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Anisamide-targeted PEGylated gold nanoparticles designed to target prostate cancer mediate: enhanced systemic exposure of siRNA, tumour growth suppression and a synergistic therapeutic response in combination with paclitaxel in mice

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ABSTRACT

Small interfering RNA (siRNA) has recently illustrated therapeutic potential for malignant disorders. However, the clinical application of siRNA-based therapeutics is significantly retarded by the paucity of successful delivery systems. Recently, multifunctional gold nanoparticles (AuNPs) as non-viral delivery carriers have shown promise for transporting chemotherapeutics, proteins/peptides, and genes. In this study, AuNPs capped with polyethylenimine (PEI) and PEGylated anisamide (a ligand known to target the sigma receptor) have been developed to produce a range of positively charged anisamide-targeted PEGylated AuNPs (namely Au-PEI-PEG-AA). The Au_{110}-PEI-PEG_{5000}-AA construct effectively complexed siRNA *via* electrostatic interaction, and the resultant complex (Au_{110}-PEI-PEG_{5000}-AA.siRNA) illustrated favourable physicochemical characteristics, including particle size, surface charge, and stability. *In vitro,* Au_{110}-PEI-PEG_{5000}-AA selective bound to human prostate cancer PC-3 cells, inducing efficient endosomal escape of siRNA, and effective downregulation of the *RelA* gene. *In vivo*, prolonged systemic exposure of siRNA was achieved by Au_{110}-PEI-PEG_{5000}-AA resulting in significant tumour growth suppression in a PC3 xenograft mouse model without an increase in toxicity. In addition, a combination of siRNA-mediated NF-κB knockdown using Au_{110}-PEI-PEG_{5000}-AA with Paclitaxel produced a synergistic therapeutic response, thus providing a promising therapeutic strategy for the treatment of prostate cancer.

1. Introduction

Prostate cancer is a leading cause of cancer-related fatalities for male population, in 2018 a total of 29,430 new cases and 164,690 deaths from this malignant disorder are forecasted to occur in the United States [1]. Recent advances in understanding the molecular pathology underlying prostate carcinogenesis have provided significant opportunities for the application of gene-based therapeutic strategies [2]. Small interfering RNA (siRNA), which results in sequence-specific post-transcriptional gene silencing in mammalian cell lines, has shown impressive anti-cancer potential [3]. However, the paucity of successful delivery systems dramatically retards the clinical translation of siRNA-based therapeutics for cancer [4].

Recently, the development of novel organic and inorganic materials has revolutionised the field of siRNA delivery for treatment of solid tumours [5] [6] [7] and haematopoietic malignancies [8] [9]. As a result, a variety of non-viral siRNA delivery nanoparticle (NP) formulations have been developed for prostate cancer therapy [10]. Despite the promise, significant challenges such as inefficient siRNA encapsulation or complexation, *in vitro* and *in vivo* NP instability, non-specific cell binding, poor endosomal or lysosomal escape, and low gene knockdown efficacy, remain to be overcome before siRNA-based therapeutics can be widely accepted for use in patients with prostate cancer.

Among the diverse range of nanoparticulate delivery carriers, gold NPs (AuNPs) have been utilised to develop siRNA nanomedicines mainly due to favourable physicochemical properties [11]. Previously, spherical positively charged AuNPs were synthesised using surfactant-free methods in the present of L-cysteine methyl ester hydrochloride [$HSCH_2CH(NH_2)COOCH_3 \cdot HCl$] [12] with poly (ethylenimine) (PEI) [13] as the capping agents. Although these positively charged AuNPs (Au-L-cysteine and Au-PEI) demonstrated effective gene silencing in vitro, activity

was curtailed due to to non-specific binding to serum proteins [12] [13]. When Au-$_L$-cysteine was further modified with polyethylene glycol (PEG), stability in serum-containing medium was improved [14]; however, these PEGylated AuNPs displayed poor cellular uptake due to the "PEG dilemma" phenomenon [15]. Bi-functional PEG moieties on AuNPs have therefore been exploited to facilitate conjugation of targeting ligands, resulting in cell-specific internalisation [16].

Informed by previous studies, a range of novel spherical AuNPs were developed in this study for targeted delivery of siRNA in the treatment of prostate cancer. The spherical Au core was initially coated with PEI to achieve a cationic surface (Au-PEI) capable of complexing siRNA. To enhance stability in physiological environments and mediate selective uptake in prostate cancer cells, Au-PEI was further modified by PEGylated anisamide (AA, a ligand known to target the sigma receptor overexpressed on the prostate cancer cell membrane [17] [18]) to generate a PEGylated Au-AA targeted construct, Au-PEI-PEG-AA. The resultant complex of Au-PEI-PEG-AA with siRNA against *RelA* gene (a gene product from the NF-κB transcription factor complex) was investigated alone and in combination with paclitaxel for therapeutic efficacy in a prostate carcinoma xenograft mouse model.

2. Materials and methods

2.1. Materials

PC-3 (human prostate cancer cell line) and CT26 (mouse colon cancer cell line) were purchased from the American Type Culture Collection (ATCC, USA). Negative control siRNA (siNeg) (sense sequence 5'-UUC UCC GAA CGU GUC ACG U-3', no modification), FAM-labelled siRNA (siFAM) [sense sequence 5'-UUC UCC GAA CGU GUC ACG U-3', modified by carboxyfluorescein (FAM) on 5' of sense

sequence], and RelA siRNA (siRelA) (sense sequence 5'-CCA UCA ACU AUG AUG AGU U-3') were purchased from GenePharma Co., Ltd., Shanghai, China. These HPLC-purified siRNAs were prepared in RNase-free water following manufacturer's recommendations.

Tetrachloroauric acid trihydrate ($HAuCl_4.3H_2O$), L-ascorbic acid ($C_6H_8O_6$), *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC.HCl), N-hydroxysuccinimide (NHS), *N*,*N*-Diisopropylethylamine (DIPEA) purified by re-distillation, branched PEI solutions (*Mw* = 2 kDa, 50 % w/v), *p*-Anisic acid (99.5%), dry dichloromethane (DCM), dry pentane, magnesium sulfate ($MgSO_4$), and thiol PEG Amine ($HS\text{-}PEG_{7500}\text{-}NH_2$ and $HS\text{-}PEG_{3500}\text{-}NH_2$) were purchased from Sigma-Aldrich. In addition, $HS\text{-}PEG_{5000}\text{-}NH_2$ was obtained from PEG Creative Work. All chemicals were used as received without any further purification.

2.2. Synthesis of AuNPs

Purified H_2O (resistivity ≈ 18.2 MΩ cm) was used as a solvent for AuNP synthesis. Glassware was cleaned with aqua regia (3 parts of concentrated HCl and 1 part of concentrated HNO_3), rinsed with distilled water, ethanol and acetone, and dried overnight before use.

2.2.1. Optical spectra

The optical absorption spectra were obtained using the Evolution 60 UV-Visible spectrophotometer with a Xenon Flash Lamp (300-1100 nm range, 0.5 nm resolution) (Thermo Fisher Scientific).

2.2.2. Scanning electron microscopy (SEM)

AuNP samples were deposited onto a Silicon wafer and air-dried prior to analysis using a FEI 630 NanoSEM equipped with an Oxford INCA energy dispersive X-ray

(EDX) detector operated at 5 kV.

2.2.3. Nuclear magnetic resonance (NMR)

NMR was recorded in deuterated chloroform ($CDCl_3$) using a Bruker NMR (400 or 300 MHz) at ambient temperature.

2.2.4. Dynamic light scattering (DLS)

Particle size and zeta potential were measured in deionised water (0.2 μm membrane-filtered) using the Malvern Nano-ZS (Malvern Instruments, UK) at 25 °C with the default non-invasive back scattering (NIBS) technique at a detection angle of 173°.

2.2.5. Synthesis of Au-PEI

Au-PEI with ~ 25 nm of diameter (Au_{25}-PEI): 2.6 mL of freshly prepared $HAuCl_4.3H_2O$ aqueous solution (19.8 mM) was added into 47.5 mL of water at 30 °C, followed by the addition of 1.55 mL of PEI aqueous solution (5 mM). When PEI was added into the solution, the colour changed from light to deep yellow indicating the interaction between the amino groups of PEI and the Au metal ions, similar to that previously observed [13], 0.83 mL of aqueous L-ascorbic acid (250 mM) was added into the solution with stirring at room temperature (RT) until the colour changed to deep red.

Au-PEI with ~ 60 nm of diameter (Au_{60}-PEI): 50 mL of freshly prepared $HAuCl_4.3H_2O$ aqueous solution (0.25 mM) were added into 0.1 mL of PEI aqueous solution (5 mM) at RT, 0.775 mL of L-ascorbic acid (100 mM) were further added into the solution and stirred at RT for 41 h.

