t.; b) TFA, CH_2Cl_2, r.t.

Figure 2. Physicochemical evaluation of the cyclodextrin.siRNA complexes. **(a)** Complexation of siRNA (0.5 μg) with G-CD, G-CD-PEG, and G-CD-PEG-AA at different mass ratios (MRs), (**b**) Morphology of G-CD-PEG-AA.siRNA complex (scale bar = 500 nm) (MR75), (**c**) Particle sizes of CDs and CD.siRNA complexes (MR75), (**d**) Zeta potentials of CDs and CD.siRNA complexes (MR75) (* $P<0.05$ compared to unformulated CDs), (**e**) Stability of naked siRNA (0.5 μg) and siRNA complexed with G-CD-PEG-AA (MR75) after incubation for 24- and 48-hour in fetal bovine serum at 37 °C.

Figure 3. In vitro investigations of cyclodextrins and cyclodextrin.siRNA complexes in prostate cancer cell lines. (**a**) Viability (MTT) of PC-3 cells treated with CDs (mean ± SD, n = 3), (**b**) Fluorescein-positive PC-3 cells (%, mean ± SD) after G-CD-PEG-AA.fluorescein-siRNA (MR75, 50 nM siRNA) transfection with or without 3-hour pretreatment with 40 μM haloperidol (* $P<0.05$ compared to the pretreatment control), (**c**) Intracellular distribution of G-CD-PEG-AA.fluorescein-siRNA (MR75, 100 nM siRNA) in TRAMP C1 cells at time intervals of 30 minute, 4 hour and 24 hour post-transfection using confocal microscopy. The images, left to right represented fluorescein, LysoTracker Red, merged images of fluorescein and LysoTracker Red, and transmission light. (**d**) Luciferase reporter gene silencing in PC-3 cells by naked anti-luciferase siRNA, anti-luciferase siRNA formulated with and without anisamide, formulated scrambled siRNA. (NS = no significance, * $P<0.05$ compared to formulated scrambled siRNA) (mean ± SD, n = 3).

Figure 4. Influence of anti-tumour therapy following intravenous administration of G-CD-PEG-AA.VEGF siRNA complex (MR75) (siRNA dose = 0.5 mg/kg mouse body weight) in the TRAMP C1 mouse tumour model. (**a**) Tumour growth (mean±SD, n = 5 mice/group) evaluated by tumour volume (NS = no significance, * $P<0.05$ compared to G-CD-PEG-AA on its own), (**b**) Tumour VEGF mRNA knockdown (mean±SD, n = 5 mice/group) (NS = no significance, * $P<0.05$ compared to negative controls), (**c**) Body weight concurrent with tumour treatments (mean±SD, n = 5 mice/group), and Liver enzyme levels; (**d**) Alanine aminotranferease (ALAT), and, (**e**) aspartate aminotransferase (ASAT) (mean±SD, n = 5 mice/group).

