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# Graphene nanoribbons as a drug delivery agent for lucanthone mediated therapy of glioblastoma multiforme

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**Abstract**

We report use of PEG-DSPE coated oxidized graphene nanoribbons (O-GNR-PEG-DSPE) as agent for delivery of anti-tumor drug Lucanthone (Luc) into Glioblastoma Multiformae (GBM) cells targeting base excision repair enzyme APE-1 (Apurinic endonuclease-1). Lucanthone, an endonuclease inhibitor of APE-1, was loaded onto O-GNR-PEG-DSPEs using a simple non-covalent method. We found its uptake by GBM cell line U251 exceeding 67% and 60% in APE-1-overexpressing U251, post 24 h. However, their uptake was ~38% and 29% by MCF-7 and rat glial progenitor cells (CG-4), respectively. TEM analysis of U251 showed large aggregates of O-GNR-PEG-DSPE in vesicles. Luc-O-GNR-PEG-DSPE was significantly toxic to U251 but showed little/no toxicity when exposed to MCF-7/CG-4 cells. This differential uptake effect can be exploited to use O-GNR-PEG-DSPEs as a vehicle for Luc delivery to GBM, while reducing nonspecific cytotoxicity to the surrounding healthy tissue. Cell death in U251 was necrotic, probably due to oxidative degradation of APE-1.

***From the Clinical Editor:*** This study reports on the utility of PEG-DSPE coated oxidized graphene nanoribbons as anti-tumor drug delivery agents of Lucanthone into Glioblastoma Multiformae cells targeting base excision repair enzyme APE-1, demonstrating promising anti-tumor effects with good preservation of healthy cells.


*Key words:* Apurinic endonuclease-1; Thioxanthenones; Lucanthone; Graphene nanoribbons; GBM; CG-4; Rat glial progenitor cells

APE-1, the primary base excision repair (BER) enzyme of mammalian system is over-expressed in a variety of tumors.[1] Although there is evidence both for and against a correlation between APE-1 levels and radioresistance in tumors,[2] an inverse relationship between the expression level of APE-1 and radiation and chemotherapy responses has been observed in medulloblastoma and primitive neuroectodermal tumors.[1] *In vitro* studies have also shown that APE-1 contributes to the glioma cell resistance in response to alkylating agents therapy, and its endonuclease activity is increased by oxidative stress.[3] Previously, we[4] and others[5,6] had demonstrated a correlation between base excision repair protein APE-1 and radiation sensitivity with GBM cell cultures. Also, we have shown that thioxanthenones such as lucanthone (CAS 479-50-5) and hycanthone (CAS 3105-97-3) inhibit the APE-1 endonuclease function in GBM cell lines with higher or over-expressed APE-1 levels without affecting its DNA substrate binding function.[7] As the next step, it is essential to determine whether we can use this mechanistic insight to cause tumor regression in mouse tumor models. However, as APE-1 is present both in normal and tumor cells, a way to target these thioxanthenones to GBM and other tumors specifically with no/minimal damage to the surrounding normal tissue is needed.

Graphene, a two dimensional, single layer, hexagonal lattice of carbon atoms has attracted much attention due to its unique chemical and physical properties.[8] Studies have also established

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that graphene can be used in various biomedical applications such as imaging and drug delivery.[9–11] The large surface of graphene can be chemically modified with a wide variety of molecules that can enhance biocompatibility,[12] solubility,[13] or allow the targeting to specific cell types and hence proves to be a good platform for biomedical use.[14] Reports show that oxidized graphene nanoplatelets synthesized by modified Hummer's method (chemical oxidation of graphite followed by ultrasonic cleavage) and coated with the amphiphilic polymer 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol (DSPE-PEG) can load high amounts of aromatic molecules such as the drug doxorubicin and release them into tumor cells.[13,15] The loading of the drug is achieved through the pi-stacking, a non-covalent interaction between electrons in adjacent pi bonds.[13,15]

Recently, Kosynkin and co-workers have pioneered a method that allows the synthesis of oxidized graphene nanoribbons (O-GNRs) in macroscopic amounts by the longitudinal unzipping of multi walled carbon nanotubes.[16] Our recent *in vitro* studies indicate that these nanoparticles coated with PEG-DSPE (hereafter called O-GNR-PEG-DSPE) may also be suitable for cell specific drug delivery.[17] In this paper, we report the efficacy of O-GNR-PEG-DSPE to load and deliver Luc to the GBM cell line U251.

