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Co-delivery of doxorubicin and siRNA using octreotide-conjugated gold nanorods for targeted neuroendocrine cancer therapy[†]

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Abstract

A multifunctional gold (Au) nanorod (NR)-based nanocarrier capable of co-delivering small interfering RNA (siRNA) against achaete-scute complex-like 1 (ASCL1) and an anticancer drug (doxorubicin (DOX)) specifically to neuroendocrine (NE) cancer cells was developed and characterized for combined chemotherapy and siRNA-mediated gene silencing. The Au NR was conjugated with (1) DOX, an anticancer drug, via a pH-labile hydrazone linkage to enable pH-controlled drug release, (2) polyarginine, a cationic polymer for complexing siRNA, and (3) octreotide (OCT), a tumor-targeting ligand, to specifically target NE cancer cells with overexpressed somatostatin receptors. The Au NR-based nanocarriers exhibited a uniform size distribution as well as pH-sensitive drug release. The OCT-conjugated Au NR-based nanocarriers (Au-DOX-OCT, targeted) exhibited a much higher cellular uptake in a human carcinoid cell line (BON cells) than non-targeted Au NR-based nanocarriers (Au-DOX) as measured by both flow cytometry and confocal laser scanning microscopy (CLSM). Moreover, Au-DOX-OCT-ASCL1 siRNA (Au-DOX-OCT complexed with ASCL1 siRNA) resulted in significantly higher gene silencing in NE cancer cells than Au-DOX-ASCL1 siRNA (non-targeted Au-DOX complexed with ASCL1 siRNA) as measured by an immunoblot analysis. Additionally, Au-DOX-OCT-ASCL1 siRNA was the most efficient nanocarrier at altering the NE phenotype of NE cancer cells and showed the strongest anti-proliferative effect. Thus, combined chemotherapy and RNA silencing using NE tumor-targeting Au NR-based nanocarriers could potentially enhance the therapeutic outcomes in treating NE cancers.

Introduction

Neuroendocrine (NE) cancers including carcinoid, islet cell tumors, pheochromocytoma, and medullary thyroid cancer are hormone secreting neoplasms that can cause significant patient morbidity, such as uncontrollable diarrhea, hypertension, flushing, skin rashes, and heart failure.¹⁻⁴ NE cancers are the second most prevalent tumor in the gastrointestinal tract after colorectal cancer.⁵ Most patients with NE cancers will have metastatic disease at the

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time of presentation.⁶ Surgical resection is the only curative option but most patients are not candidates for operative intervention due to widespread metastases or the degree of hepatic involvement by the NE cancers. Moreover, other forms of therapy including chemoembolization, radiofrequency ablation, cryoablation, and chemotherapy have had limited efficacy.^{7–12} Therefore, besides surgery, there are no curative treatments for NE cancers and their metastases, emphasizing the need for the development of other forms of therapy.

Recent advances in nanomedicine have the potential to significantly enhance the therapeutic outcomes of cancer treatment including NE cancers.^{13–18} Drug/agent nanocarriers are desirable tumor-targeting vehicles because many tumors present fenestrated neovasculature and poor lymphatic drainage, which allows them to accumulate preferentially over time at the tumor site (passive targeting).^{16,18,19} To further improve the delivery efficiency and cancer specificity, nanocarriers with active tumor-targeting capability are required.^{20, 21}

To date, nanoparticles have been largely designed to encapsulate and deliver a single therapeutic agent. However, there is a growing interest in developing multi-agent co-delivery nanocarriers that can encapsulate multiple types of payloads and simultaneously deliver them to targeted disease sites in a specific and controlled manner for combined therapy. These multi-agent co-delivery nanocarriers take advantage of the synergetic effects of different treatment mechanisms to dramatically improve overall therapeutic outcomes. It has already been demonstrated that combining a chemotherapeutic agent with small interfering ribonucleic acid (siRNA), or the combination of two or more different chemotherapeutic agents in a single nanoparticle, can significantly enhance therapeutic efficacy.^{22–28}

Small interfering RNA (siRNA) has been studied extensively to treat various genetic diseases, including cardiovascular diseases and various cancers, since it can inhibit specific protein expression by suppressing a target gene selectively.^{29–35} Moreover, unlike chemotherapeutics, siRNA exhibits a high specificity and a low non-specific toxicity. However, siRNA cannot easily cross the cellular membrane due to its polyanionic nature and is susceptible to enzymatic degradation. Thus, RNA-based therapeutics have lagged behind other treatment alternatives due to the lack of safe and effective carriers for *in vivo* siRNA delivery. Generally, the ideal carrier for siRNA bind and condense siRNA, provide protection against degradation, specifically direct siRNA to target cells, facilitate its intracellular uptake and escape from endosome/lysosome into cytosol, and finally promote efficient gene silencing.^{33, 36, 37}

Due to their tunable sizes and optical properties, as well as their chemical versatility, gold nanoparticles including gold (Au) nanorods (NRs) have been investigated for a broad spectrum of biomedical applications, including targeted gene/drug delivery, localized photothermal therapy, and contrast agents for optical and X-ray computed tomography (CT) imaging.^{35, 38–43}