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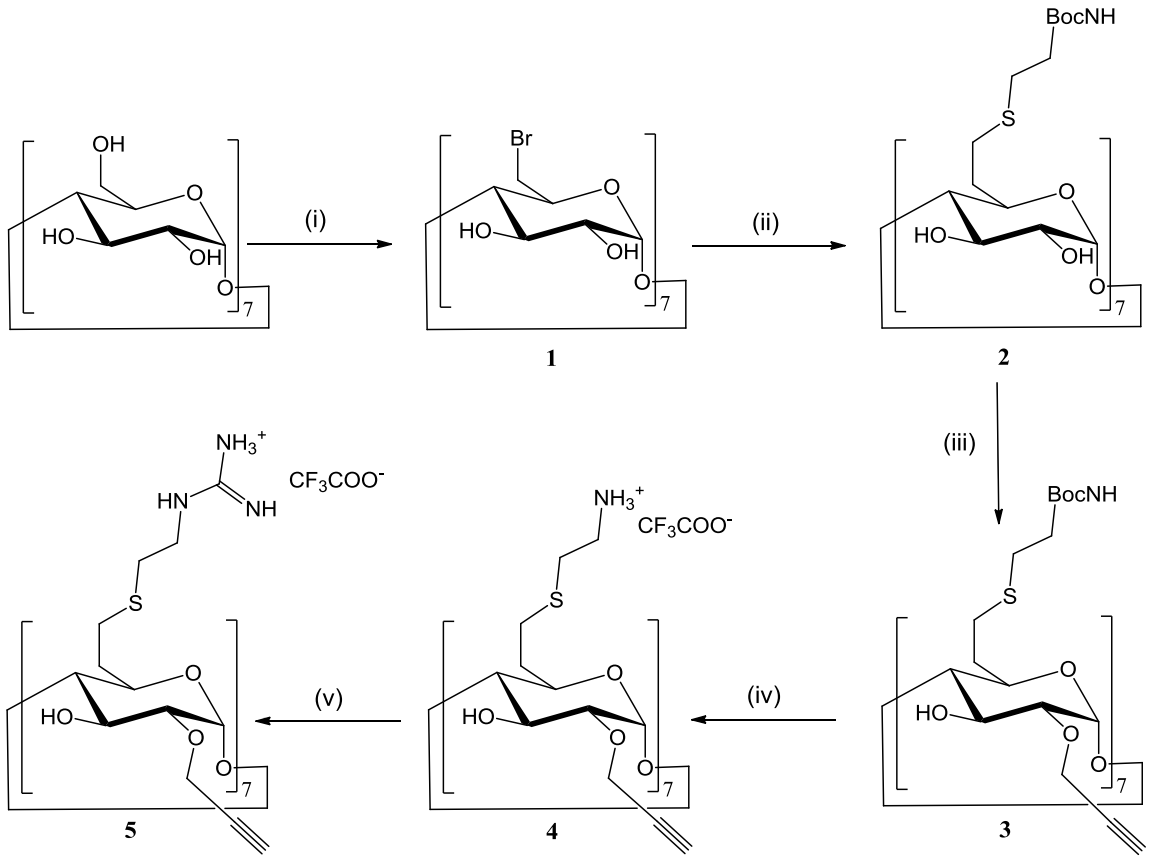
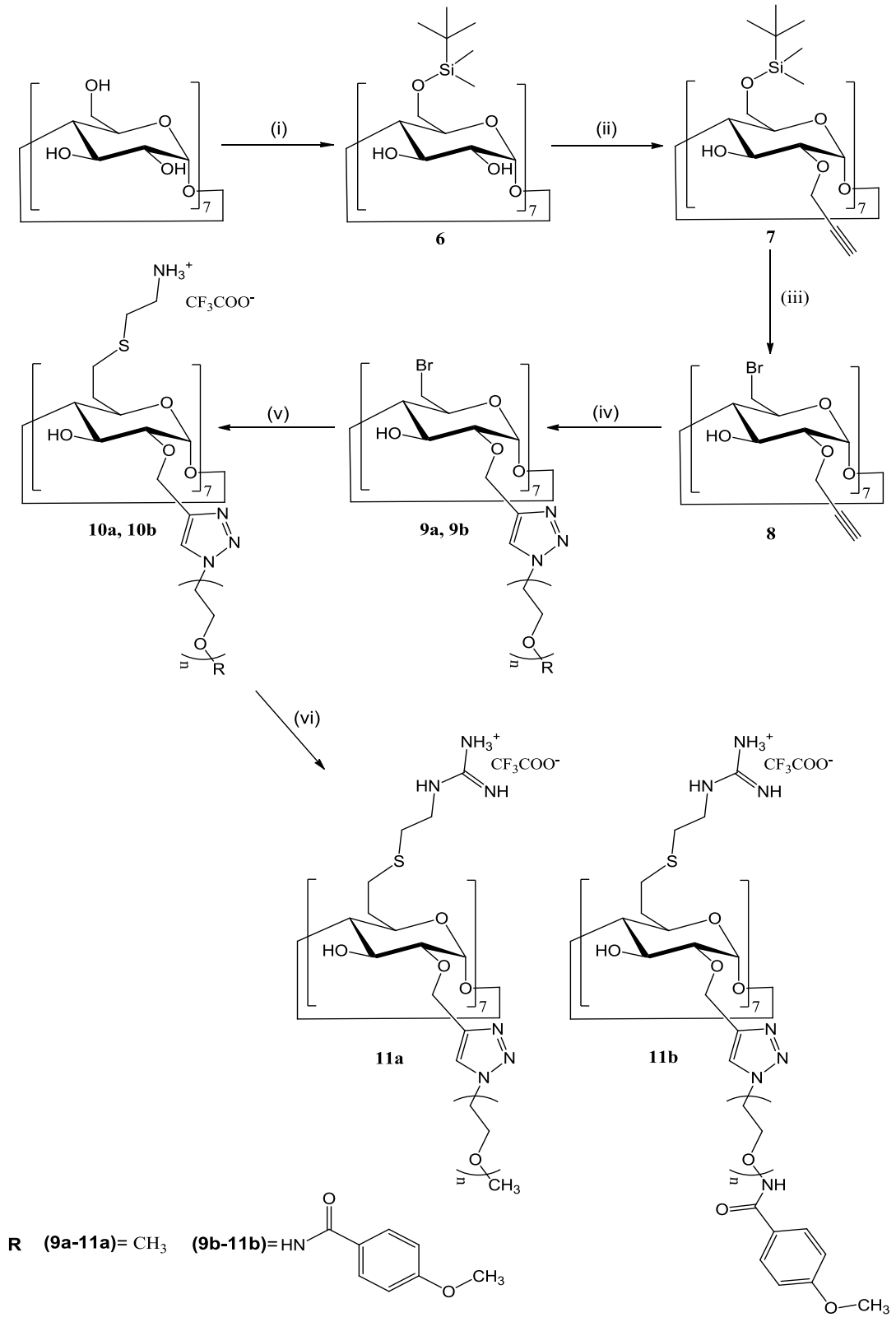