Au-PEI with ~ 95 nm of diameter (Au_{95}-PEI): 50 mL of freshly prepared $HAuCl_4.3H_2O$ aqueous solution (0.25 mM) were added into 0.025 mL of PEI aqueous solution (5

mM) at RT. Subsequently, 0.775 mL of $_L$-ascorbic acid (100 mM) were added into the solution and stirred at RT for 20 h.

Au-PEI with ~ 110 nm of diameter (Au_{110}-PEI): 50 mL of freshly prepared $HAuCl_4.3H_2O$ aqueous solution (1 mM) were added into 0.3 mL of PEI aqueous solution (5 mM) at RT. Subsequently, 0.775 mL of $_L$-ascorbic acid (100 mM) was added into the solution and stirred at RT for 3 h.

2.2.6. Synthesis of NHS-activated anisic acid

NHS-activated anisic acid was produced as previously described [13]. Briefly, EDC.HCl (1.5 eq, 1.9 g, 9.9 mM) was added to the *p*-anisic acid solution (1 g, 6.572 mM) in dry DCM (250 ml) under Argon, followed by the addition of NHS (1.45 eq, 1.1 g, 9.56 mM). The reaction mixture was stirred for ~ 42 h under Argon at RT. The organic phase was washed twice with water followed by a wash with brine, dried over $MgSO_4$, filtered on Whatman filter paper, and evaporated. The activated ester thus obtained was left to stir in 30 mL of dry pentane for ~ 48 h, filtered, dried under vacuum, and used without further purification. The yield of the anisic-NHS ester (AA-NHS) was approximately 90%. The product was analysed using NMR spectroscopy [(400 MHz, $CDCl_3$): δ ppm 8.08-8.11 (d, 2H, ArH-CO-), 6.97-6.99 (d, 2H, ArH-OCH_3), 3.89 (s, 3H, OCH_3), 2.91 (s, 4H CH_2-CH_2 in NHS ring)] [13].

2.2.7. Synthesis of PEGylated anisamide (PEG-AA)

SH-PEG_{3500}-AA: 140 mg of SH-PEG_{3500}-NH_2 (0.04 mM) were dried under vacuum for 10 min at RT, using? a flow of nitrogen (N_2), and dried under vacuum for 5 min at RT. The SH-PEG_{3500}-NH_2 powder was then dissolved in 4 mL DCM under N_2 and 4 mL of AA-NHS (20 mg, 0.08 mM) was added and stirred in dry DCM at 0 °C. Subsequently, 120 µL of DIPEA (0.705 mM) in dry DCM was added and incubated with stirring under N_2 at RT for 48 h. The resultant SH-PEG_{3500}-AA was precipitated at 0 °C using 100 mL cold diethyl ether/ethanol solution (v/v = 99/1). The

SH-PEG_{3500}-AA was collected at 0 °C by centrifugation at 11,000 rpm and dried overnight under the vacuum. The SH-PEG_{3500}-AA (~ 90% yield) was characterised by NMR in $CDCl_3$: δ ppm 7.78-7.80 (d, 2H, ArH-CO-), 6.91-6.93 (d, 2H, ArH-OMethyl), 3.85 (s, Ar-OCH_3), 3.65 (O-CH_2-CH_2 of PEG), 2.9 (CH2-C=O). The degree of anisamide attachment was ~ 95% as calculated from the peak integration ratio of phenyl proton at δ 7.78 to methylene protons at δ 3.65.

SH-PEG_{5000}-AA: 100 mg of SH-PEG_{5000}-NH2 (0.02 mM) were dried under the vacuum for 10 min at RT, using N_2, and dried under the vacuum for 5 min at RT. The SH-PEG_{5000}-NH_2 powder were then dissolved in 4 mL of DCM under N_2 and 2 mL of AA-NHS (8.5 mg, 0.034 mM) in dry DCM was added and stirred at 0 °C. Subsequently, 50 µL of DIPEA (0.615 mM) in dry DCM was added and stirred under N_2 at RT for 48 h. The resultant SH-PEG_{5000}-AA was precipitated at 0 °C using 100 mL cold diethyl ether/ethanol solution (v/v = 99/1). The SH-PEG_{5000}-AA was collected at 0 °C by centrifugation at 11,000 rpm and dried overnight under the vacuum. The SH-PEG_{5000}-AA (~ 90% yield) was characterised by NMR in $CDCl_3$: δ ppm 7.79-7.81 (d, 2H, ArH-CO-), 6.90-6.92 (d, 2H, ArH-OMethyl), 3.85 (s, Ar-OCH_3), 3.65 (O-CH_2-CH_2 of PEG), 2.9 (CH2-C=O). The degree of anisamide attachment was ~ 100% as calculated from the peak integration ratio of phenyl proton at δ 7.78 to methylene protons at δ 3.65.

SH-PEG_{7500}-AA: 150 mg of SH-PEG_{7500}-NH2 (0.02 mM) were dried under the vacuum for 10 min at RT, using N_2, and dried under the vacuum for 5 min at RT. The SH-PEG_{7500}-NH_2 powder were then dissolved in 4 mL of DCM under the N_2 and 2 mL of AA-NHS (10 mg, 0.04 mM) in dry DCM was added and stirred at 0 °C. Subsequently, 60 µL of DIPEA (0.705 mM) in dry DCM was added and stirred at RT for 48 h. The resultant SH-PEG_{7500}-AA was precipitated at 0 °C using 100 mL cold diethyl ether/ethanol solution (v/v = 99/1). The SH-PEG_{7500}-AA was collected at 0 °C by centrifugation at 11,000 rpm for 5 min and dried overnight under the vacuum. The SH-PEG_{7500}-AA (~ 90% yield) was characterised by NMR in $CDCl_3$: δ ppm 7.78-7.80

(d, 2H, ArH-CO-), 6.91-6.93 (d, 2H, ArH-OMethyl), 3.85 (s, Ar-OCH_3), 3.65 (O-CH_2-CH_2 of PEG), 2.9 (CH2-C=O). The degree of anisamide attachment was ~ 95% as calculated from the peak integration ratio of phenyl proton at δ 7.78 to methylene protons at δ 3.65.

2.2.8. PEGylation of Au-PEI (Au-PEI-PEG and Au-PEI-PEG-AA)

The Au-PEI was PEGylated by adding SH-PEG-NH_2 (100 μM) or SH-PEG-AA (100 μM), in which the final concentrations were fixed approximately at a molar ratio of 1:250 for SH-PEG-NH_2/SH-PEG-AA and $HAuCl_4$. This reaction was continued with stirring for ~ 6 to 16 h at RT. The resultant Au-PEI-PEG and Au-PEI-PEG-AA were collected by centrifugation at 11,000 rpm for 5 min. As a result, ~ 60% of the initial SH-PEG-NH_2 and SH-PEG-AA were conjugated?? onto the Au surface, this was confirmed using UV-vis spectroscopy (Fig. S4). In addition, the successful PEGylation was confirmed using DLS and SEM.

2.3. Cytotoxicity

PC-3 and CT26 cells were maintained in RPMI-1640 medium (CORNING) supplemented with 10% FBS and a Penicillin-Streptomycin Nystatin solution (Biological Industries). Five thousand PC-3 or CT26 cells were seeded per well in 96-well culture plates one day before transfection. AuNPs ([c] = 1, 5, 10, 20, 50, 100, 250, 500 and 1000 μg/mL) were added to the cells for 72 h under normal growth conditions. Following incubation, cells were incubated with 20 μL 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) stock (5 mg/mL in PBS) in 200 μL fresh growth medium for 4 h at 37 °C. ~ 100 to 200 μL DMSO was added to dissolve the purple formazan products. The results were read at 570 nm using a microplate reader.

2.4. Preparation and physicochemical characterisation of AuNP.siRNA complexes

Solutions of Au-PEI, Au-PEI-PEG, and Au-PEI-PEG-AA (1 μg/μL) were added to siRNA solutions at different weight ratios (WRs) of AuNPs to siRNA, followed by 2 h sonication at 55-60 °C. The efficiency of AuNPs to complex siRNA was assessed using gel retardation assay [16]. Briefly, solutions of AuNPs and siRNA (0.2 μg siNeg) at various WRs were loaded onto 1% (w/v) agarose gels in Tris-Borate-EDTA (TBE) buffer containing the GelStain (Transgen Biotech, China). Electrophoresis was performed at 90 V for 20 min and the resultant gels were photographed under UV.

Measurements of particle size and zeta potential of AuNP.siRNA complexes were performed as described in section 2.2.5. The concentration of siRNA was fixed at 1 μg/mL.

Complexes containing 0.2 μg siNeg at WR40 were incubated for 1, 2, 4 and 8 h in 50% (v/v) fetal bovine serum (FBS; Gibco) at 37 °C. After incubation, samples were heated for 5 min at 80 °C for serum inactivation. Samples were then treated with excess heparin (1000 IU/mL) at RT for 1 h to release siRNA from AuNPs. Samples were loaded onto 1.5% (w/v) agarose gels containing the GelStain. Electrophoresis was performed at 90 V for 20 min and the resultant gels were photographed under UV.

