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Multifunctional gold-nanoparticles: A nanovectorization tool for the targeted delivery of novel chemotherapeutic agents

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ABSTRACT

Due to their small size and unique properties, multifunctional nanoparticles arise as versatile delivery systems easily grafted with a vast array of functional moieties, such as anticancer cytotoxic chemotherapeutics and targeting agents. Here, we formulated a multifunctional gold-nanoparticle (AuNP) system composed of a monoclonal antibody against epidermal growth factor receptor (EGFR) (anti-EGFR D-11) for active targeting and a Co(II) coordination compound $[CoCl(H_2O)(phendione)_2][BF_4]$ (phendione = 1,10-phenanthroline-5,6-dione) (TS265) with proven antiproliferative activity towards cancer cells (designated as TargetNanoTS265). The efficacy of this nanoformulation, and the non-targeted counterpart (NanoTS265), were evaluated in vitro using cancer cell models and in vivo using mice xenografts. Compared to the free compound, both nanoformulations (TargetNanoTS265 and NanoTS265) efficiently delivered the cytotoxic cargo in a controlled selective manner due to the active targeting, boosting tumor cytotoxicity. Treatment of HCT116-derived xenografts tumors with TargetNanoTS265 led to 93% tumor reduction. This simple conceptual nanoformulation demonstrates the potential of nanovectorization of chemotherapeutics via simple assembly onto AuNPs of BSA/HAS-drug conjugates that may easily be expanded to suit other cargo of novel compounds that require optimized controlled delivery to cancer target.

1. Introduction

The development of AuNPs is at the core of many of these recent innovative systems, as multifunctional scaffolds capable to integrate diagnosis, drug monitoring, targeted delivery, and controlled drug release functions into a single platform [1,2]. In recent years the growing focus of oncology onto targeted therapies has placed AuNPs as attractive candidates for the delivery of drug payloads directly into cancer cells due to its physicochemical properties including relatively high biocompatibility and ease conjugation to biomolecules, capable of improving drug delivery and increased targeting efficacy [3–8]. Indeed, AuNPs have been effectively used to deliver i) drugs and contrast agents that otherwise exhibit low solubility and poor pharmacokinetics and ii) compounds that are naturally susceptible to enzymatic degradation, as well as those that display low intracellular penetration (e.g. siRNA) [8,9]. These AuNP based systems may be of the utmost importance to overcome current limitations in cancer therapy, such as side effects and acquisition of multidrug resistance [10]. Our group pioneered the use of AuNPs functionalized with hairpin ssDNA structures labeled with a fluorophore - gold nanobeacon (Au-nanobeacon) – to effectively silence any possible RNA mediated pathway inside the cell with low toxicity [11,12]. This system was shown to be effective in vivo in a promising approach to combat multidrug resistant tumors, combining in the same particle an antitumor agent (5-fluorouracil) with silencing of *MRP1*, a gene associated with acquired resistance in several tumors. In vivo results showed a remarkable tumor size reduction, from the synergic effect of the two agents [13]. AuNPs can be surface functionalized with active ligands at high densities (100× higher than that attainable with conventional liposomes) [14]. By capitalizing on the receptor-ligand

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molecular interaction, AuNPs drug payload and other therapeutic molecules have been described to be internalized into the cells by receptor-mediated endocytosis [15–17]. Nevertheless, the cell type and the nanoparticles´physicochemical properties (e.g. size, shape, or surface functionalities) can affect the internalization mechanism [18,19] and depending on the mechanisms that cells use to internalize the nanoparticles, its compartmentalization may vary, as does its biological fate and the release of the drug [20]. Among the several documented molecular alterations present at the surface of cancer cells, the increase expression of the epidermal growth factor receptor (EGFR) is well documented regarding cancer treatment, and may be targeted using a monoclonal antibody such as anti-EGFR D-11 [20,21].

The Co(II) coordination compound $CoCl(H_2O)(phendione)_2][BF_4]$ (phendione = 1,10-phenanthroline-5,6-dione) – TS265, shows high cytotoxic effect against cancer cell lines particularly colorectal and hepatocellular carcinoma cell lines and a much lower cytotoxicity towards normal human fibroblasts [22]. We have previously demonstrated that TS265 induces cell cycle arrest in S phase with subsequent cell death by apoptosis, via the promotion of the upregulation of pro-apoptotic Bax and cell-cycle-related p21; reduces expression of proteins typically upregulated in tumors and is able to produce double-strand breaks in a concentration-dependent manner. Steady-state and time-resolved fluorescence spectroscopy studies demonstrated a strong and specific interaction of the complex with human serum albumin (HSA), suggesting that this complex can be efficiently transported by albumin in the blood plasma [22–24]. The higher cytotoxicity towards HCT116 tumor cells compared with normal human fibroblasts prompt us to further enhance its cytotoxic potential by using gold nanoparticles as multifunctional nanovectorization system.

Here, we have designed a conceptual nanovectorization tool for targeted chemotherapy using TS265 as a model drug. The conceptual design of the multifunctional nanovectors involves the synthesis of ~14 nm AuNPs readily functionalized with bifunctional PEG (SH-EG(8)-(CH2)2-COOH) to provide for stability and stealth capability in vivo, bovine serum albumin (BSA) to convey TS265 (due to the previous knowledge that TS265 interacts with albumin [24]) and the anti-EGFR D-11 monoclonal antibody as a targeting moiety towards cancer cells (due to the high expression of EGFR in cancer cells [20,21]). The novel nanoformulation (TargetNanoTS265) was evaluated in NSCLC cell lines, colorectal cancer cell lines and immunocompromised mice in order to understand its targeting and therapeutic potential.