OH
HO
OH
O
O
7
(i)
Br
HO
OH
O
O
7
1
(ii)
BocNH
S
HO
OH
O
O
7
2
(iii)
BocNH
S
HO
O
O
O
7
3
(iv)
NH3+
CF3COO-
S
HO
O
O
O
7
4
(v)
NH3+
CF3COO-
HN
NH
S
HO
O
O
O
7
5

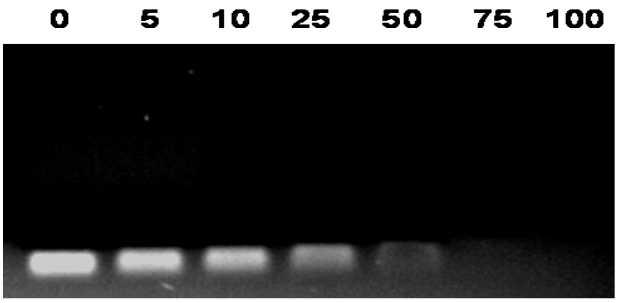
a

Figure 1.

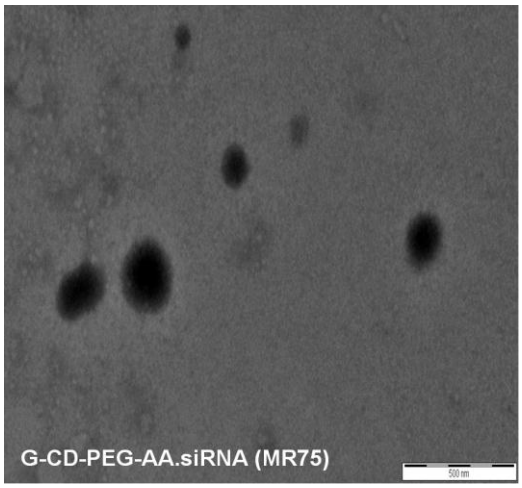


b

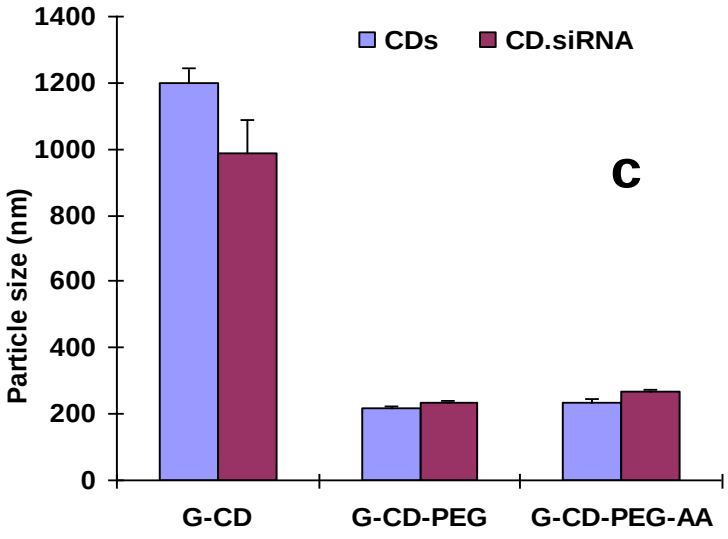
Figure 1.