In addition, complexes (WR40) were incubated in 50% (v/v) FBS at 37 °C for 24 h, and the particle sizes were measured using Malvern Nano-ZS as described in section 2.2.5. FBS alone and complexes incubated in deionised water at 37 °C for 24 h were used as controls. The concentration of siRNA was fixed at 1 μg/mL.

2.5. Cellular uptake

PC-3 and CT26 cells were respectively seeded at 1 x 10^5 cells per well in 24-well culture plates one day before transfection. Cells were treated with AuNPs complexes (WR40) containing 50 nM siFAM and incubated for 8 h under normal growth

conditions. Following incubation, cells were washed twice with pre-warmed PBS and trypsinised. After 1000 rpm centrifugation for 5 min, the supernatant was discarded and cells were re-suspended in 1000 μL ice-cold PBS. 10,000 cells were measured for each sample according to the Becton Dickinson FACS-calibur manual.

2.6. Intracellular trafficking

PC-3 cells (1 x 10^5 cells per well) were seeded in 6-well culture plates with glass bottoms (JET BIOFIL®, China) for 24 h. Cells were treated with Au-PEI-PEG-AA complexes (WR40) containing 50 nM siFAM and incubated for 4 h under the normal growth conditions. Following this, cells were incubated in LysoTracker® Deep Red (75 nM; Invitrogen) at 37 °C for 30 min. Fresh growth medium was added to the cells prior to confocal microscopic analysis using an Olympus FV 1000 microscope.

2.7. Gene knockdown

PC-3 cells (1 x 10^5 cells per well) were seeded in 24-well culture plates. Following 24 h incubation, cells were transfected by the Au-PEI-PEG-AA formulation (WR40) containing 100 nM siRelA for 24 h. After this, total RNA was isolated from cells using TransZol UP (Transgen Biotech, China). First-strand cDNA was generated from total RNA samples using TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (Transgen Biotech, China). Gene expression was assessed by real-time quantitative PCR (qPCR) using TransStart® Top Green qPCR SuperMix (Transgen Biotech, China). Assays were carried out using primers for RelA (forward: 5'-GCC TCA TCC ACA TGA ACT TGT GGG-3'; reverse: 5'-ACC ATG GTC TGG GCA AGG ACT GGG -3') and GAPDH (forward: 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse: 5'-TCC ACC ACC CCC TGT TGC TGT A-3'). Amplification was carried out under 40 cycles of denaturation at 94 °C (5 s) and annealing at 60 °C (30 s) (StepOnePlus™ Real-Time PCR System, Applied Biosystems™). The

quantitative level of each RelA mRNA was measured as a fluorescent signal corrected according to the signal for GAPDH mRNA.

In addition, PC-3 cells (1 x 10^5 cells per well) were seeded in 24-well culture plates. Following 24 h incubation, cells were transfected by Au-PEI-PEG-AA formulation (WR40) containing 100 nM siRelA for 48 h. Subsequently, cells were lysed using RIPA buffer [50 mM Trish (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS, sodium orthovanadate, sodium fluoride, EDTA, and leupeptin], and protein concentrations were quantified using the Easy II BCA Protein Quantitative Kit (GenStar, China). ~ 30-50 μg of proteins per sample were loaded onto an SDS-PAGE and electrophoresed at 80 V for 0.5 h and subsequently at 120 V for 1.5 h. Proteins were then transferred to a PVDF membrane (Immobilon®-P Transfer Membrane; Millipore) at 200 mA for 1.5 h. Membranes were incubated with appropriate antibodies [RelA (#1574i53) and β-actin (#8427f55), purchased from Affinity, USA] at RT for 12 h. Antibody reactive bands were detected using MicroChemi (DNR Bio-Imaging Systems Ltd., Israel). Densitometry analysis of bands was performed using ImageJ, and all results were normalised to the β-actin control.

2.8. *In vitro* anti-cancer effects

PC-3 cells (100,000 per well) were seeded in 24-well culture plates for one day. Au-PEI-PEG-AA complexes (WR40) containing 100 nM siRelA were applied to the cells for 24 h. Cells were subsequently incubated with Annexin V-FITC and propidium iodide (PI) supplied from TransDetect® Annexin V-FITC/PI Cell Apoptosis Detection Kit (Transgen Biotech, China). The apoptotic cells were analysed using flow cytometry (Becton Dickinson FACS-calibur).

PC-3 cells (5,000 per well) were seeded in 96-well culture plates. After 24 h, Au-PEI-PEG-AA complexes (WR40) containing 100 nM siRelA were added to cells

and incubated for 48 and 72 h. Following incubation, cells were treated with the TransDetect® Cell Counting Kit (CCK) (Transgen Biotech, China) and incubated for 2 h before measuring the absorbance at 450 nm.

In addition, PC-3 cells (5,000 per well) were seeded in 96-well culture plates. After 24 h, Au-PEI-PEG-AA complexes (WR40) containing 100 nM siRelA were added to cells and incubated for 4 h. After this, cells were further treated with Paclitaxel (PTX, purchased from Hainan Choitec Pharmaceuticals Co., Ltd.; [c] = 50, 100 and 200 nM) for 20 h. Following incubation, cells were treated with the TransDetect® Cell Counting Kit (CCK) solution (Transgen Biotech, China) and incubated for 2 h before measuring the absorbance at 450 nm.

2.9. Animal experiments

The animal ethics committee of Jilin University approved all experiments. All mice were maintained in a pathogen free animal facility for at least 2 weeks before the experiments.

2.10. *In vivo* toxic studies

Male BALB/C mice (6-8 weeks) were purchased from Changchun Institute of Biological Products, China. Animals (n = 4 per group) were intravenously injected with the siNeg (~ 1 mg/kg) complexed with Au-PEI-PEG or Au-PEI-PEG-AA (WR40), 100 μL of saline (negative control) and 100 μg of poly polyinosinic:polycytidylic acid [poly (I:C)] (positive control). Blood samples were collected at 2 h post injection. Serum IFN-α was measured by the IFN alpha Mouse ELISA Kit (Invitrogen).

In addition, mice (n = 4 per group) were intravenously injected with Au-PEI-PEG-AA formulations (WR40) containing siRelA (~ 1 mg/kg). After 4 h, animals were treated with intraperitoneal injection of PTX (~ 10 mg/kg). The body weight of mice was

recorded regularly. On the endpoint, major tissues (the heart, liver, spleen, lung and kidney) were collected and analysed using the haematoxylin and eosin (H & E) staining assay. In addition, blood samples were collected and analysed using a haemacytometer, in order to determine the haematological toxicity [19].

2.11. Pharmacokinetics

Male BALB/C mice (~ 20-22 g) (n = 4 per group) were intravenously injected with siFAM either alone (~ 1 mg/kg) or complexed with Au-PEI-PEG or Au-PEI-PEG-AA (WR40). Blood samples (~ 30 μL) were collected at 1, 3, 5, 10, 15, 30 and 60 min. The extraction and quantification of siFAM were carried out as described in [20]. The extraction efficiency of siFAM from Au-PEI-PEG and Au-PEI-PEG-AA was 94% and 96% respectively. The concentration of siFAM in plasma obtained from standard curves was corrected using these determined extraction efficiencies. Pharmacokinetic parameters were calculated using DAS 2.0.

2.12. Tissue distribution

The xenograft model was established by subcutaneous injection of 5 x 10^6 PC-3 cells into the flank of male BALB/c nude mice (5-6 weeks, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.). Tumour growth was recorded regularly, and the volume was calculated using a 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 the volume reached ~ 250 mm^3, siFAM either alone (~ 1 mg/kg) or formulated with Au-PEI-PEG or Au-PEI-PEG-AA was intravenously injected into tumour bearing mice (n = 4 per group). Two hours post treatment, mice were sacrificed, and the organs (heart, liver, spleen, lung and kidneys) and tumours were collected. As described in [21], the liver was homogenised in 1mL lysis buffer, and other tissues were homogenised in 300 μL lysis buffer. The homogenised samples were incubated at 65 °C for 10 min. siFAM in the supernatant was extracted and measured as

described in section 2.11. The dose of siRNA accumulated in tissues was quantified from a strand curve obtained by spiking known amounts of siFAM alone or siFAM complexed with AuNPs in tissues from untreated mice [21].

2.13. *In vivo* anti-tumour study

When the tumour volume reached ~ 150 mm^3, siRelA (~ 1 mg/kg) formulated with Au-PEI-PEG-AA was intravenously injected into animals (n = 4 per group) at Day 1, 3 and 5. Four hours post-injection, PTX was intravenously injected at a dose of ~ 10 mg/kg into the tumour bearing mice. Tumour growth and body weight were recorded regularly, and the tumour volume was calculated as described above. Treatments of saline, PTX alone and siNeg (~ 1 mg/kg) formulated with Au-PEI-PEG-AA were used as controls.