2. Experimental section

2.1. Compounds

The metal compound $[Co(Phendione)_2(H_2O)Cl]BF_4]$ (TS265) was synthesized and characterized as previously described [22]. Paclitaxel (PTX) was purchased from Sigma-Aldrich (Spain). Millipore® water was used for the preparation of all aqueous solutions. Human Serum albumin (HSA, 96–99% with a molecular mass of 66–67 kDa) and Bovine Serum Albumin (BSA, 98% with a molecular mass of 66 kDa) were purchased from Sigma-Aldrich.

2.2. Interaction studies of TS265 with albumin

HSA and BSA stock solutions were prepared by gently dissolving the protein in phosphate buffer pH 7.0 with 0.15 M NaCl, gently swirled for 45 min to allow the protein to hydrate and fully dissolve. The concentration of each stock solution was determined by UV spectrophotometry using the molar extinction coefficient at 278 nm (HSA = 36.850 M^{-1} cm^{-1} and BSA = 43.824 M^{-1} cm^{-1}) [24]. Spectroscopic and UV-VIS measurements were carried out on individually prepared samples to ensure the same pre-incubation time at (37.0 ± 0.5) °C in each essay. For Spectroscopic measurements HSA and BSA concentrations were kept constant at 2.0 μM and 0.13 μM, respectively, while the concentration of the complex ranged from 0 to 375 μM. For UV–VIS measurements BSA concentration was kept constant at 2.0 μM, while the concentration of the TS265 complex ranged from 0 to 60 μM. Samples were incubated at 37 °C for 1 h.

2.3. Spectroscopic measurements

UV–Visible absorption spectra were recorded at room temperature on a UV–VIS spectrophotometer (UVmini 1240, Shimadzu, Germany) in the range 230–500 nm with 1 cm path quartz Suprasil® cuvettes as described previously [22,24]. Fluorescence measurements were carried out on a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, Spain) at room temperature as described previously [24].

2.4. Cell culture

A549 (ATCC® CCL-185™) and HCT116 (ATCC® CCL-247™) tumor cell lines obtained from ATCC (Chicago, IL) were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, New York, EUA) and supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Invitrogen) and 1% (v/v) antibiotic/antimycotic (Invitrogen, New York, EUA). NCI-H1975 [H-1975, H1975] (ATCC® CRL-5908™) human lung adenocarcinoma cells were grown in RPMI (Roswell Park Memorial Institute) 1640 medium (RPMI; Invitrogen, New York, EUA) supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution (Invitrogen). Cells were maintained in 75 cm^2 culture flasks (VWR) at 37 °C in a 99% humidified atmosphere of 5% (v/v) CO_2 (CO_2 Incubator Leec, UK). Primary human fibroblasts were cultured as previously described [22].

2.5. Gold nanoparticles synthesis and assembly of Au-nanoconjugates

AuNPs were synthesized by the citrate reduction method described by Lee and Meisel [25] and characterized by UV–VIS spectroscopy, Transmission electron microscopy (TEM) and dynamic light scattering (DLS). AuNPs functionalization with PEG (AuNP@PEG) was performed following incubation the AuNPs solution (10 nM) with 0.028% (w/v) Sodium dodecyl sulfate (SDS), and SH-EG(8)-(CH2)2-COOH (Iris-Biotech) for a period of 16 h under agitation at room temperature. The excess of PEG chains was removed by centrifugation at 14000 g for 30 min at 4 °C and the degree of PEG coverage on the AuNPs' surface evaluated via Ellman's Assay [9,12].

AuNP@PEG were functionalized with Human Serum Albumin (HSA) (AuNP@PEG@HSA) or Bovine Serum Albumin (BSA) (AuNP@PEG@BSA) (Sigma, MW 66,120 kDa) by a process based on a EDC/NHS reaction. Briefly, a master mix of 21 nM of the synthesized AuNP@PEG, 1.25 mg/mL sulfo-NHS (Sigma, MW 217.13 Da) and 0.312 mg/mL EDC (Sigma, MW 191.70 Da) in 10 mM pH 6 MES buffer (2-(N-morpholino)ethanesulfonic acid) (Sigma, MW 195.24 Da). The master mix was incubated for 30 min and then centrifuged at 14000 g for 30 min at 4 °C. The supernatant was removed and replaced by 2.5 mM pH 6 MES buffer. The master mix was incubated with

10 μg/mL of BSA or HSA for 16 h. Afterwards the suspensions were washed twice with MES buffer to remove the excess BSA/HSA, through centrifugation at 14000 g for 30 min at 4 °C. Supernatants were also recovered and tested for protein concentration using Bradford Assay (Thermo Scientific). AuNP@PEG were also functionalized with *anti*-EGFR D-11 (Santa Cruz Biotechnology, USA) and with the same BSA concentration 1 h prior to antibody addition (AuNP@PEG@anti-EGFR_BSA) under the previously described conditions. Anti-EGFR D-11 antibody was added to reaction mix at a final concentration of 33 μg/mL. Subsequently, 6 nM AuNP@PEG@BSA, 6 nM AuNP@PEG@HSA and 6 nM AuNP@PEG@anti-EGFR_BSA were mixed separately with 50 μM of TS265 and incubated for 1 h at 4 °C to obtain AuNP@PEG@BSA@TS265, AuNP@PEG@HSA-TS265, AuNP@PEG@anti-EGFR_BSA@TS265, respectively. After this period, solutions were centrifuged at 14000 g for 30 min at 4 °C, to remove excess of TS265. The amount of TS265 in the