0
5
10
25
50
75
100

a

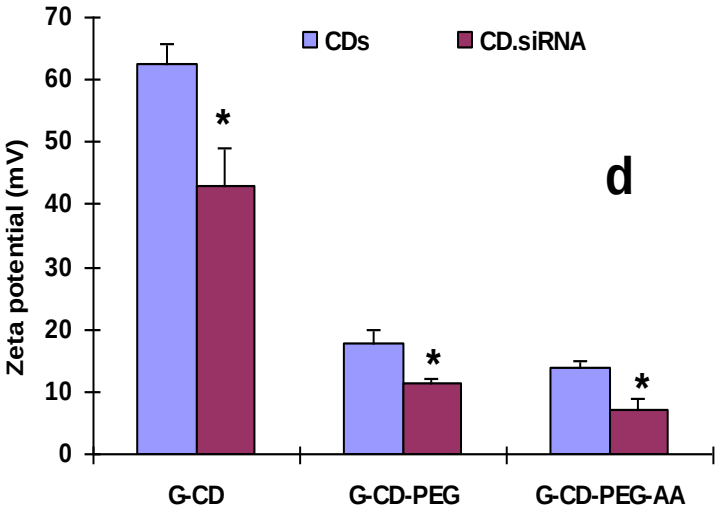

G-CD-PEG-AA.siRNA (MR75)

b


1400
1200
1000
800
600
400
200
0
Particle size (nm)
CDs
CD.siRNA
G-CD
G-CD-PEG
G-CD-PEG-AA

c

Figure 2.


70
60
50
40
30
20
10
0
Zeta potential (mV)
CDs
CD.siRNA
d
*
*
*
G-CD
G-CD-PEG
G-CD-PEG-AA

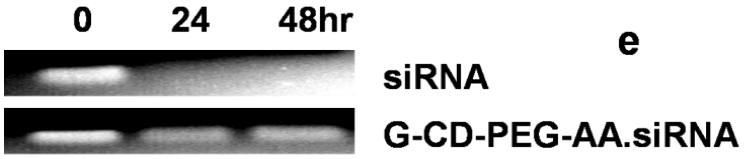
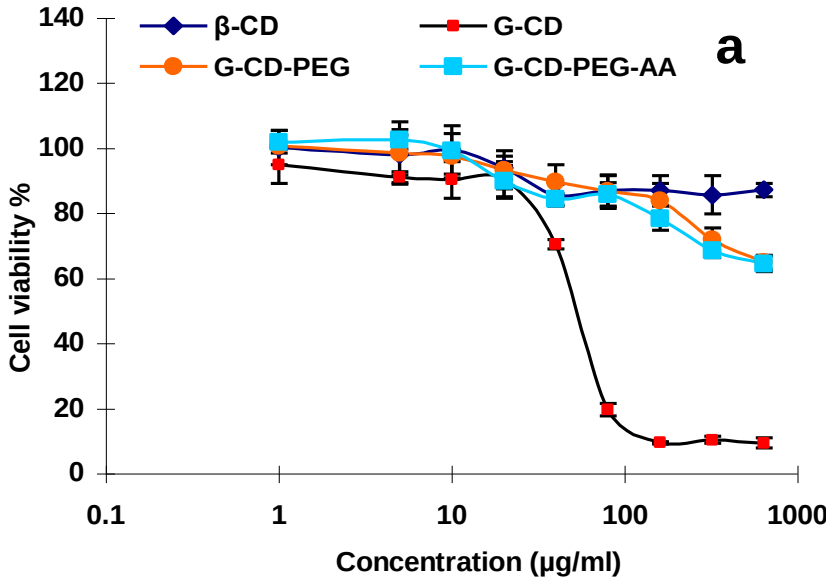

0
24
48hr
e
siRNA
G-CD-PEG-AA.siRNA

Figure 2.