As described earlier, nanocarriers conjugated with active tumor-targeting ligands (e.g., peptides, antibodies, aptamers, etc.) that provide specific nanocarrier–cell surface interactions demonstrate enhanced cellular uptake by cancer cells which can lead to improved therapeutic efficacy and reduced peripheral toxicity.^{14, 16, 44} The majority of NE cancers express somatostatin receptors (SSTRs types 1–5).^{6, 45, 46} Treatment with somatostatin analogs such as octreotide (OCT, a cyclic octapeptide alcohol (D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol))), pasirisotide, and lanreotide have been shown to effectively suppress NE cancer hormone secretion through binding of SSTRs.^{43, 46–48} Recent data suggest that somatostatin analogs also have a mild tumor-suppressive effect and

may slightly prolong tumor-free progression.⁴⁷ Moreover, somatostatin analogs labeled with radioisotopes have been successfully developed for imaging NE cancers (somatostatin scintigraphy or octreoscan) and therapeutic purposes (peptide receptor radionuclide therapy).

In this study, we have developed a multifunctional Au NR-based nanocarrier capable of co-delivering an anticancer drug (i.e., doxorubicin (DOX)) and siRNA (against ASCL1) and specifically targeting NE cancer cells with overexpressed SSTRs using OCT as the active-tumor targeting ligand (Fig. 1). DOX was conjugated onto the Au NRs via pH-sensitive bonds to achieve pH-controlled drug release in the tumor cells. siRNA was complexed onto the cationic polyarginine segments conjugated onto the Au NRs via electrostatic interaction. It is expected that tumor-targeted chemotherapy combined with siRNA-mediated gene silencing can significantly enhance therapeutic efficacy against human NE cancers.

Materials and methods

Materials

Tetrachloroauric (III) acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (99.9%)), sodium borohydride (NaBH_4 , 99%), L-ascorbic acid (AA, 99+%), cetyltrimethylammonium bromide (CTAB, 99%), silver nitrate (AgNO_3 , 99+%), anhydrous dimethyl sulfoxide (DMSO), thioglycolic acid (TGA), methyl thioglycolate (MTG), anhydrous hydrazine, 4-dimethylamino pyridine (DMAP), N-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), poly-L-arginine (PLR, M_w : 15–70 kDa), and ethylenediamine-tetraacetic acid (EDTA) were purchased from Sigma Aldrich, USA, and used without further purification. Ultrapure deionized water (DI water, Milli-Q Water Systems) was used for all solution preparations and experiments. Glassware used for preparing Au NRs was cleaned by soaking in aqua regia followed by washing with DI water. The heterobifunctional poly (ethylene glycol) (PEG) derivatives, i.e., hydroxyl-PEG-carboxyl (OH-PEG-COOH, M_w : 5000 Da) and methoxyl-PEG-hydroxyl (MPEG-OH, M_w : 5000 Da), were purchased from JenKem Technology, USA. The anticancer drug, doxorubicin-HCl (DOX), was purchased from Tecoland Corporation, USA. Octreotide acetate (M_w : 1019.26 Da) was purchased from Aroz Technologies (USA). All other chemicals and reagents used were of analytical reagent grade. Phosphate and acetate buffered solutions were prepared in our laboratory.

Synthesis and purification of bare Au NRs

Bare Au NRs were prepared via a well-established two-step seed-mediated growth method with a slight modification.^{48, 49} Briefly, 600 μl of ice-cold 10 mM NaBH_4 solution was quickly added to 250 μl of 0.01 M HAuCl_4 dissolved in a 0.1 M CTAB surfactant solution. The solution was stirred for 2 min. Afterwards, it was allowed to react for 2 h to form the CTAB-capped Au nanoparticles to be used as seeds for the synthesis of Au NRs. To synthesize the Au NRs, 95 ml of CTAB solution (0.1 M) was added to a 200 ml flask. Afterwards, 950 μl of AgNO_3 solution (0.01 M) mixed in 5 ml of HAuCl_4 solution (0.01 M) was added to the CTAB solution. Subsequently, 550 μl of freshly prepared L-ascorbic acid solution (0.1 M) were added to the above mixture as a weak reducing agent. After swirling for 3 min, the gold color disappeared, indicating that the gold (III) was reduced to gold (I). Lastly, 100 μl of CTAB-capped Au nanoparticle seeds were added. The solution was left to stand overnight. The resulting Au NRs were separated from the reaction solution via centrifugation at 14,000 rpm for 12 min, washed with deionized water twice to remove any residual reactants, and separated from water via centrifugation at 14,000 rpm for about 15 min using a Beckman Coulter Avanti J-26-XPI centrifuge (Beckman, Germany).

Tethering methyl thioglycolate (MTG) and thioglycolic acid (TGA) onto Au NRs (1)

In order to conjugate PLR and DOX onto the surface of Au NRs, methyl thioglycolate (MTG) and thioglycolic acid (TGA) were first tethered onto the Au NRs via their thiol groups. The feed molar ratio was set at Au NRs:MTG:TGA = 1:3000:3000.⁵⁰ 30 μ l of the MTG and TGA mixture (both at 1 mM) was added to 10 ml of 1 nM Au NRs in a mixture of water and methanol solution (1:1 molar ratio). The resulting mixture was allowed to react for 12 h in order to form stable thiol monolayers. Thereafter, the reaction mixture was centrifuged at 14,000 rpm for 15 min and redispersed in 10 ml of DI water.