2.14. Statistical analysis

Data were calculated as the mean ± standard deviation (SD). An unpaired Student's t-test (two-tailed) was used to test the significance of differences between two mean values. A one-way ANOVA (Bonferroni's Post-Hoc test) was used to test the significance of differences in three or more groups. In addition, a two-way ANOVA (Bonferroni's Post-Hoc test) was used to test the significance of differences in measurements of body weight, pharmacokinetics, and tumour growth. In all experiments, $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Synthesis and physicochemical characterisation of Au-PEI and Au-PEI-PEG-AA

Due to the ease of synthesis and biocompatibility gold nanoparticles (AuNPs) have been widely studied to formulate siRNA nanomedicines [11]. In this study, spherical AuNPs were used as a scaffold to produce positively charged anisamide-targeted PEGylated NPs for delivery of siRNA into prostate cancer. As previously reported [13], AuNPs were initially modified with branched PEI (*Mw* = 2 kDa) to achieve positively charged Au-PEI. It has been reported that PEI with high *Mw* (e.g. > 10 kDa) can generate higher transfection efficiency relative to low *Mw* PEI (e.g. 2 kDa) [22]. However, low *Mw* PEI is less toxic and more biocompatibile compared to high *Mw* PEI [23]. Consequently, PEI 2 kDa was to chosen to bind siRNA to the AuNPs and to facilitate intracellular release (see section 3.4.2).

Au-PEI NPs with a range of particle sizes namely; Au_{25}-PEI, Au_{60}-PEI, Au_{95}-PEI and Au_{110}-PEI, were synthesised by the conjugation of PEI onto the surface of AuNPs. Conjugation was confirmed by UV-vis spectroscopy indicating that a red shift of ~ 50 nm accompanied by peak broadening which was clearly observed when the particle size increased from ~ 25 to 100 nm (Fig. 1a). The Au_{110}-PEI presented a spherical shape with a diameter of ~ 110 nm (Fig. 1b). The Au_{25}-PEI, Au_{60}-PEI and Au_{95}-PEI demonstrated similar morphology with diameters of ~ 25, 60 and 95 nm, respectively (Fig. S1). In contrast to SEM results, the particle size of Au-PEI obtained from the DLS was slightly larger (Fig. 1c and Table S1), indicating that the AuNPs were successfully coated with PEI. In addition, the positive surface charge of the Au-PEI gradually decreased as the particle size of the AuNPs increased (Fig. 1d and Table S1).

The anisic acid was activated by reacting with NHS to form an anisic-NHS ester (AA-NHS), which was confirmed using NMR (Fig. S2). The AA-NHS reacted with the amino group ($-NH_2$) of SH-PEG-NH_2 to form a stable amide linkage, producing SH-PEG-AA (Fig. S3). The Au-PEI was subsequently chemically modified with SH-PEG-AA to form cationic anisamide-targeted PEGylated AuNPs

(Au-PEI-PEG-AA). Results in Fig. 2a indicate that the particle size of Au-PEI-PEG-AA with the same core size (e.g. Au_{110}-PEI) was clearly increased with the PEG *Mw* (Table. S1). In contrast, the zeta potential of Au-PEI-PEG-AA with the same core size (e.g. Au_{110}-PEI) was gradually decreased with increasing the PEG *Mw* (Fig. 2b and Table S1). In addition, a thin 'halo-like' layer was clearly observed on the surface of Au-PEI-PEG-AA (Fig. 2c), most likely due to the attachment of PEG chains. In summary, these results imply or show?? that the PEGylated anisamide was successfully conjugated to the surface of Au-PEI.

3.2. Cytotoxicity of Au-PEI-PEG and Au-PEI-PEG-AA

It is well established that physicochemical characteristics (i.e. particle size, surface charge, particle shape and surface coating [24]) can significantly affect the toxicity of delivery NPs [11]. The *in vitro* toxicity of Au-PEI-PEG and Au-PEI-PEG-AA was assessed using human prostate cancer PC-3 cells (Table S2). Results show that the attachment of PEG with increasing Mw to AuNPs with the same core size (e.g. Au_{110}-PEI) significantly enhanced cell viability; for example, IC50 values of Au_{110}-PEI-PEG_{3500}-AA, Au_{110}-PEI-PEG_{5000}-AA and Au_{110}-PEI-PEG_{7500}-AA in PC-3 cells were approximately 390, 440 and 500 μg/mL respectively. In addition, the cytotoxicity of AuNPs with the same PEG length was significantly reduced when the particle size was increased; for instance, IC50 values of Au_{25}-PEI-PEG_{5000}-AA, Au_{60}-PEI-PEG_{5000}-AA, Au_{95}-PEI-PEG_{5000}-AA and Au_{110}-PEI-PEG_{5000}-AA in PC-3 cells were approximately 100, 200, 330 and 440 μg/mL respectively. Similar IC50 values were also recorded for AuNPs in CT26 cells (data not shown).

Recently, NPs have been modified with multifunctional ligands that can simultaneously enable delivery to target tissues/cells [25] [26] [27] and release drugs *via* stimuli-responsive means either extracellularly and/or intracellularly [28] [29] [30], in order to achieve personalised and effective therapeutic regimens. However, potential toxicity may be caused by interaction with specific cell receptors, cell

membranes, or intracellular organelles [11]. The sigma receptor is known to be overexpressed on the cell membrane of various human (PC-3, DU-145 and LNCaP) and mouse (Tramp C1) prostate cancer cell lines [31] [32] [33]. It is interesting to note that anisamide-targeted AuNPs demonstrated slightly higher PC-3 cell death compared to non-targeted counterparts (Table S2). In contrast, no significant difference in cytotoxicity was found between Au-PEI-PEG and Au-PEI-PEG-AA in CT26 cells (a cell line where the sigma receptor is less well expressed [34]) (data not shown). Therefore, these results suggest that the cytotoxic profiles of Au-PEI-PEG-AA may be dependent on the density of sigma receptors in cell lines (see discussion in section 3.4). On the basis of the cytotoxicity results, the concentrations of Au-PEI-PEG and Au-PEI-PEG-AA used for the following *in vitro* experiments ensured > 90% of cell viability.

3.3. Complexation of siRNA with Au-PEI-PEG and Au-PEI-PEG-AA

The formation of AuNP and siRNA complexes *via* the electrostatic interaction between cationic PEI and anionic siRNA was assessed using gel a retardation assay (Fig. 3a, S5, S6 and S7). Results show that full binding of siRNA with Au_{110}-PEI-PEG_{5000} and Au_{110}-PEI-PEG_{5000}-AA was achieved from WR40 of AuNPs and siRNA onwards (Fig. 4a). However, the AuNPs with smaller diameters did not bind siRNA as efficiently as the bigger counterparts (Fig. S6), most likely due to the fact that the bigger Au core, particularly with the spherical shape, may provide more binding space for small nucleic acids with a stiff rod-like structure (e.g. siRNA) (Fig. 1??). In addition, it is interesting to note that AuNPs with shorter PEGylation (*Mw* = 3500 kDa) achieved full complexation with siRNA at WR20 onwards, but full binding of siRNA was achieved only from WR40 onwards by AuNPs with longer PEGylation (*Mw* = 5000 and 7500 kDa) (Fig. S7). As the AuNPs are modified with the same grafting density (i.e. ~ 60%, Fig. S4), a possible explanation is that the PEG

with the higher *Mw* (proportional to the chain length) on the Au surface may fold back and overlap each other thus negatively affecting the siRNA binding efficiency.

The ability of Au-PEI-PEG-AA to protect siRNA from serum nucleases was evaluated in 50% FBS-containing medium at 37 °C (Fig. 3b). Unlike the naked siRNA which degraded significantly after 1 h incubation (data not shown), the siRNA formulated with Au_{110}-PEI-PEG_{5000}-AA (WR40) was resistant to serum degradation upto 8 h. In addition, following incubation in 50% FBS-containing medium at 37 °C, the Au_{110}-PEI-PEG_{3500}-AA.siRNA formulation aggregated at WR40, in contrast the AuNP formulations (WR40) with longer PEG chains (*Mw* = 5000 and 7500 kDa) significantly inhibited the binding of serum proteins (Fig. 3c). Based on these physicochemical characteristics (Fig. 3, S5, S6 and S7), the Au_{110}-PEI-PEG_{5000}-AA.siRNA formulation (WR40) was used for all further *in vitro* and *in vivo* experiments.

