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Evaluation of cytotoxicity of a purified venom protein from *Naja kaouthia* (NKCT1) using gold nanoparticles for targeted delivery to cancer cell

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ABSTRACT

In our earlier report, gold nanoparticle (GNP) and snake venom protein toxin NKCT1 were conjugated and primary characteristics were done. In this communication, further characteristics of GNP-NKCT1 were done with TGA, BET, Zeta potential, ICP-MS, FTIR, XPS, and *in vitro* release kinetics for its physicochemical, molecular nature and bonding. TGA and ICP-MS showed that the number of conjugation was 40 ± 5 to 90 ± 8 NKCT1 per gold nanoparticles. FTIR and XPS corresponding to (C═O), (N—H), (S—S) reformulated the conjugation of GNP with NKCT1. The efficacy of GNP-NKCT1 on cancer cells were analyzed by MTT assay which demonstrated superior cytotoxic effects as compared to native NKCT1. IC_{50} dose of GNP-NKCT1 was less than 4 μg/ml in cancer cell lines, whereas in case of NKCT1 it was average 8 μg/ml. Twice dose of IC_{50} of GNP-NKCT1 even showed less toxicity compared to unconjugated NKCT1, towards normal epithelial or fibroblast cell and also in peripheral blood mononuclear lymphocytes. Flow cytometry analysis revealed that percentage of apoptotic C6 cells was much higher in GNP-NKCT1 treatment (54.58%) than that of NKCT1 treatment (26.79%). Flow cytometric analysis of cell cycle using GNP-NKCT1 on C6 cancer cells revealed that it arrested the cell cycle at Go/G_1 phases. In diethylnitrosamine (DEN) induced *in vivo* hepatocarcinoma mice, the activities of hepatic enzymes- aspartate transaminase (AST) and alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and activities of antioxidant enzymes- superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) were restored by GNP-NKCT1. This study indicated the capability of gold nanoparticles in enhancing the cancer cell uptake of NKCT1 and also suggested that GNP-NKCT1 might be a good source of anti-carcinoma or anti-sarcoma targeted agent.

1. Introduction

Nanotechnology has provided research level breakthrough in biotechnology, diagnostics, drug delivery and cancer treatment [1,2]. For optimum drug action, the most efficient method of administration is to deliver the drug to the desired site of action in the body, removing or minimizing side effects at non target sites. Many reports stated nano-engineered drug carrier systems, such as micelles, liposome, dendrimer, cyclodextrin, silicon and metal nanoparticles have higher advantages of carrier capacity, efficient incorporation, targeted delivery and control release [3]. Gold nanoparticles have shown great promise as therapeutics, therapeutic delivery vector, and intracellular imaging agents. It is also extremely important since gold nanoparticles are easily functionalized which allows the production of new drugs with chemical groups that target cancer cells and due to high electron density of Au, allow for enhanced imaging [4–6]. Other specific biomedical applications of gold nanoparticles involve chemotherapy (via drug delivery) and directed thermal irradiation of tumors, with both approaches representing a promise for cancer treatment [7]. However, due to their 'nano' size, their entry is easily facilitated into various cells posing one of the greatest difficulties in using these nanoparticles for targeted delivery to specific tissues [8]. To overcome this problem, researchers have been conjugating these nanoparticles with various peptide and ligands to develop strategies for targeted delivery [9].

It was already proved that nanoparticles carrying synthetic drugs had shown great promise in treating cancer patients. When

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anticancer agent was loaded with nanoparticles, concentrations of those agents were increased in cancer tissue. In our previous communication, we conjugated a cytotoxic protein toxin NKCT1 (isolated and purified from *Naja kaouthia* snake venom) with gold nanoparticles which showed enhanced antileukemic effect [10]. NKCT1 is 6.7 KDa protein, which showed cytotoxic effect on human leukemic cell line. It contains 60 amino acids and the amino acid sequence is LKCNKLVPLFYKTCPAGKNLCYKMFMVSNKTVPVKRGCIDVCPKNSLVLKYVCCNT RCN (Uniport entry name CX1 NAJKA, accession P0CH80). NKCT1 also exhibited 29.1% killing of human normal lymphocyte cell. From drug development and administration point of view, this was highly toxic. Our aim was to reduce this toxicity and increase the efficacy of this protein with the use of gold nanoparticles. To date, no studies investigated the effects of Naja kaouthia venom in combination with gold nanoparticles on cancer mainly on glioblastoma and hepatocarcinoma. The current study focused on physicochemical and molecular characteristics of GNP-NKCT1, their apoptotic potential on different cancer types, and toxicity towards normal fibroblast and epithelial cell line.

2. Materials & method

2.1. Chemicals

Dulbecco's modified Eagle medium, RPMI1620 medium, Fetal bovine serum, Trypsin, penicillin and streptomycin were purchased from Gibco (Gaithersburg, USA). $HAuCl_4$ salt, $NaBH_4$, Poly ethylene glycol, Imatinib mesylate, RNase, MTT, DMSO, EtBr, Agarose were purchased form Sigma Co. (St. Louis, USA). Annexin V-FITC kit was bought from BD Biosciences (San Jose, CA). Lyophilized *Naja kaouthia* crude venom (NKV) was purchased from the Calcutta snake park, Kolkata, India. All other chemicals were purchased from local manufacturer and were of analytical grade.