Conjugation of poly-L-arginine (PLR) onto the Au NRs (2)

PLR segments were conjugated onto the Au NRs via an amidation reaction between the amine groups of PLR and the carboxylic acid groups of TGA tethered onto the Au NRs surfaces. Briefly, PLR (0.1 g) was dissolved in 25 ml of 50 mM sodium tetraborate buffer (STBB, pH 8.5). The resulting solution was stirred vigorously for approximately 30 min and subsequently filtered through a 0.22 μ m Durapore® membrane (Sterile Millex GV, Sigma–Aldrich, Buchs, Switzerland) into a 45 ml Corning tube. In order to conjugate PLR onto the Au NRs, the carboxyl groups anchored onto the Au NRs were first converted to NHS ester groups via a reaction with NHS. To conduct this reaction, an appropriate stoichiometric amount of functionalized Au NRs (100 mg) and condensing agents EDC (1 mg) and NHS (1 mg) were reacted for 18 h and then slowly added to the above PLR solution while it was continuously stirred. After another 12 h of vigorous stirring at room temperature, the solution was transferred to a dialysis tube (Spectr/Por dialysis tubing, molecular weight cut-off of 8 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The synthesized product was dialyzed for 24 h in 10 mM phosphate buffered saline (PBS, pH 7.0), followed by an additional 24 h of dialysis in deionized water. The product was then freeze dried for 48 h.

Conjugation of DOX onto functionalized Au NRs (3–4)

DOX was synthesized using a two-step reaction procedure.⁵¹ In the first step, the methoxyl groups of the MTG linkers on the surface Au NRs (600 mg) were substituted with hydrazide groups by reacting with 10 mg (0.3 mmol) of anhydrous hydrazine in 50 ml of dry DMF at 40°C for 24 h. Thereafter, the formed intermediate product was dialyzed against a 0.25% ammonia solution for 48 h, followed by freeze drying for 48 h. In the second step, the freeze-dried product was dissolved in 25 ml of DMF, and an excess amount of DOX (60 mg) was added. The mixed solution was stirred in the dark at room temperature for 24 h to obtain DOX conjugated Au NRs. These were then subjected to dialysis against DI water for 72 h to completely remove any un-reacted DOX.

Conjugation of OCT tumor targeting ligands to PEG (5)

Octreotide (OCT) was conjugated onto heterobifunctional OH–PEG–COOH via an amidation reaction in the presence of NHS and DCC. First, OH–PEG–NHS ester was prepared by reacting OH–PEG–COOH (100 mg, 0.02 mmol) with NHS (2.76 mg, 0.024 mmol) in the presence of DCC (0.5 mg, 0.024 mmol) in freshly distilled anhydrous DMF (15 ml) for 36 h. After the byproduct dicyclohexylurea was removed via filtration, the final product was vacuum dried to remove the solvent and was then dried overnight to obtain the white powder. OCT (24.5 mg, 0.024 mmol) was conjugated to OH–PEG–NHS (60 mg, 0.012 mmol) at a feed molar ratio of 2:1 in the PBS buffer. Sodium carbonate was used to adjust the pH value to 8.5. The reaction was conducted in the dark at room temperature for 24 h.

PEG conjugation onto functionalized Au NRs (6)

A mixture of MPEG–OH (3.5 mg, 0.0007 mmol) and OH–PEG–OCT (1.77 mg, 0.0003 mmol) was conjugated onto functionalized Au NRs (50 mg) via an esterification between the carboxylic acid groups of PLR and hydroxyl groups of PEG. The reaction was conducted in the presence of DMAP (1.41 mg) and DCC (2.39 mg) under a nitrogen atmosphere for 12 h. After the by-product dicyclohexylcarbodiurea was removed by filtration, the product was precipitated with cold diethyl ether. The precipitate was then dialyzed against deionized water for 48 h using cellulose tubing (molecular weight cut-off, 12 kDa) and freeze dried. The resulting OCT-conjugated Au NR-based nanocarrier is referred to as Au-DOX-OCT (Fig. 1E). Once siRNA is complexed onto Au-DOX-OCT, it is referred to as Au-DOX-OCT-siRNA as shown in Fig. 1F.

In addition, in order to demonstrate the synergistic antitumor efficiency of ASCL1 siRNA, DOX, and OCT, two other types of Au NR-based nanocarriers—namely, Au NRs conjugated with only PLR–PEG (i.e., Au, Fig. 1A), as well as Au NRs conjugated with both DOX and PLR–PEG but without OCT targeting ligands (i.e., Au-DOX, Fig. 1C)—have also been synthesized following similar procedures as described above for Au-DOX-OCT and were used as controls for this study. Once siRNA is complexed onto Au and Au-DOX, they are referred to as Au-siRNA (Fig. 1B) and Au-DOX-siRNA (Fig. 1D), respectively.

Characterization

The morphology and size of the Au NRs nanoparticles were measured using transmission electron microscopy (TEM) (LEO912-OMEGA, Zeiss, Germany). To prepare the TEM sample, a drop of Au NR solution containing 1 wt.% of phosphotungstic acid was deposited onto a 200 mesh copper grid coated with carbon and dried at room temperature. Absorbance measurements were carried out using a Varian Cary 300 Bio UV-visible spectrophotometer. The calibration curve of absorbance for different concentrations of DOX was determined at 485 nm.