3.4. *In vitro* mechanistic studies using AuNP.siRNA complexes

3.4.1. Cellular uptake

Recently, a variety of delivery NPs modified with the anisamide or anisamide-derived targeting ligands have been developed to deliver nucleic acids in the treatment of cancer [35] [36] [37] [38] [39] [40]. Here, the selective cellular uptake of FAM labelled siRNA (siFAM) complexed with Au_{110}-PEI-PEG_{5000} or Au_{110}-PEI-PEG_{5000}-AA (WR40, 50 nM siRNA) was investigated in both PC-3 (a sigma receptor overexpressing cell line [18]) and CT26 (a cell line where the sigma receptor is less well expressed [34]). As shown in Fig. 4a, Au_{110}-PEI-PEG_{5000}-AA significantly enhanced the uptake of siRNA into PC-3 cells (~ 45%) compared to the untargeted Au_{110}-PEI-PEG_{5000} (~ 15%). In the case of CT26 cells cellular uptake by Au_{110}-PEI-PEG_{5000}-AA was significantly reduced (~ 20%) and was only slightly higher than that obtained by Au_{110}-PEI-PEG_{5000} (~ 15%) (Fig. 4a). These results

indicate that the anisamide-targeted AuNP.siRNA construct has an increased binding affinity for PC-3 cells suggesting the cellular uptake is principally mediated *via* the sigma receptor.

The sigma receptor consists of two main subclasses namely sigma-1 receptor (σ1R) and sigma-2 receptor (σ2R) [41]. The sigma receptor was earlier detected in the central nervous system of mammals [42] and recently, both sigma receptor subtypes have been found to overexpress in human cancer cell lines and patient tumour tissues [41]. NPs targeting σ1R have shown promise and recently, a number of studies have demonstrated the successful application of σ1R in drug delivery for cancer therapy [38] [39] [40]. In addition, due to overexpression of σ2R on the cell membrane of various human and mouse tumour cell lines [44] [45] [46], it also has potential as a targeting ligand for diagnostic imaging and therapy. Compounds targeting σ2R are currently under investigation for the diagnosis of breast cancer (clinical trial NCT02762110) and for the treatment of Alzheimer's disease (clinical trial NCT02907567). A debate on the role of σ1R as the receptor for targeted drug delivery has been raised, however the tumour-targeting specificity of σ1R cannot be denied in the absence of more conclusive studies [43].

Due to the lack of appropriate pharmacological inhibitors that selectively block the sigma receptors [43], it is difficult to determine whether single subtypes or both are associated with the receptor-mediated internalisation of Au-PEI-PEG-AA, this will be the focus of future work.

3.4.2. Intracellular trafficking

Following ligand-receptor mediated internalisation, NPs are normally trapped in endosomes where the pH becomes slightly acidic (~ 5 to 6). Subsequently, NPs are transported into lysosomes in which the pH drops further (~ 4.5) and various degradative enzymes may dissociate NP formulations and degrade siRNA. It has been

reported by Gilleron and colleagues that a lipid-based NP delivery system (LNP) released siRNA from endosomes into the cytoplasm at low efficiency (1-2%) [47]. In addition, Sahay et al. reported that ~ 70% of internalised siRNA formulated with LNPs underwent exocytosis from lysosomes [48]. These studies indicate that delivery nanocarriers must efficiently release siRNA from the endosomes or lysosomes, in order to successfully initiate the RNAi machinery in the cytoplasm.

It has been reported that AuNPs with certain shapes (e.g. Au nanoshells and nanocages) can absorb the light in the near-infra red (NIR) to induce photothermal effects that can assist the endosomal escape of siRNA [49]. In addition, modification of AuNPs with functional groups (e.g. pH-sensitive and enzymes-responsive moieties) may also assist the endosomal release of siRNA [11]. For example, PEI is known to be efficiently in facilitating endosomal escape *via* the "proton sponge effect" whereby the acidic pH in endosomes protonates PEI causing an increase in osmotic pressure and subsequent membrane rupture [50].

The intracellular trafficking of anisamide-targeted AuNPs was studied in PC-3 cells using FAM-labelled siRNA (Fig. 4b). After 4 h incubation with Au_{110}-PEI-PEG_{5000}-AA.siFAM (WR40, 50 nM siRNA), fluorescence (green) was detected inside the cells, mainly in the cytoplasm and without co-localisation with the lysosomal marker (red) (Fig. 4b). These results indicate that Au_{110}-PEI-PEG_{5000}-AA achieved efficient endosomal escape of sRNA within 4? h, most likely due to the PEI-mediated "proton sponge effect", these results are similar to those previously reported for other PEI-modified AuNPs [13] [16].

3.4.3. Gene knockdown

The nuclear factor κ-B (NF-κB) is a substantially investigated transcription factor that has been implicated in cell proliferation, angiogenesis and metastasis of prostate carcinoma [2]. The NF-κB is a combination of five gene products (RelA, RelB, c-Rel,

NF-κB1 and NF-κB2) [51]. Here, the endogenous gene silencing of anti-RelA siRNA (100 nM) using Au-PEI-PEG-AA (WR40) was studied in PC-3 cells (Fig. 5). Following 24 h incubation, siRelA formulated with Au_{110}-PEI-PEG_{5000}-AA significantly ($p < 0.01$) reduced the RelA mRNA level relative to negative controls including naked siRelA, siRelA formulated with Au_{110}-PEI-PEG_{5000}, and siNeg formulated with Au_{110}-PEI-PEG_{5000}-AA (Fig. 5a). In addition, siRelA formulated with Au_{110}-PEI-PEG_{5000}-AA also significantly ($p < 0.01$) down-regulated RelA protein expression after 48 h incubation relative to the aforementioned negative controls (Fig. 5b and S8). It is worth noting that siRelA formulated with Au_{110}-PEI-PEG_{5000}-AA resulted in significantly ($p < 0.05$) greater gene silencing compared to Lipofectamine® 2000 (a commercially available lipid-based transfection reagent used as a positive control in this study) (Fig. 5 and S8), indicating the therapeutic potential of the Au_{110}-PEI-PEG_{5000}-AA construct as a nanoparticulate siRNA delivery system for prostate cancer.

3.4.4. *In vitro* anti-cancer efficacy

Following efficient NF-κB knockdown, the anisamide-targeted formulation (WR40, 100 nM siRelA) induced significant levels of apoptosis in PC-3 cells ($p < 0.01$, 24 h incubation) compared to negative controls (Fig. 6a). Due to the apoptotic effects, Au_{110}-PEI-PEG_{5000}-AA.siRelA significantly slowed down the proliferation of PC-3 cells ($p < 0.01$; ~ 40% and 60% reductions after 48 h and 72 h incubation, respectively); in contrast, no significant anti-proliferative effect was observed with the negative controls (Fig. 6b). These results suggest that the anti-cancer effects of the AuNPs are related to *RelA* gene silencing, and are not due to cell death or cytotoxicity.

When the NF-κB transcription factor is activated, tumour cells are known to become resistant to conventional chemo- and radiotherapy [52]. Recently, a combination strategy has been applied to re-sensitise cancer cells to chemotherapeutics by

suppressing the NF-κB signalling pathway [53]. It has been reported that transfection of anti-RelA siRNA improved the sensitivity of PC-3 cells to chemotherapeutics (docetaxel and ciplatin) [54], due most likely to the downregulation of anti-apoptosis genes such as Bcl-2 and Bcl-xL (these are NF-κB downstream genes) [55]. In this study, when combined with Paclitaxel (PTX, an alkaloid with microtubule-targeting capacity, widely utilised to treat a variety of cancers [56]), the downregulation of NF-κB mediated by siRelA using Au_{110}-PEI-PEG_{5000}-AA achieved a synergistic anti-proliferative effect in comparison to either PTX or Au_{110}-PEI-PEG_{5000}-AA.siRNA alone (Fig. S8), indicating the therapeutic potential of this combination strategy in the treatment of prostate cancer.

3.5. *In vivo* studies using AuNP.siRNA complexes

3.5.1 Toxicity

As described in section 3.2, Au-PEI-PEG and Au-PEI-PEG-AA did not cause significant cytotoxicity in vitro. The in vivo toxicity of AuNPs was further assessed using mice (n = 4 mice per group) (Fig. 7a, S9 and S10; Table S3). Two hours post intravenous administration of a single bolus? dose, AuNP.siRNA formulations (WR40, ~ 1 mg/kg siRNA) no increase in interferon alpha (IFN-α) levels were detected (Fig. S9). In addition, following treatment with either Paclitaxel (PTX, ~ 10 mg/kg) alone, or Au_{110}-PEI-PEG_{5000}-AA.siRelA (WR40, ~ 1 mg/kg siRNA) alone, or a combination of AuNPs and PTX no significant decrease in body weight was detected over a 2 week period compared to the saline control group (Fig. 8a). At the endpoint (Day 14), the heart, liver, spleen, lung and kidneys were collected and analysed using H & E staining. No significant histological differences between samples from mice treated with PTX, AuNP.siRNA or the combination versus the saline control were detected (Fig. S10). In addition, the peripheral blood was analysed using the haemacytometer to further examine systemic toxicity (Table S3), the results indicate no significant stats? haematological toxicity following the test treatments. In

summary, these preliminary murine toxicology studies on the Au_{110}-PEI-PEG_{5000}-AA.siRNA formulation (WR40) indicate no significant signs of systemic toxicity.