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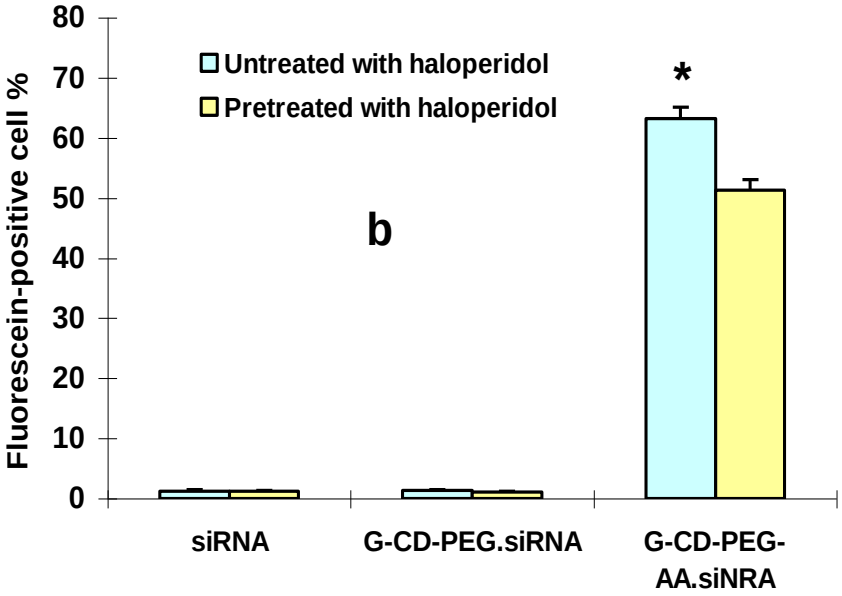