Determination of the DOX loading content

The DOX loading content (DLC), defined as the weight percentage of DOX in the Au NR-based nanocarriers, was quantified by UV-Vis analysis. First, DOX was released completely from the nanocarriers in a solution of 0.1 N HCl to cleave the hydrazone bonds. The absorbance of DOX at 485 nm was measured to determine the DLC in the solution using an established calibration curve. The DLC measurements were performed in triplicate for each sample.

In vitro drug release study

Drug release studies were performed in a glass apparatus at 37 °C in acetate buffer (pH 5.3 and 6.6) and phosphate buffer (pH 7.4) solutions. Next, 50 mg of Au NR-based nanocarriers was dispersed in 5 ml of medium and placed in a dialysis bag with a molecular weight cut-off of 2 kDa. The dialysis bag was immersed in 95 ml of the release medium and kept in a horizontal laboratory shaker (100 rpm) under constant temperature. Samples of 2 ml volume were periodically removed and the same volume of fresh medium was added. The amount of released DOX was analyzed with a spectrophotometer at 485 nm. Drug release studies were performed in triplicate for each sample.

Gel retardation assay

The siRNA binding ability of the Au NR-based nanocarriers was studied by agarose gel electrophoresis. The siRNA and Au NR-based nanocarrier complexes were prepared at four different ratios between the weight concentration of the Au nanocarriers and the molar

concentration of the ASCL1 siRNA (i.e., 21 mg/l: 32 μ M, 42 mg/l: 32 μ M, 56 mg/l: 32 μ M, and 84 mg/l: 32 μ M). Electrophoresis was carried out on a 1% agarose gel with a current of 100 V for 30 min in a TAE buffer solution (40 mM Tris-HCl, 1 v/v % acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide and then analyzed on a UV illuminator to show the position of the complexed siRNA band relative to that of naked siRNA.

Cellular uptake studies of Au NR-based nanocarriers

Based on DOX auto-fluorescence (488 nm excitation and 575 nm emission), the cellular uptake of the Au NR-based nanocarriers was analyzed using both flow cytometry and confocal laser scanning microscopy (CLSM). For flow cytometry, BON cells were seeded in 6-well plates at a density of 3×10^5 cells/ml and incubated overnight in a DMEM medium with 10% FBS under standard conditions. Then the cells were treated with targeted nanocarriers (Au-DOX-OCT) or non-targeted nanocarriers (Au-DOX) at a DOX concentration of 1 μ g/ml and further incubated for 30 or 120 min. Thereafter, the cells were washed with PBS to remove unincorporated nanocarriers and lifted using a non-enzymatic cell dissociation solution and stained for 30 min with Hoechst 33342 (10 μ g/ml) at 37 °C. DOX uptake was analyzed using a FACS Calibur four-color analysis cytometer (Becton Dickinson, San Jose, CA) and FlowJo analysis software (Tree Star, Inc., Ashland, OR). For each sample at least 1×10^5 cells were analyzed and DOX fluorescence was displayed on a linear scale. For the CLSM studies, BON cells were seeded (10×10^5) onto 96-well high optical quality plates and grown overnight. Cells were treated with targeted nanocarriers (Au-DOX-OCT) or non-targeted nanocarriers (Au-DOX) at a DOX concentration of 1 μ g/ml followed by incubation for 30 and 120 min. The cells were then washed with warm PBS, fixed with 1.5% formaldehyde for 15 min, and washed again with double-distilled H₂O. Then the cells in the optical plate were mounted with Prolong Gold antifade reagent and DOX uptake was observed with a Nikon A1R-Si high speed spectral laser scanning confocal inverted microscope (Nikon, Melville, NY) with emission filters detecting fluorescent light at 575 nm. Digital monochromatic images were acquired using NIS-Elements BR Software.

Validation of anti-tumor properties of Au NR-based nanocarriers conjugated with ASCL1 siRNA

Antitumor properties of the family of Au NR-based nanocarriers (e.g. Au, Au-siRNA, Au-DOX, Au-DOX-siRNA, Au-DOX-OCT or Au-DOX-OCT-siRNA) were evaluated using the MTT cell proliferation assay and immunoblotting for the NET marker (ASCL1). For the MTT assay, BON cells were plated in quadruplicate on 96-well plates and incubated overnight. The following day, the media was replaced with fresh media containing Au, Au-ASCL1 siRNA, Au-DOX, Au-DOX-ASCL1 siRNA, Au-DOX-OCT or Au-DOX-OCT-ASCL1 siRNA at a DOX concentration of 1 μ g/ml. The cells were further incubated up to 72 h with subsequent treatment at 24 and 48 h. Cells grown in pure culture medium served as the viability control. The MTT assay was performed by aspirating the treatment media and adding 25 μ l of serum-free media containing a 0.5 mg/ml MTT reagent to each well and incubating it at 37 °C for 4 h. After incubation, 75 μ l of DMSO were added to each well and mixed thoroughly. The multi-well plates were then measured at 570 nm using a spectrophotometer (Quant, Bio-Tek Instruments, Winooski, VT). For Western blot analysis, cells were seeded in 6-well plates and treated for 24 h with a family of Au NR-based nanocarriers as described for the MTT assay. To isolate protein, cells were washed with PBS and harvested according to a previously described protocol.⁵² Total cellular protein concentrations were determined by a BCA assay and 20 μ g of denatured cellular extracts were resolved by 10% SDS-PAGE gels. Protein bands in the gel were then transferred to a nitrocellulose membrane and incubated with the appropriate primary antibody. The antibody dilutions were as follows: 1:2000 for ASCL1 and 1:10000 for GAPDH. Membranes were

incubated overnight at 4 °C and washed the next day with buffer (1xPBS, 0.05% Tween 20). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies were used for secondary incubation. Proteins were then visualized with chemiluminescent substrates Immunstar (GAPDH) or Femto (ASCL1).