3.5.2 Pharmacokinetics

The incorporation of PEG (PEGylation) into NPs is known to prevent aggregation and adsorption of blood components (i.e. serum proteins) [57] and therefore has become one of the most efficient approaches to improve the half-life of NPs in systemic circulation [58]. The circulation time of PEGylated NPs is influenced by the PEG *Mw* (proportional to the chain length), increases in circulation times of ~ 4, 7 and 17 min have been reported for micelles incorporating 5, 10, and 20 kDa of PEG, respectively [59]. For PEGylated liposomes, conflicting effects have been reported. In one study increased circulation was achieved when the PEG *Mw* was increased from 750 Da to 5 kDa [60], in contrast another study found negligible effects with PEG *Mw* ranging from 350 Da to 2 kDa [61] suggesting that longer PEG lengths are required to prolong in vivo exposure.

As described in Fig. 3c, AuNP formulations with longer PEGylation (*Mw* = 7500) demonstrated better *in vitro* stability compared to shorter PEGylation (*Mw* = 3500 and 5000). However, longer PEGylation (*Mw* = 7500) negatively affected the siRNA binding efficiency in comparison to shorter PEGylation (*Mw* = 3500 and 5000) (Fig. S7). Under the experimental conditions used in this study, Au_{110}-PEI-PEG_{5000}-AA could efficiently complex siRNA achieving a formulation with favourable physicochemical properties, and consequently the pharmacokinetic profile of this construct was evaluated.

The plasma concentrations versus time for siFAM alone (~ 1 mg/kg) and siFAM complexed with Au_{110}-PEI-PEG_{5000} or Au_{110}-PEI-PEG_{5000}-AA (WR40) (n = 4 mice per group) are shown in Fig. 7b. Plasma concentrations of free siRNA decreased

rapidly, with only residual levels detected less than 15 min post administration. In contrast, the siRNAs complexed with PEGylated AuNPs were more slowly cleared from the plasma, over 60 min post administration (Fig. 7b).

The pharmacokinetic parameters were generated by fitting to a one-compartmental model (Table 1). The rank order in systemic exposure (area under the curve, AUC) was Au_{110}-PEI-PEG_{5000}-AA ≈ Au_{110}-PEI-PEG_{5000} > siFAM, where the AUC values calculated for AuNP.siRNA formulations were significantly ($p < 0.05$) higher than free siFAM. In addition, PEGylated formulations significantly ($p < 0.05$) reduced clearance (CL) values compared to free siFAM, no significant difference was observed between targeted and non-targeted formulations (Table 1). Corresponding with a decrease in CL, a trend towards longer circulating time (i.e. the half-life, $t_{1/2}$) was observed with a rank order of Au_{110}-PEI-PEG_{5000}-AA > Au_{110}-PEI-PEG_{5000} > siFAM, where the $t_{1/2}$ value of Au_{110}-PEI-PEG_{5000}-AA.siFAM (6.20 min) was significantly higher than those of Au_{110}-PEI-PEG_{5000}.siFAM (4.98 min, $p < 0.05$) and siFAM (0.46 min, $p < 0.001$). In summary, these results show that the Au_{110}-PEI-PEG_{5000}-AA.siRNA formulation achieved a 13.5 fold increase in systemic exposure of siRNA relative to the naked control.

3.5.3 Tissue distribution

It is known that the leaky blood vessels in solid tumours provide access to circulating NPs with particle size < 500 nm *via* the "enhanced penetration and retention" (EPR) effect [62]. However, NPs above 100 nm are generally recognised by the MPS (e.g. liver Kupffer cells) [63]. In addition, positively charged NPs, due to non-specific adsorption of serum proteins, may be entrapped in the lung [63]. It has been reported that the majority of drugs delivered by NPs was found inside the liver, lung and spleen [64] and in contrast, minimal drug distribution was observed in tumours [e.g. < 5% of injection dose (ID)/g] [65] [66].

In this study, the tissue distribution of siRNA was analysed *ex vivo* 2 h after intravenous injection of a single dose containing siFAM alone (~ 1 mg/kg) or siFAM complexed with Au_{110}-PEI-PEG_{5000} or Au_{110}-PEI-PEG_{5000}-AA (WR40) (n = 4 mice per group) (Fig. 7c). It is not surprising that free siRNA did not show efficient retention in excised tissues (< 7% ID/g). In contrast, the siRNA delivered by AuNP formulations was mainly found in the liver (~ 40% ID/g), tumour (~ 15% ID/g) and lung (~ 10% ID/g), thus the accumulation of siRNA in the tumour was greatly improved (Fig. 7c) in comparison with previous studies [65] [66]. Although not statistically different?, the tumour uptake of the targeted AuNP tended to be greater versus the untargeted NP implying that the addition of anisamide targeting ligand may further assist the tissue uptake [21].

3.5.4 *In vivo* anti-tumour effects

It is well known that the NF-κB signalling pathway plays a key role in development, maintenance, and progression of solid tumours and haematological malignancies [67] [68], therefore presenting a promising target for cancer therapy [52]. siRNA-based blockage of NF-κB has resulted in *in vitro* and *in vivo* anti-proliferative and anti-metastasis effects [69] [70] [71] [72]. In this study, the therapeutic efficacy of Au_{110}-PEI-PEG_{5000}-AA complex (WR40) containing anti-RelA siRNA (~ 1 mg/kg) in PC-3 xenograft mice was assessed following intravenous administration (n = 4 mice per group) (Fig. 7d). Results show that the Au_{110}-PEI-PEG_{5000}-AA complex containing siRelA significantly ($p < 0.05$) retarded tumour growth relative to Au_{110}-PEI-PEG_{5000}-AA complex containing negative control siRNA and the saline control group. This anti-tumour effect was mainly due to the *RelA* gene knockdown (Fig. S11).

Recently, it has been reported that intraperitoneal injections of anti-RelA siRNA decreased the expression of NF-κB in gastric cancer, enhanced the sensitivity of tumour cells to PTX, and thereby achieved a synergistic therapeutic effect with PTX

in tumour bearing mice [73]. Since PTX is also the first line chemotherapeutic for prostate cancer, the rational for the combination of AuNP.siRNA formulation and PTX in prostate cancer therapy was assessed in this study (Fig. 7d). When a combination of the Au_{110}-PEI-PEG_{5000}-AA.siRelA complex and PTX was given to diseased mice, the anti-tumour efficacy was significantly ($p < 0.01$) higher than either of the individual monotherapies (Fig. 7d). This synergistic effect confirmed the hypothesis that the inactivation of NF-κB (Fig. S11) can enhance the sensitivity of tumour cells to PTX, which is most likely due to the downregulation of anti-apoptosis genes regulated by NF-κB [55] (Fig. 6a). In addition, the combination of the Au_{110}-PEI-PEG_{5000}-AA.siRelA complex and PTX did not induce any significant toxicity (Fig. 7a, S9, S10 and Table S3), indicating the potential for clinical translation of this combination strategy in the treatment of prostate carcinoma.

4. Conclusions

A range of positively charged anisamide-targeted PEGylated AuNPs (namely Au-PEI-PEG-AA) were developed for delivery of therapeutic siRNA in the treatment of prostate carcinoma. One of these Au-PEI-PEG-AA, namely Au_{110}-PEI-PEG_{5000}-AA could effectively complex siRNA *via* the electrostatic interaction, and the resultant complexation (Au_{110}-PEI-PEG_{5000}-AA.siRNA) demonstrated favourable particle size, surface charge, and stability. The *in vitro* studies show cell specific internalisation indicating the function of the anisamide targeting ligand. Following intravenous administration the tumour reduction was reflected in the siRNA-mediated knockdown of NF-κB. More importantly, a synergistic therapeutic effect was promoted by a corresponding downregulation of target gene in combination with chemotherapeutics, without showing significant toxicity. These results imply that the anisamide-targeted

AuNP vector provides a promising strategy for combination therapy in the treatment of prostate cancer.

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References:

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018, CA: a cancer journal for clinicians 68(1) (2018) 7-30.

[2] K.A. Fitzgerald, J.C. Evans, J. McCarthy, J. Guo, M. Prencipe, M. Kearney, W.R. Watson, C.M. O'Driscoll, The role of transcription factors in prostate cancer and potential for future RNA interference therapy, Expert Opin Ther Targets 18(6) (2014) 633-49.

[3] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411(6836) (2001) 494-8.

[4] J. Guo, L. Bourre, D.M. Soden, G.C. O'Sullivan, C. O'Driscoll, Can non-viral technologies knockdown the barriers to siRNA delivery and achieve the next generation of cancer therapeutics?, Biotechnology advances 29(4) (2011) 402-17.

[5] Z. Wang, G. Liu, H. Zheng, X. Chen, Rigid nanoparticle-based delivery of anti-cancer siRNA: challenges and opportunities, Biotechnology advances 32(4) (2014) 831-43.

[6] H.J. Kim, A. Kim, K. Miyata, K. Kataoka, Recent progress in development of siRNA delivery vehicles for cancer therapy, Adv Drug Deliv Rev 104 (2016) 61-77.