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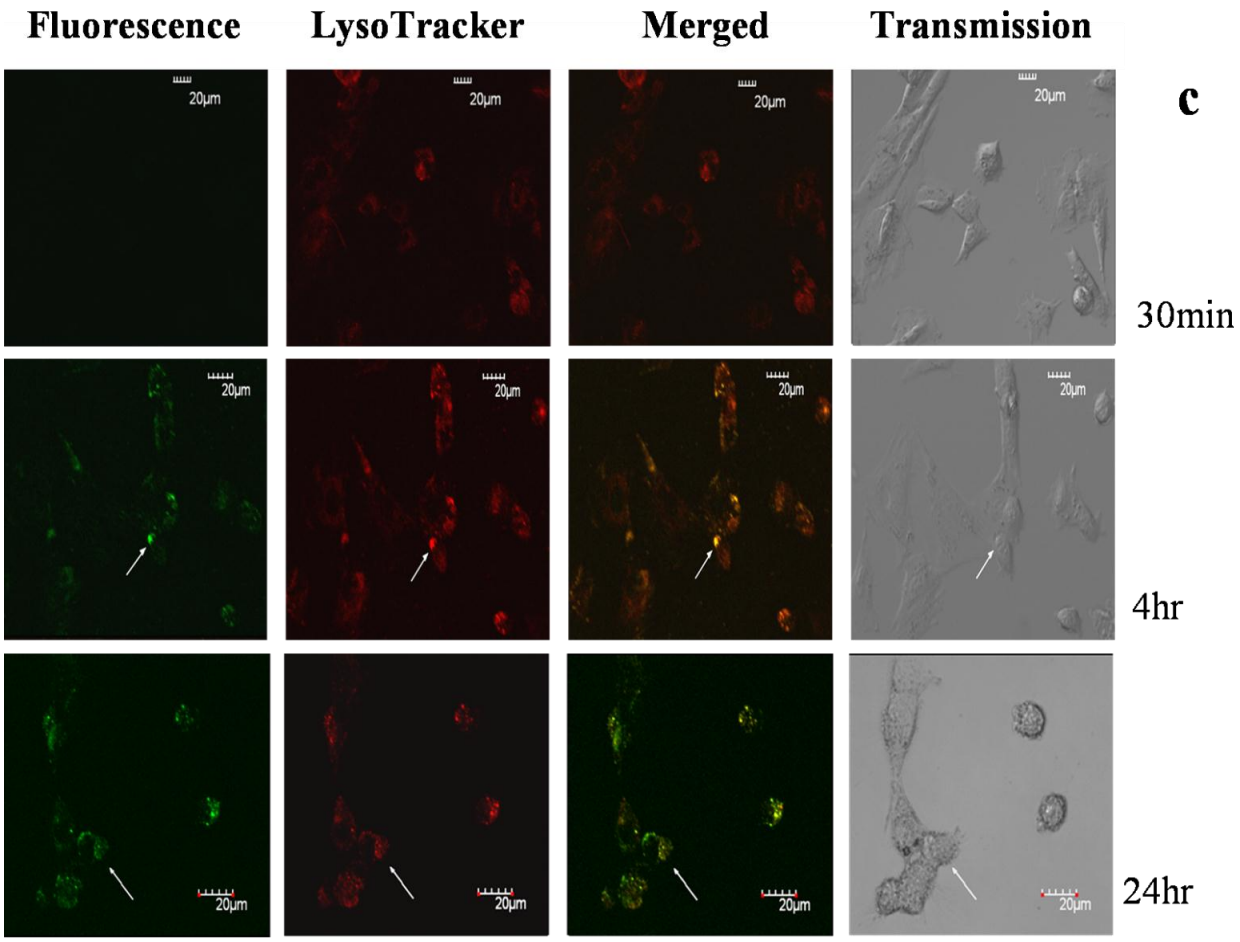
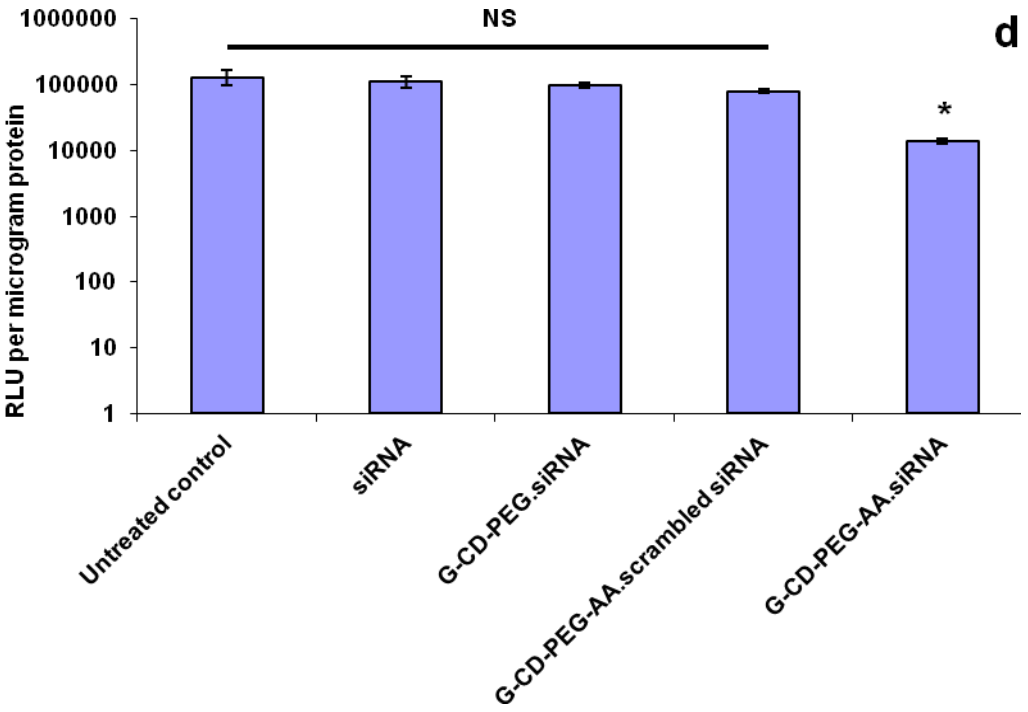

Fluorescence
LysoTracker
Merged
Transmission
c
30min
4hr
24hr
20μm

Figure 3.