Comparison of gene silencing efficiency using liposomes and Au NR-based nanocarriers complexed with ASCL1 siRNA

The gene silencing efficiency using the liposomal transfection agent (Lipofectamine™2000) and Au NR-based nanocarriers were compared through protein isolation and Western blot analysis. Approximately 24 h before transfection, BON cells were seeded in 6-well plates with a density of 3×10^5 cells/ml and incubated in a DMEM medium with 10% FBS under standard conditions. For the gene silencing experiments using Au NR-based nanocarriers complexed with ASCL1 siRNA, cells were treated with Au-siRNA, Au-DOX-siRNA or Au-DOX-OCT-siRNA at a concentration of 32 μ M of ASCL1 siRNA (with a corresponding DOX concentration of 1 μ g/ml for the latter two nanocarriers). Transfection with Lipofectamine™2000 was performed according to the manufacturer's instructions. The cells were transfected for 4 h followed by incubation with fresh DMEM medium with 10% FBS for 20 h longer. The amount of siRNA per well was 40, 80, or 100 μ M. Proteins were harvested 24 h after transfection and used for ASCL1 and GAPDH Western blot analysis as described.

Results and Discussion

Synthesis and characterization of the Au NR-based nanocarriers

The Au NR-based nanocarriers were prepared according to Scheme 1. First, bare (i.e., unfunctionalized) Au NRs were synthesized according to a well-established, seed-mediated growth method.^{48,49} The UV/IR absorption spectrum and a representative TEM image of the bare Au NRs are shown in Fig. 2. The bare Au NRs had a length and diameter of approximately 50 and 10 nm, respectively (Fig. 2A), and exhibited two absorption bands: a weak short-wavelength band around 520 nm (due to transverse electronic oscillation) and a strong long-wavelength band around 808 nm (due to longitudinal oscillation of electrons) (Fig. 2B). It is well-known that near infrared (NIR) light can be transmitted deep into the tissue without causing any significant damage. As such, Au NRs can be potentially used for photothermal therapy.

In order to functionalize the Au NRs, two thiolated linkers, methyl thioglycolate (MTG) and thioglycolic acid (TGA), were first anchored onto the Au NRs through thiol moiety. MTG linkers were employed for DOX (the model anticancer drug in this study) conjugation, while the MTA linkers were used to conjugate poly-L-arginine (PLR), which is used for siRNA complexing. The negatively charged siRNA molecules were complexed onto the cationic PLR molecules via electrostatic interaction.

The average number of MTG and AET arms attached to a given Au NR was estimated as follows:⁵³ the volume of one Au NR, assuming 50 nm for the length and 10 nm for the diameter (based on TEM images), is $V = \pi r^2 l = \pi \times (5 \text{ nm})^2 \times 50 \text{ nm} = 3,927 \text{ nm}^3$, which means that the theoretical average mass per Au NR (m) is $7.58 \times 10^{-17} \text{ g}$ ($\rho_{\text{Au}} = 19,300 \text{ kg m}^{-3}$). Hence, the molecular weight of the Au NRs (i.e., the weight of 1 mole of Au NRs) is $M = m \times N_A \approx 45,631,600 \text{ g mol}^{-1} = 4.56 \times 10^7 \text{ g mol}^{-1}$ ($N_A = \text{Avogadro constant}$). The increase in mass after coupling MTG and TGA was approximately 0.65%, which was measured by comparing the weight of the freeze-dried Au NRs before and after MTG and TGA conjugation in triplicate, indicating that the molecular weight of the MTG and TGA functionalized Au NRs rose to $45,928,205 \text{ g mol}^{-1}$. As such, the average MTG and TGA mass per mole of Au NRs was about 296,605 g. Assuming that the molar ratio of the MTG

and TGA linker attached to the Au NR was the same as the molar ratio in the reaction mixture, and based on the average molecular weight of the MTG and TGA linkers ($(92 + 106)/2 = 99 \text{ g mol}^{-1}$), the total number of MTG and TGA linkers per Au NR was estimated to be $296,605/99 = 2996$. Therefore, the number of MTG and AET anchored onto each Au NRs was estimated to be approximately 1,500 each.^{53–55}

Next, DOX was conjugated through an acid-sensitive hydrazone linkage via a two-step reaction.^{51, 56} First, the methoxyl groups of the MTG linkers were substituted by hydrazide groups by an ester-amide exchange aminolysis (EAE) reaction. In the second step, an excess amount of DOX was conjugated to the hydrazide group through a pH-sensitive hydrazone linkage.