2.2. Cell culture

NIH-3T3 (Normal epithelial cell line) and HeK-293 (Normal fibroblast cell line) were purchased from National facility for Animal Tissue and cell culture, Pune India. Cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, $NaHCO_3$ (1.5 g/L), Penicillin (100 units) and streptomycin (10 μg/ml). C6, U87 (Gliobastoma cell line), MCF7 (Breast carcinoma cell line), HepG2 (Hepatocarcinoma cell line) were bought from Chittaranjan National Cancer Institute and cultured on complete DMEM media. Cells were grown to confluence at 37 °C in a humidified atmosphere of 5% CO_2 inside an incubator (Heal Force, China).

2.3. Animals

Male Swiss albino mice (20 ± 2 g) were purchased from authenticated supplier and housed in standard polypropylene cages at controlled temperature (24 °C ± 2 °C), with light conditions (12 h light and dark cycle) and relative humidity (60% ± 5%). The animals were provided with synthetic dry pellet diet, green vegetables, water *ad libitum*. The experiments were conducted according to the departmental animal ethics committee for the purpose of control and supervision of experiments on animals. All animal experiments were approved by the animal ethics committee, Department of Physiology, University of Calcutta and were in accordance with the guideline of the committee for the purpose of control and supervision of experiments on animal (CPCSEA), Government of India (Animal Ethical Committee approval no. PHY/CU/IAEC/102/IFD/SAD/2278/2011–2012 dt: 21.09.2011).

2.4. Synthesis of GNP and conjugation of NKCT1 and purification of GNP-NKCT1

The gold nanoparticles were prepared by sodium boro-hydrate reduction method with modifications and GNP-NKCT1 was afterward purified first in a 0.45 μm syringe filter and then by dialysis as previously described by Bhowmik et al., [10].

2.5. Characterization of GNP-NKCT1

2.5.1. Zeta potential of GNP-NKCT1

The zeta potential (Zetasizer 3000, Malvern Instruments, UK) measurements of the 2–20 nm sized GNP and 25–220 nm sized GNP-NKCT1 consisted of 4 repeats of each solutions. The pH of the both GNP and conjugated one were adjusted to 7.2. Because the zeta potential measurements were performed in an aqueous solution, the smolouchowski approximation was used to calculate the zeta potential from measured electrophoretic mobility [11].

2.5.2. BET surface area studies

The surface area analysis of the samples at the liquid nitrogen temperature (78 k) was recorded on the BET surface area analyzer, procured from Quanta chrome Instruments limited (Nova 2000e, Quanta chrome, USA) [12]. The N_2 adsorption was measured from a six-point isotherm in a relative pressure range of 0.05–0.3 at 77.3 K. The assumption for the cross-sectional area of N_2 was taken to be 16.2(°A) 2 and the density used was 3.65 g/cm^3. The sample was prepared by heating at 150 °C for 1 h while simultaneously a flow of N_2 gas across the sample tube seeps away the liberated contaminants. The particle size distribution of the powder was measured by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano ZS laser particle size analyzer. The instrument was equipped with a He-Ne laser source ($\lambda = 633$ nm) and at scattering angle of 1730. The dispersion concentration was around 0.1 g/l. The suspension was prepared by dispersing the powder in distilled water and treated for 6 min in an ultrasonic bath to obtain a well-dispersed suspension.

2.5.3. X-ray photo electron spectroscopy

X-ray photo electron spectra were acquired on a Jeol JPS 9200 spectrometer equipped with an MgKα X-ray source (1253.6 eV). The source was operated at 10 kV/20 mA and calibrated using $Au4f_{7/2}$ (84.0 eV) and $Ag3d_{5/2}$ (368.2 eV) from foil samples. The samples were introduced into an ultrahigh vacuum chamber of the spectrometer between 5×10^{-9} and 2×10^{-8} Torr and measured at 25 °C (RT). The analyzed area was a circle of 0.8 eV/step for general spectra and 23.5 eV of pass energy and o.1 eV/step for the spectra of the different elements [13].

2.5.4. Fourier transform infrared spectroscopy

The FTIR investigations were carried out with a PerkinElmer FTIR spectrophotometer in the range of middle infrared of 4000–400 cm^{-1}, using the KBR pellet technique, which involved mixing throughly the material to be tested with KBR before forming a pellet at high pressure. Sixteen scans and the resolution of 4 cm^{-1} were characterized for these spectra [14].

2.5.5. Estimation of NKCT1 per GNP

2.5.5.1. By absorption spectra. The concentrations of peptides per nanoparticles were calculated by UV-Vis tritation of GNP (1 mM) using increasing NKCT1 concentration (10 μg/ml to 100 μg/ml). The NKCT1 and GNP solutions were incubated for 30 min at RT. The GNP was removed by 0.2 μm filter (Milipore) and the concentration of conjugated peptide in solution was measured from the absorbance at 540 nm. From a linear fit of data points the number of NKCT1 per