## Methods

### *Reagents*

Cell line U251 and reagents used for measuring endonuclease activity were as described previously.[7] CG-4, rat glial progenitor cell line that remains a progenitor for only about 20–25 passages was kind gift from Dr. Toru Ogata from Research Institute, Namiki, Tokorozawa-City, Japan. Luc obtained from Dr. S. Archer (Sterling-Winthrop Research Institute, Rensselaer, NY) were maintained at 4 °C under hygroscopic conditions, and dissolved in 1.2 mg/mL PEG-DSPE (in double distilled water) just prior to reactions. Plasmids consisting of full length APE-1 in pCMV10 were a kind gift from Dr. Bruce Demple (Stony Brook University, NY). Multi-walled carbon nanotubes and propidium iodide (PI) were obtained from Sigma Aldrich. All cell culture components were obtained from GIBCO. Annexin V/PI staining kits were obtained from Trevigen.

### *Cell culture*

U251 transfected with either the blank plasmid pCMV10 (CMV/U251) or full length APE-1 in pCMV10 (AI-5/CMV/U251) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 800 μg/ml of G418. CG-4 were grown in 70% of DMEM F12 containing 1X penicillin-streptomycin (100 μg/ml Streptomycin + 100 U of penicillin) (PS) with 1X N2 supplement (containing 1 mM Transferrin, 0.06 mM Insulin, 0.002 mM progesterone, 10 mM putresceine and 0.003 mM selenite) and 30% of B104 conditioned medium. MCF-7 were grown at 37 °C in a humidified atmosphere of 5% $CO_2$ in RPMI medium supplemented with 10% fetal bovine serum and 1X PS.

### *O-GNR synthesis*

O-GNRs were synthesized from multi-walled carbon nanotubes (MWCNTs)(Sigma-Aldrich, Length = 5-9 μm) using the oxidative longitudinal unzipping method.[16] Briefly, MWCNTs (150 mg) were suspended in 30 ml concentrated (96%) $H_2SO_4$. After 4 h, 4.75 mM $KMnO_4$ was added slowly and stirred for an h followed by further stirring for another h at 55–70 °C in an oil bath. This solution was poured on ice (400 ml) containing 5 mL 30% $H_2O_2$ and the ice-$H_2O_2$ slurry was allowed to melt. The solution obtained was centrifuged at 3000 rpm for 30 minutes, after which the supernatant was discarded. The pellet obtained was then washed with 36% HCl. Ethanol and ether washes were used for flocculation and the final product (O-GNR) was obtained as pellet after centrifugation (30 minutes, 3000 rpm). This product was dried overnight in a vacuum oven at 60 °C.

O-GNRs were characterized using atomic force microscopy (AFM) and transmission electron microscopy (TEM). AFM images were obtained using a Nano Surf Easy Scan 2 AFM (NanoScience Instruments Inc, Phoenix, AZ) operating in tapping mode using a V-shaped cantilever and TEM images were obtained using a Tecnai Bio Twin G transmission electron microscope (FEI, Hillsboro, OR), at 80 kV.

### *Luc loading on O-GNR-PEG-DSPE*

Powdered O-GNRs were dispersed in a solution of 1.2 mg/mL 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)) (PEG-DSPE), at a concentration of 1 mg/mL. This dispersion was bath sonicated for 30 minutes to produce O-GNR-PEG-DSPE. 1 mg/mL solution of Luc in 1.2 mg/mL PEG-DSPE served as a Luc stock solution. 200 μL of the O-GNR-PEG-DSPE solution and 400 μL of the Luc solution were combined in a 20 mL glass vial, and the total volume was made up to 1 mL using a stock solution of 1.2 mg/mL PEG-DSPE and stirred at 4 °C for 24 h. After this loading period, unincorporated Luc was separated out from the loaded O-GNRs by centrifugation at 13000 RPM for 1 hour. In order to calculate the loading efficiency, the absorbance of the supernatant was measured at 328 nm using an Evolution 300 UV–vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and compared to a standard curve. The loading efficiency was calculated by subtracting the unloaded Luc from the total available Luc. Loaded O-GNR-PEG-DSPEs were left in pellet form until used.

### *Uptake of O-GNR-PEG-DSPE in the U251 and CG-4 cell lines (using flow cytometry)*

O-GNR-PEG-DSPEs were loaded with PI and purified using the same method used for Luc loading. CMV/U251 and A1-5/CMV/U251 were grown in 10 cm dishes at 37 °C and 5% $CO_2$ in DMEM. Cells were either incubated with PI-loaded O-GNR-PEG-DSPEs at a concentration of 40 μg per mL (previously reported to be a non-toxic concentration) (15) of media, or left untreated. After 24 h, cells were trypsinized, resuspended in FACS buffer (1X PBS containing 20% fetal bovine serum) and placed on ice. Flow cytometry was performed immediately after all samples were prepared using a FACS Calibur Cell Sorter (BD Biosciences, San Jose, CA).

### *Transmission electron microscopy*

Six well plates with surfaces covered with ACLAR® film (Electron Microscopy Sciences, Hatford, PA) were plated with CMV/U251 and MCF-7 cells at a density of $5 \times 10^5$ cells per