In order to conjugate OCT, the active tumor-targeting ligand for NE cancers, onto the Au NRs, OCT was first conjugated onto a heterobifunctional PEG derivative, i.e., OH-PEG-COOH (M_w : 5000 Da), to form OH-PEG-OCT. Then, OH-PEG-OCT was conjugated onto the Au NRs via an esterification reaction between the hydroxyl group and the carboxyl group in the presence of DCC and DMAP. Methoxyl-PEG-hydroxyl (MPEG-OH, M_w : 5000 Da) was also conjugated onto the Au NRs in order to adjust the solubility and stability of the resulting Au NR-based nanocarriers in physiological conditions, as well as to help achieve the desirable OCT molar ratio. Specifically, the molar ratio of OH-PEG-OCT and MPEG-OH was set at 3:7, with the total molar amount being equal to that of the carboxyl groups of PLR. In addition, non-targeted Au NR-based nanocarriers without OCT conjugation (i.e., Au-DOX, Fig. 1C), as well as Au NRs conjugated with only PLR-PEG (i.e., Au, Fig. 1A), were also prepared in a similar fashion to be used as a control.

Drug loading level and *in vitro* drug release

The amount of DOX incorporated into the Au NR-based nanocarriers was determined by UV analysis after cleaving the hydrazone linkers between the nanocarriers and DOX. The DLC was calculated to be 1.19 wt.%. In order to test the pH-sensitivity of the Au NR-based nanocarriers, *in vitro* drug release studies were performed under simulated physiological conditions at pH values of 5.3, 6.6, and 7.4 at 37 °C. As shown in Fig. 3, the pH value of the medium had a strong effect on the DOX release rate from the Au NR-based nanocarriers. The drug release at pH 7.4 was negligible, with an initial burst release of about 5.7% and a total of 11.3% drug release after 45 h. However, at pH values of 5.3 and 6.6, the DOX release rate was much faster, with approximately 90.3% and 79.6% of the drug released within 45 h, respectively, showing that DOX release from Au NR-based OCT-targeting ligand on the cellular uptake and intracellular distribution of Au NR-based nanocarriers in NE cancer cells (BON GI carcinoid) were analyzed using both flow cytometry and CLSM. Quantitative flow cytometric analysis (Fig. 5A) showed that the cellular uptake of Au-DOX-OCT (targeted) was 14% and 44% higher than Au-DOX (non-targeted) after 30 and 120 min incubation, respectively (Fig. 5B). In contrast, our control cell line, WI 38 pulmonary fibroblasts, which was negative for the presence of SSTRs, showed that the cellular uptake was nearly the same for Au-DOX-OCT and Au-DOX (Fig. S1 in the ESI[†]). These results indicated that conjugating nanocarriers in an acidic environment was governed by the acid-cleavable hydrazone linkages. As such, the pH-sensitive Au NR-based nanocarriers will minimize premature drug release during circulation in the bloodstream (pH 7.4), yet provide a sufficient amount of drug to effectively kill cancer cells once the Au NR-based nanocarriers are internalized into the endocytic compartments where the pH value ranges from 4.5 to 6.5. This feature will greatly enhance tumor-directed, therapeutic efficacy while

[†]Electronic Supplementary Information (ESI) available: Additional flow cytometry histogram profiles of DOX fluorescence and ASCL1 knockdown results. See DOI: 10.1039/b000000x/

minimizing the undesirable side-effects. These findings agree with our previous studies on intracellular environment-sensitive drug delivery systems.^{50, 56–58}

Gel retardation assay

The RNA binding ability of the Au NR-based nanocarriers was studied through a gel retardation assay. As shown in Fig. 4 (lanes 3–10), both targeted and non-targeted Au NR-based nanocarriers were able to bind ASCL1 siRNA through an electrostatic interaction between the positively charged PLR linkages and the negatively charged siRNA molecules. Complete retardation of siRNA mobility was achieved at a weight concentration of Au nanocarriers versus molar concentration of the ASCL1 siRNA = 84 mg/l: 32 μ M (Fig. 4, lane 6 for Au-DOX-OCT-siRNA and lane 10 for Au-DOX-siRNA, respectively). Based on these results, we used a weight concentration of Au NR-based nanocarriers/molar concentration of siRNA = 84 mg/l: 32 μ M for further investigations (e.g., MTT and ASCL1 knock-down analysis) on Au NR-based nanocarriers complexed with ASCL1 siRNA as detailed below.

Cellular uptake

Using the auto-fluorescence feature of DOX, the effects of the OCT onto Au NR-based nanocarriers increased the cellular uptake of nanocarriers in NE cancer cells that overexpressed SSTRs on their surface. This observation was further confirmed by CLSM. As shown in Fig. 6, the DOX fluorescence signal was more intense in cells incubated with Au-DOX-OCT than in cells incubated with Au-DOX for both 30 and 120 min. Thus, it is evident that the chemotherapeutic drug DOX was transferred into the cells more efficiently by OCT-conjugated Au NR-based nanocarriers. In addition, after 30 min of incubation, DOX fluorescence was observed mainly in the cytoplasm for both Au-DOX-OCT and Au-DOX nanocarriers (Fig. 6A and B). However, with a longer incubation time (120 min), a stronger fluorescent signal was located in the nuclear region of the cells treated with Au-DOX-OCT (Fig. 6D). In contrast, the fluorescence signal in cells treated with Au-DOX was mainly shown in the perinuclear region of cells (Fig. 6A and C). The nuclear localization of DOX has been previously shown to guarantee successful inhibition of DNA replication and cancer cell death.⁵⁹ These cellular uptake experiments clearly demonstrated that the presence of OCT on Au NR-based nanocarriers substantially increased the intracellular transport of DOX and that the OCT-conjugated Au NR-based nanocarriers were efficient in delivering and releasing DOX to the nucleus.