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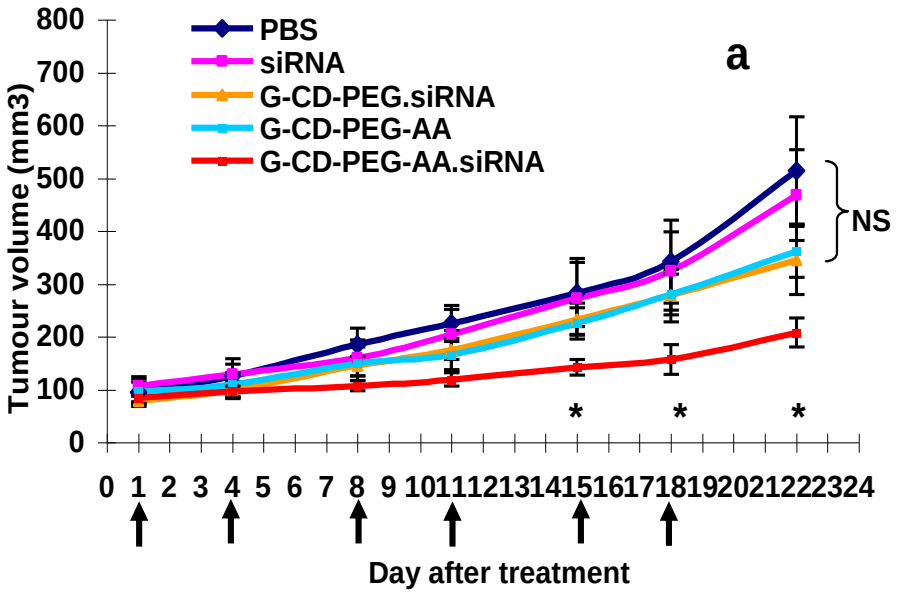

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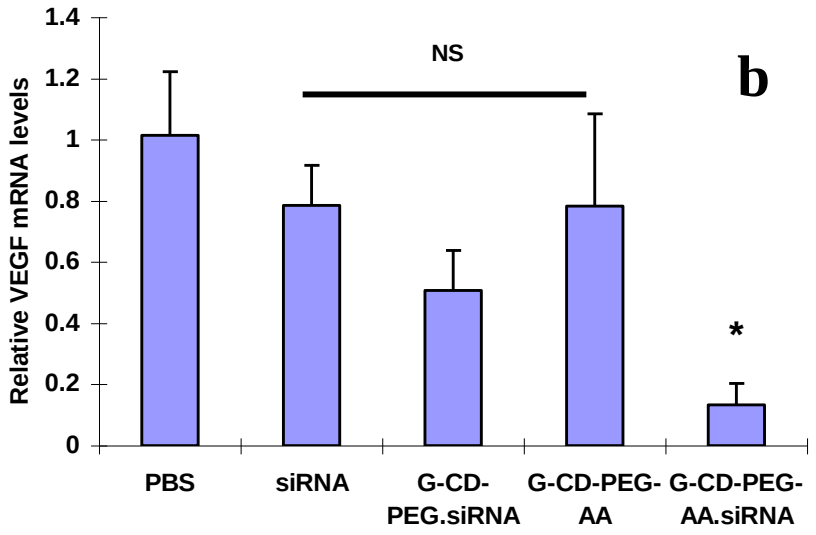
b
NS
*
Relative VEGF mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
siRNA
G-CD-PEG.siRNA
G-CD-PEG-AA
G-CD-PEG-AA.siRNA

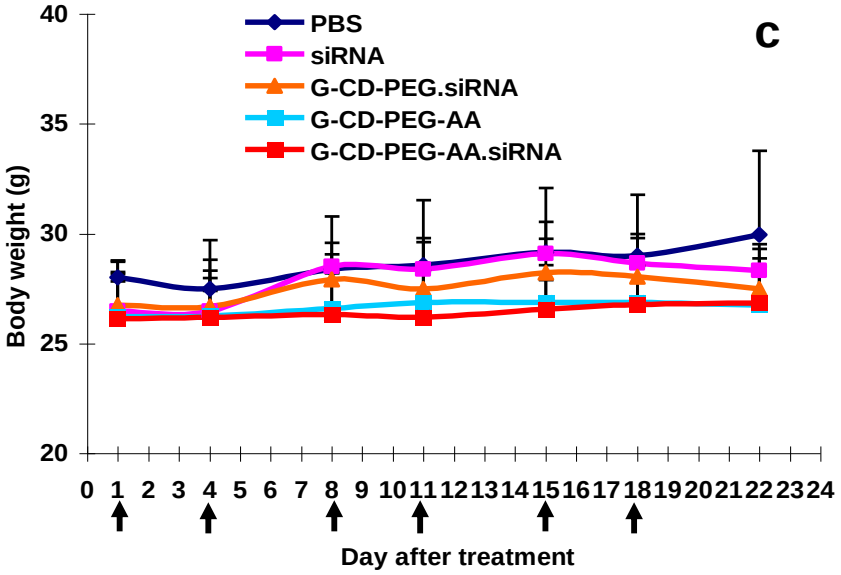
c
PBS
siRNA
G-CD-PEG.siRNA
G-CD-PEG-AA
G-CD-PEG-AA.siRNA
Body weight (g)
40
35
30
25
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Day after treatment

Figure 4.