[7] P. Resnier, T. Montier, V. Mathieu, J.P. Benoit, C. Passirani, A review of the current status of siRNA nanomedicines in the treatment of cancer, Biomaterials 34(27) (2013) 6429-43.

[8] J. Guo, M.R. Cahill, S.L. McKenna, C.M. O'Driscoll, Biomimetic nanoparticles for siRNA delivery in the treatment of leukaemia, Biotechnology advances 32(8) (2014) 1396-409.

[9] H. Uludag, B. Landry, J. Valencia-Serna, K.C. Remant-Bahadur, D. Meneksedag-Erol, Current attempts to implement siRNA-based RNAi in leukemia models, Drug discovery today 21(9) (2016) 1412-1420.

[10] J. Guo, J.C. Evans, C.M. O'Driscoll, Delivering RNAi therapeutics with non-viral technology: a promising strategy for prostate cancer?, Trends in molecular medicine 19(4) (2013) 250-61.

[11] J. Guo, K. Rahme, K.A. Fizgerald, J.D. Holmes, C.M. O'Driscoll, Biomimetic gold nanocomplexes for gene knockdown: Will gold deliver dividends for small interfering RNA nanomedicines?, Nano Res 8(10) (2015) 3110-40.

[12] J. Guo, M.J. Armstrong, C.M. O'Driscoll, J.D. Holmes, K. Rahme, Positively charged, surfactant-free gold nanoparticles for nucleic acid delivery, RSC Advances 5 (2015) 17862-71.

[13] K.A. Fizgerald, K. Rahme, J. Guo, J.D. Holmes, C.M. O'Driscoll, Anisamide-targeted gold nanoparticles for siRNA delivery in prostate cancer – synthesis, physicochemical characterisation and in vitro evaluation, Journal of Materials Chemistry B 4 (2016) 2242-52.

[14] K. Rahme, J. Guo, J.D. Holmes, C.M. O'Driscoll, Evaluation of the physicochemical properties and the biocompatibility of polyethylene glycol-conjugated gold nanoparticles: A formulation strategy for siRNA delivery, Colloids and surfaces. B, Biointerfaces 135 (2015) 604-12.

[15] S. Mishra, P. Webster, M.E. Davis, PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles, European journal of cell biology 83(3) (2004) 97-111.

[16] J. Guo, C.M. O'Driscoll, J.D. Holmes, K. Rahme, Bioconjugated gold nanoparticles enhance cellular uptake: A proof of concept study for siRNA delivery in prostate cancer cells, International journal of pharmaceutics 509(1-2) (2016) 16-27.

[17] R. Banerjee, P. Tyagi, S. Li, L. Huang, Anisamide-targeted stealth liposomes: a potent carrier for targeting doxorubicin to human prostate cancer cells, International journal of cancer 112(4) (2004) 693-700.

[18] J. Guo, J.R. Ogier, S. Desgranges, R. Darcy, C. O'Driscoll, Anisamide-targeted cyclodextrin nanoparticles for siRNA delivery to prostate tumours in mice, Biomaterials 33(31) (2012) 7775-84.

[19] Z.Z. Yang, J.Q. Li, Z.Z. Wang, D.W. Dong, X.R. Qi, Tumor-targeting dual peptides-modified cationic liposomes for delivery of siRNA and docetaxel to gliomas, Biomaterials 35(19) (2014) 5226-39.

[20] A.M. O'Mahony, S. Desgranges, J. Ogier, A. Quinlan, M. Devocelle, R. Darcy, J.F. Cryan, C.M. O'Driscoll, In vitro investigations of the efficacy of cyclodextrin-siRNA complexes modified with lipid-PEG-Octaarginine: towards a formulation strategy for non-viral neuronal siRNA delivery, Pharmaceutical research 30(4) (2013) 1086-98.

[21] S.D. Li, Y.C. Chen, M.J. Hackett, L. Huang, Tumor-targeted delivery of siRNA by self-assembled nanoparticles, Mol Ther 16(1) (2008) 163-9.

[22] W.T. Godbey, K.K. Wu, A.G. Mikos, Poly(ethylenimine) and its role in gene delivery, J Control Release 60(2-3) (1999) 149-60.

[23] S. Son, R. Namgung, J. Kim, K. Singha, W.J. Kim, Bioreducible polymers for gene silencing and delivery, Acc Chem Res 45(7) (2012) 1100-12.

[24] S.M. Moghimi, A.C. Hunter, T.L. Andresen, Factors controlling nanoparticle pharmacokinetics: an integrated analysis and perspective, Annual review of pharmacology and toxicology 52 (2012) 481-503.

[25] R. Prades, S. Guerrero, E. Araya, C. Molina, E. Salas, E. Zurita, J. Selva, G. Egea, C. Lopez-Iglesias, M. Teixido, M.J. Kogan, E. Giralt, Delivery of gold nanoparticles to the brain by conjugation with a peptide that recognizes the transferrin receptor, Biomaterials 33(29) (2012) 7194-205.

[26] Y. Yi, H.J. Kim, P. Mi, M. Zheng, H. Takemoto, K. Toh, B.S. Kim, K. Hayashi, M. Naito, Y. Matsumoto, K. Miyata, K. Kataoka, Targeted systemic delivery of siRNA to cervical cancer model using cyclic RGD-installed unimer polyion complex-assembled gold nanoparticles, J Control Release 244(Pt B) (2016) 247-256.

[27] R. Deng, N. Shen, Y. Yang, H. Yu, S. Xu, Y.W. Yang, S. Liu, K. Meguellati, F. Yan, Targeting epigenetic pathway with gold nanoparticles for acute myeloid leukemia therapy, Biomaterials 167 (2018) 80-90.

[28] L. Han, J. Zhao, X. Zhang, W. Cao, X. Hu, G. Zou, X. Duan, X.J. Liang, Enhanced siRNA delivery and silencing gold-chitosan nanosystem with surface charge-reversal polymer assembly and good biocompatibility, ACS Nano 6(8) (2012) 7340-51.

[29] F. Perche, Y. Yi, L. Hespel, P. Mi, A. Dirisala, H. Cabral, K. Miyata, K. Kataoka, Hydroxychloroquine-conjugated gold nanoparticles for improved siRNA activity, Biomaterials 90 (2016) 62-71.

[30] P. Zhang, C. Wang, J. Zhao, A. Xiao, Q. Shen, L. Li, J. Li, J. Zhang, Q. Min, J. Chen, H.Y. Chen, J.J. Zhu, Near Infrared-Guided Smart Nanocarriers for MicroRNA-Controlled Release of Doxorubicin/siRNA with Intracellular ATP as Fuel, ACS Nano 10(3) (2016) 3637-47.

[31] C.S. John, B.J. Vilner, B.C. Geyer, T. Moody, W.D. Bowen, Targeting sigma receptor-binding benzamides as in vivo diagnostic and therapeutic agents for human prostate tumors, Cancer Res 59(18) (1999) 4578-83.

[32] A. Marrazzo, J. Fiorito, L. Zappala, O. Prezzavento, S. Ronsisvalle, L. Pasquinucci, G.M. Scoto, R. Bernardini, G. Ronsisvalle, Antiproliferative activity of phenylbutyrate ester of haloperidol metabolite II [(+/-)-MRJF4] in prostate cancer cells, Eur J Med Chem 46(1) (2011) 433-8.

[33] N.A. Colabufo, C. Abate, M. Contino, C. Inglese, M. Niso, F. Berardi, R. Perrone, PB183, a sigma receptor ligand, as a potential PET probe for the imaging of prostate adenocarcinoma, Bioorg Med Chem Lett 18(6) (2008) 1990-3.

[34] S.D. Li, S. Chono, L. Huang, Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles, J Control Release 126(1) (2008) 77-84.

[35] Y. Chen, S.R. Bathula, Q. Yang, L. Huang, Targeted nanoparticles deliver siRNA to melanoma, J Invest Dermatol 130(12) (2010) 2790-8.

[36] Y. Wang, H.H. Su, Y. Yang, Y. Hu, L. Zhang, P. Blancafort, L. Huang, Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy, Mol Ther 21(2) (2013) 358-67.

[37] B.L. Rodriguez, J.M. Blando, P.D. Lansakara, Y. Kiguchi, J. DiGiovanni, Z. Cui, Antitumor activity of tumor-targeted RNA replicase-based plasmid that expresses interleukin-2 in a murine melanoma model, Mol Pharm 10(6) (2013) 2404-15.

[38] K.A. Fitzgerald, M. Malhotra, M. Gooding, F. Sallas, J.C. Evans, R. Darcy, C.M. O'Driscoll, A novel, anisamide-targeted cyclodextrin nanoformulation for siRNA delivery to prostate cancer cells expressing the sigma-1 receptor, Int J Pharm 499(1-2) (2016) 131-145.