Cytotoxicity

To study the anti-proliferative effect of Au NR-based nanocarriers complexed with siRNA, the ability of such nanocarriers to inhibit the growth of carcinoid BON cells was examined *in vitro*. The optimal complexation ratio for ASCL1 siRNA and Au NR-based nanocarriers was determined by an agarose gel retardation assay as described earlier (Fig. 4). An MTT assay showed that Au-DOX-OCT-siRNA exhibited the strongest cytotoxic effect ($p < 0.001$) at all time points (24, 48, and 72 h) among all nanocarriers tested, thus demonstrating the synergistic effects of the three components (i.e., DOX, OCT, and ASCL1 siRNA; Fig. 7). In addition, the cytotoxicity of the Au NR-based nanocarriers complexed with ASCL1 siRNA demonstrated a higher cytotoxicity than their corresponding counterparts (Au-ASCL1 siRNA versus Au, Au-DOX-ASCL1 siRNA versus Au-DOX, and Au-DOX-OCT-ASCL1 siRNA versus Au-DOX-OCT) measured at each time point. These results suggested that the Au NR-based nanocarriers could effectively deliver siRNA to the cytoplasm and that siRNA delivery was enhanced by the presence of OCT-targeting ligands on the surface of the nanocarriers. Moreover, the results demonstrated that silencing the ASCL1 oncogene at the message level resulted in significant suppression of cancer cell viability. It is known that ASCL1 inhibition results in an antitumor effect. ASCL1-RNAi has been shown to possess

growth inhibitory effects *in vivo* and *in vitro* on lung cancer cell lines with NE features (SCLC and LCNEC) through induction of cell cycle arrest and apoptosis. ASCL1 itself acts as a transcriptional repressor of multiple tumor suppressor genes. In particular, it exerts its effects upon Wnt/ β -catenin signaling inhibitors DKK1 and DKK3.⁶⁰ Additionally, siRNA against ASCL1 significantly reduces NE hormones such as synaptophysin and tryptophan hydroxylase 1 (TPH1) enzyme, which have been implicated in serotonin biosynthesis in GI carcinoids.⁶¹ Moreover, up-regulation of ASCL1 is characteristic of progressive astrocytoma.⁶² It has also been demonstrated that siRNA against ASCL1 reduces chromogranin A (CgA) levels in NE cancers such as medullary thyroid cancer.⁶³

Gene knockdown

Following the growth inhibitory effect, we examined the degree of ASCL1 knockdown at the protein level in BON cells using Au NR-based nanocarriers with and without ASCL1 siRNA complexation. As Western blot analysis demonstrates (Fig. 8), the ASCL1 protein expression level was reduced by transfection of BON cells with all three types of nanocarriers complexed with ASCL1 siRNA (i.e., Au-ASCL1 siRNA, Au-DOX-ASCL1 siRNA and Au-DOX-OCT-ASCL1 siRNA) compared to their counterparts without ASCL1 siRNA complexation (i.e., Au, Au-DOX and Au-DOX-OCT). Importantly, increasing the inhibition of protein expression was observed in the following order: Au-ASCL1 siRNA, Au-DOX-ASCL1 siRNA, and Au-DOX-OCT-ASCL1 siRNA. However, ASCL1 protein was effectively silenced only by Au-DOX-OCT-siRNA, thus indicating that ligand (OCT) enhanced co-delivery of the chemotherapeutic (DOX) and ASCL1 siRNA was the most potent treatment in altering the malignant phenotype. In addition, targeted Au NR-based nanocarriers without ASCL1 siRNA complexation (i.e., Au-DOX-OCT) reduced but did not silence ASCL1 expression (Fig. 8), thus indicating that the transfection of ASCL1 siRNA with the assistance of OCT-conjugated Au-NR based nanocarriers is crucial for knocking down the tumor marker. Lastly, we compared the silencing efficiency of targeted Au NR-based nanocarriers complexed with 32 μ M of ASCL1 siRNA with naked ASCL1 siRNA (40, 80, and 100 μ M) transfected with a liposomal transfection agent (Lipofectamine). Western blot analysis showed (Fig. S2 in the ESI[†]) that the ASCL protein knockdown by Au-DOX-OCT-siRNA at 32 μ M ASCL1 siRNA concentration was comparable to naked ASCL1 siRNA at a concentration of 100 μ M. This result is consistent with the previous results that Au NR-based nanocarriers possess an excellent capability of entering cancer cells and delivering siRNA.⁴³