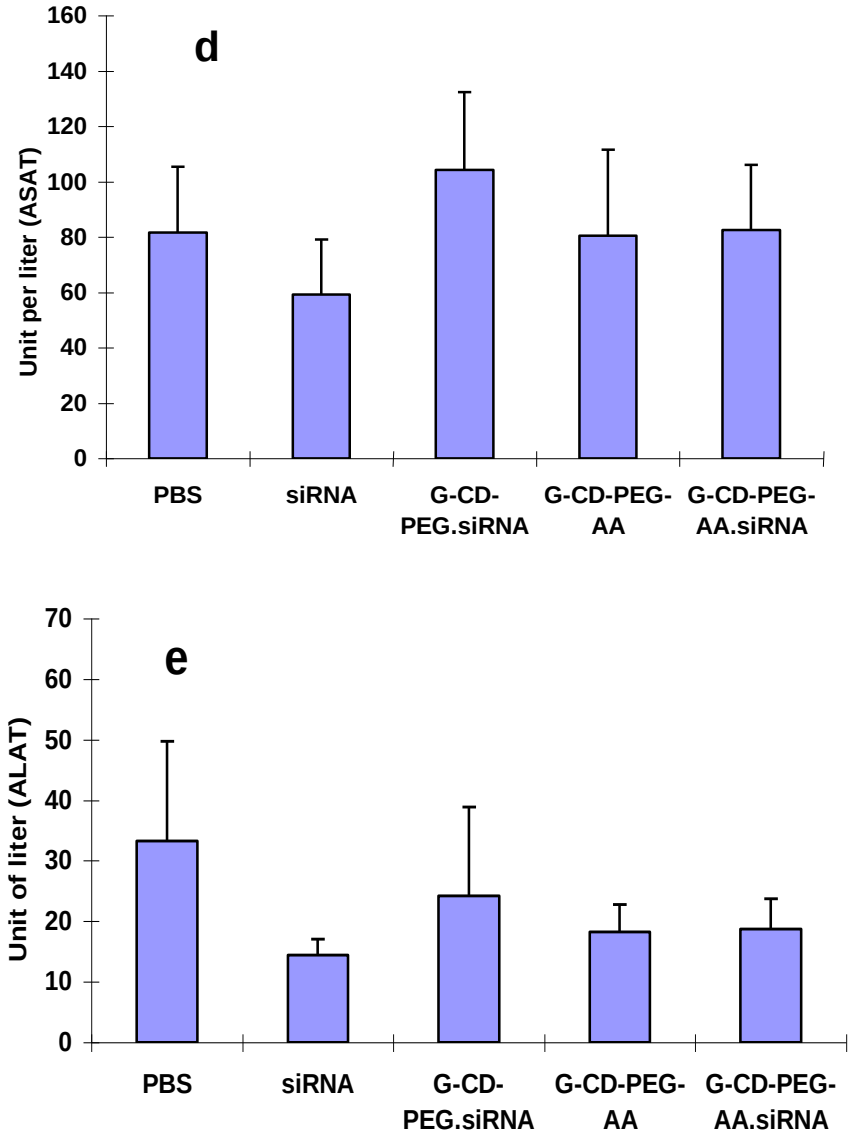
d
Unit per liter (ASAT)
160
140
120
100
80
60
40
20
0
PBS
siRNA
G-CD-PEG.siRNA
G-CD-PEG-AA
G-CD-PEG-AA.siRNA
e
Unit of liter (ALAT)
70
60
50
40
30
20
10
0
PBS
siRNA
G-CD-PEG.siRNA
G-CD-PEG-AA
G-CD-PEG-AA.siRNA

Figure 4.