[39] Q. Liu, H. Zhu, K. Tiruthani, L. Shen, F. Chen, K. Gao, X. Zhang, L. Hou, D. Wang, R. Liu, L. Huang, Nanoparticle-Mediated Trapping of Wnt Family Member 5A in Tumor Microenvironments Enhances Immunotherapy for B-Raf Proto-Oncogene Mutant Melanoma, ACS Nano 12(2) (2018) 1250-1261.

[40] W. Song, L. Shen, Y. Wang, Q. Liu, T.J. Goodwin, J. Li, O. Dorosheva, T. Liu, R. Liu, L. Huang, Synergistic and low adverse effect cancer immunotherapy by immunogenic chemotherapy and locally expressed PD-L1 trap, Nat Commun 9(1) (2018) 2237.

[41] C.G. Rousseaux, S.F. Greene, Sigma receptors [sigmaRs]: biology in normal and diseased states, J Recept Signal Transduct Res (2015) 1-62.

[42] W.R. Martin, C.G. Eades, J.A. Thompson, R.E. Huppler, P.E. Gilbert, The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog, J Pharmacol Exp Ther 197(3) (1976) 517-32.

[43] A. Dasargyri, C.D. Kumin, J.C. Leroux, Targeting Nanocarriers with Anisamide: Fact or Artifact?, Adv Mater 29(7) (2017).

[44] C. Zeng, S. Vangveravong, J. Xu, K.C. Chang, R.S. Hotchkiss, K.T. Wheeler, D. Shen, Z.P. Zhuang, H.F. Kung, R.H. Mach, Subcellular localization of sigma-2 receptors in breast cancer cells using two-photon and confocal microscopy, Cancer Res 67(14) (2007) 6708-16.

[45] H. Kashiwagi, J.E. McDunn, P.O. Simon, Jr., P.S. Goedegebuure, J. Xu, L. Jones, K. Chang, F. Johnston, K. Trinkaus, R.S. Hotchkiss, R.H. Mach, W.G. Hawkins, Selective sigma-2 ligands preferentially bind to pancreatic adenocarcinomas: applications in diagnostic imaging and therapy, Mol Cancer 6 (2007) 48.

[46] S.U. Mir, I.S. Ahmed, S. Arnold, R.J. Craven, Elevated progesterone receptor membrane component 1/sigma-2 receptor levels in lung tumors and plasma from lung cancer patients, Int J Cancer 131(2) (2012) E1-9.

[47] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stoter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape, Nat Biotechnol 31(7) (2013) 638-46.

[48] G. Sahay, W. Querbes, C. Alabi, A. Eltoukhy, S. Sarkar, C. Zurenko, E. Karagiannis, K. Love, D. Chen, R. Zoncu, Y. Buganim, A. Schroeder, R. Langer, D.G. Anderson, Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling, Nat Biotechnol 31(7) (2013) 653-8.

[49] J. Guo, K. Rahme, Y. He, L.L. Li, J.D. Holmes, C.M. O'Driscoll, Gold nanoparticles enlighten the future of cancer theranostics, Int J Nanomedicine 12 (2017) 6131-6152.

[50] G. Creusat, A.S. Rinaldi, E. Weiss, R. Elbaghdadi, J.S. Remy, R. Mulherkar, G. Zuber, Proton sponge trick for pH-sensitive disassembly of polyethylenimine-based siRNA delivery systems, Bioconjug Chem 21(5) (2010) 994-1002.

[51] X. Dolcet, D. Llobet, J. Pallares, X. Matias-Guiu, NF-kB in development and progression of human cancer, Virchows Arch 446(5) (2005) 475-82.

[52] V. Baud, M. Karin, Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls, Nat Rev Drug Discov 8(1) (2009) 33-40.

[53] P. Godwin, A.M. Baird, S. Heavey, M.P. Barr, K.J. O'Byrne, K. Gately, Targeting nuclear factor-kappa B to overcome resistance to chemotherapy, Front Oncol 3 (2013) 120.

[54] Y. Li, F. Ahmed, S. Ali, P.A. Philip, O. Kucuk, F.H. Sarkar, Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis

induced by chemotherapeutic agents in human cancer cells, Cancer Res 65(15) (2005) 6934-42.

[55] C.Y. Wang, J.C. Cusack, Jr., R. Liu, A.S. Baldwin, Jr., Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB, Nat Med 5(4) (1999) 412-7.

[56] T.M. Mekhail, M. Markman, Paclitaxel in cancer therapy, Expert Opin Pharmacother 3(6) (2002) 755-66.

[57] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, Adv Drug Deliv Rev 99(Pt A) (2016) 28-51.

[58] A. Kolate, D. Baradia, S. Patil, I. Vhora, G. Kore, A. Misra, PEG - a versatile conjugating ligand for drugs and drug delivery systems, J Control Release 192 (2014) 67-81.

[59] M. Miteva, K.C. Kirkbride, K.V. Kilchrist, T.A. Werfel, H. Li, C.E. Nelson, M.K. Gupta, T.D. Giorgio, C.L. Duvall, Tuning PEGylation of mixed micelles to overcome intracellular and systemic siRNA delivery barriers, Biomaterials 38 (2015) 97-107.

[60] A. Mori, A.L. Klibanov, V.P. Torchilin, L. Huang, Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo, FEBS Lett 284(2) (1991) 263-6.

[61] N. Dos Santos, C. Allen, A.M. Doppen, M. Anantha, K.A. Cox, R.C. Gallagher, G. Karlsson, K. Edwards, G. Kenner, L. Samuels, M.S. Webb, M.B. Bally, Influence of poly(ethylene glycol) grafting density and polymer length on liposomes: relating plasma circulation lifetimes to protein binding, Biochim Biophys Acta 1768(6) (2007) 1367-77.

[62] H. Maeda, H. Nakamura, J. Fang, The EPR effect for macromolecular drug delivery to solid tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo, Adv Drug Deliv Rev 65(1) (2013) 71-9.

[63] W. Li, F.C. Szoka, Jr., Lipid-based nanoparticles for nucleic acid delivery, Pharm Res 24(3) (2007) 438-49.

[64] R.M. Schiffelers, H.K. de Wolf, I. van Rooy, G. Storm, Synthetic delivery systems for intravenous administration of nucleic acids, Nanomedicine (Lond) 2(2) (2007) 169-81.

[65] H.K. de Wolf, C.J. Snel, F.J. Verbaan, R.M. Schiffelers, W.E. Hennink, G. Storm, Effect of cationic carriers on the pharmacokinetics and tumor localization of nucleic acids after intravenous administration, Int J Pharm 331(2) (2007) 167-75.

[66] F. Takeshita, Y. Minakuchi, S. Nagahara, K. Honma, H. Sasaki, K. Hirai, T. Teratani, N. Namatame, Y. Yamamoto, K. Hanai, T. Kato, A. Sano, T. Ochiya, Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo, Proc Natl Acad Sci U S A 102(34) (2005) 12177-82.

[67] A. Richmond, Nf-kappa B, chemokine gene transcription and tumour growth, Nat Rev Immunol 2(9) (2002) 664-74.

[68] M. Karin, Nuclear factor-kappaB in cancer development and progression, Nature 441(7092) (2006) 431-6.

[69] J. Duan, J. Friedman, L. Nottingham, Z. Chen, G. Ara, C. Van Waes, Nuclear factor-kappaB p65 small interfering RNA or proteasome inhibitor bortezomib sensitizes head and neck squamous cell carcinomas to classic histone deacetylase inhibitors and novel histone deacetylase inhibitor PXD101, Mol Cancer Ther 6(1) (2007) 37-50.

[70] J.C. Evans, J. McCarthy, C. Torres-Fuentes, J.F. Cryan, J. Ogier, R. Darcy, R.W. Watson, C.M. O'Driscoll, Cyclodextrin mediated delivery of NF-kappaB and SRF siRNA reduces the invasion potential of prostate cancer cells in vitro, Gene Ther 22(10) (2015) 802-10.

[71] R.A. Ortega, W. Barham, K. Sharman, O. Tikhomirov, T.D. Giorgio, F.E. Yull, Manipulating the NF-kappaB pathway in macrophages using mannosylated, siRNA-delivering nanoparticles can induce immunostimulatory and tumor cytotoxic functions, Int J Nanomedicine 11 (2016) 2163-77.

[72] H. Yu, C. Guo, B. Feng, J. Liu, X. Chen, D. Wang, L. Teng, Y. Li, Q. Yin, Z. Zhang, Y. Li, Triple-Layered pH-Responsive Micelleplexes Loaded with siRNA and Cisplatin Prodrug for NF-Kappa B Targeted Treatment of Metastatic Breast Cancer, Theranostics 6(1) (2016) 14-27.

[73] M. Inoue, S. Matsumoto, H. Saito, S. Tsujitani, M. Ikeguchi, Intraperitoneal administration of a small interfering RNA targeting nuclear factor-kappa B with paclitaxel successfully prolongs the survival of xenograft model mice with peritoneal metastasis of gastric cancer, Int J Cancer 123(11) (2008) 2696-701.