Conclusions

A multifunctional, OCT-conjugated, Au NR-based nanocarrier capable of co-delivering siRNA (against ASCL1) and an anticancer drug (DOX) specifically to NE cancer cells was developed for combined chemotherapy and siRNA-mediated gene silencing in NE cancers. These Au NR-based nanocarriers exhibited pH-sensitive drug release behavior, which can minimize the non-specific systemic spread of toxic drugs during circulation while maximizing the efficiency of tumor-targeted anti-cancer drug delivery. The presence of the tumor targeting ligand OCT increased the cellular uptake of Au NR-based nanocarriers (Au-DOX-OCT) substantially in a BON cell line compared to non-targeted Au NR-based nanocarriers (Au-DOX). Moreover, Au-DOX-OCT-ASCL1 siRNA (targeted Au NR-based nanocarriers complexed with ASCL1 siRNA) induced a significantly higher gene silencing efficiency in BON carcinoid cells than Au-DOX-siRNA (non-targeted Au NR-based nanocarriers complexed with ASCL1 siRNA). MTT assay further revealed that targeted nanocarriers with ASCL1 siRNA (Au-DOX-OCT-siRNA) were the most efficient in altering the NE phenotype of BON cells and showed the strongest anti-proliferative effect. In summary, Au NR-based nanocarriers bearing all three components—namely, a tumor-

targeting agent (OCT), a chemotherapeutic drug (DOX), and a nucleic acid (ASCL1 siRNA)—exhibited a much higher therapeutic efficacy in treating NE cancer cell lines than Au NR-based nanocarriers bearing only one or two components. Thus, Au-DOX-OCT-siRNA nanocarriers can provide a promising approach for tumor-targeted and combined RNA therapy in treating NE cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ASCL1	achaete-scute complex-like 1
Au NRs	gold nanorods
Au-DOX-OCT	Octreotide-conjugated Au nanorods conjugated with doxorubicin
Au-DOX	non-targeted Au nanorods conjugated with doxorubicin
Au-DOX-OCT-siRNA	Au nanorods conjugated with doxorubicin and octreotide, and complexed with ASCL1 siRNA
Au-DOX-siRNA	non-targeted Au nanorods conjugated with doxorubicin and complexed with ASCL1 siRNA
Au	Au nanorods only conjugated with polyarginine and poly(ethylene glycol)
Au-ASCL1 siRNA	Au nanorods only conjugated with polyarginine and poly(ethylene glycol), but complexed with ASCL1 siRNA

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Fig. 1.

Illustration of six Au NR-based nanocarriers—(A) Au only (i.e., Au NR functionalized with PLR-PEG arms only), (B) Au-siRNA (Au NR functionalized with PLR-PEG and complexed with ASCL1 siRNA), (C) Au-DOX (Au NR plus DOX), (D) Au-DOX-siRNA (Au NR, DOX, and ASCL1 siRNA), (E) Au-DOX-OCT (Au NR, DOX, and OCT targeting) and (F) Au-DOX-OCT-siRNA (Au NR, DOX, OCT targeting, and ASCL1 siRNA)—were developed to determine (1) the benefit of OCT targeting, and (2) the synergistic antitumor efficiency of ASCL1 siRNA, DOX, and OCT.

Fig. 2. Morphology and optical properties of bare Au NRs: (A) A representative TEM image showing Au NRs with an aspect ratio of 5, and (B) Optical absorption spectra of spherical gold nanoparticle seeds (size ~10 nm, the green curve) showing a surface plasmon resonance peak at 527 nm with the Au NRs (the blue curve) showing two surface plasmon resonance peaks at 520 nm and 808 nm.

Fig. 3.
DOX release profiles of Au-DOX-OCT at pH 5.3, 6.6, and 7.4.

Fig. 4. Electrophoretic mobility of siRNA after complexation with Au NR-based nanocarriers: lane 1, dsRNA ladder; lane 2, naked ASCL1 siRNA; lanes 3–6, Au-DOX-OCT-siRNA; and lanes 7–10, Au-DOX-siRNA.

Fig. 5. Flow cytometry histogram profiles (A) and bar graph representations (B) of DOX fluorescence in BON cells incubated with fresh media (control), Au-DOX, or Au-DOX-OCT for 30 min and 120 min (DOX concentration: 1 $\mu\text{g/ml}$).

Fig. 6. Confocal laser scanning microscopy (CLSM) images of BON cells incubated at 37 °C with Au-DOX or Au-DOX-OCT for 30 min (6A, 6B) and 120 min (6C, 6D) at a DOX concentration of 1 μg/ml.

Fig. 7.

BON cell viability measured by MTT colorimetric assay after treatment with Au, Au-ASCL1 siRNA, Au-DOX, Au-DOX-ASCL1 siRNA, Au-DOX-OCT or Au-DOX-OCT-ASCL1 siRNA at a DOX concentration of 1 $\mu\text{g/ml}$ and an ASCL1 siRNA concentration of 32 μM for 24, 48, and 72 hours. Data are presented as the mean $\pm$ SEM of quadruplicate experiments.

Fig. 8. Western blot analysis of neuroendocrine tumor markers (ASCL1) after treatment with Au, Au-ASCL1 siRNA, Au-DOX, Au-DOX-ASCL1 siRNA, Au-DOX-OCT, or Au-DOX-OCT-ASCL1 siRNA at a DOX concentration 1 μ g/ml and an ASCL1 siRNA concentration of 32 μ M for 24 h. GAPDH was used as the protein loading control.

Scheme 1.

Synthetic scheme of the Au NR-based nanocarrier (Au-DOX-OCT-siRNA).