Cytotoxicity and Cellular Death Modality of Surface-Decorated Gold Nanorods against a Panel of Breast Cancer Cell Lines

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ABSTRACT: Herein, the antiproliferative effect of surface-decorated gold nanorods (GNRs) was investigated against three different breast cancer cell lines. The results indicate that the cell lines exhibited different biological responses and death modalities toward the treatment. The cell lines exhibited similar cellular uptake of the nanoparticles; however, MDA-MB-231 demonstrated the highest cytotoxicity compared to other cell lines upon treatment with GNRs. The expression of the CDH1 gene, which is involved in cell adhesion and metastasis, was dramatically increased in treated MDA-MB-231 cells compared to other cell lines. Early apoptosis and late apoptosis are the dominant cellular death modalities of MDA-MB-231 cells upon treatment with GNRs.

INTRODUCTION

Current cancer treatment is based mainly on chemotherapeutic drugs and radiation; however, these treatments usually result in severe side effects due to the drastic damage caused to the surrounding healthy tissues. Besides, a high incidence of relapse and resistance may reduce the effectiveness of traditional cancer treatment approaches. Cancer treatment by utilizing a nanoparticle-based approach plays a crucial role in overcoming the limitations of conventional cancer therapy by enhancing the cellular uptake and targeting. A considerable amount of research focusing on gold nanoparticles (GNPs) as a novel nanomedicine and their potential applications in cancer therapy has been carried out in this context.1,2

GNPs applications in cancer diagnosis and treatment are due to their favorable optical and physical merits that provide potential nano-systems for cancer diagnosis, photo-imaging, and photothermal therapy. Also, GNPs are synthesized in different shapes and sizes and they possess tunable properties. Another crucial property of GNPs is the large surface area to volume ratio available for drug loading and conjugation with small chemicals or large biomolecules, thus, enhancing drug solubility, stability, and pharmacokinetic parameters.3–6

GNP’s functionalization is a key aspect in determining their colloidal stability, cytotoxicity, biocompatibility, cellular uptake, target-ability, half-life, and elimination behavior upon administration. Functionalization of GNPs may be achieved through either physical adsorption or covalent linking of ligands into the surface of nanoparticles via a thiol moiety. It is crucial to note that nanoparticles’ surface charge and size also have a tremendous effect on their cytotoxicity.6–10 For example, particles with a positive surface charge are usually more cytotoxic than negatively-charged particles due to nonspecific interactions with negatively charged cellular membranes; however, the nature of the attached ligands and their contribution to the cellular uptake and cytotoxicity could not be excluded.

GNPs and particularly nonspherical shapes like gold nanorods (GNRs) have promising applications in cancer therapy, such as photothermal therapy, and as nanocarriers for chemotherapeutic agents, where their solubility, targeting, stability, and half-life were significantly enhanced.11–13 Besides, conjugation of certain ligands to GNP’s surface could enhance their cellular uptake and consequently their cytotoxicity. Many ligands and biomolecules were utilized to enhance GNP’s cellular uptake, such as polyethylene glycol, folic acid, peptides, transferrin, glucose, phospholipids, and others.14–22

Different types of breast cancers show considerable heterogeneity despite a common tissue of origin. There has been extensive research for subtyping breast cancer at the molecular and genetic levels and to determine various clinical, pathological, and molecular factors for selection of treatment...
modalities and prognosis. For *in vitro* testing, various cell lines are available for research purposes that represent, more or less, one of these subtypes, and using these models, a nearer approximation to the selection of the best modality for treatment could be realized.

Recently, we have reported the cytotoxicity and cellular uptake of phospholipid-modified GNRs against a noninvasive breast cancer cell line (MCF-7), where the nanoparticles enhanced several cellular apoptotic events in addition to necrosis. This study further investigates the cytotoxicity and cellular death modality of phospholipid-GNRs against other types of breast cancer, namely, T47D, another luminal A subtype breast cancer cell line with different estrogen receptor (ER) alpha and beta expression, MDA-MB-231, a triple negative breast cancer cell line and ZR-75-1 cell line, a luminal B subtype breast cancer cell line, by studying the antiproliferative activity, cellular uptake, and the cellular death modality.

**RESULTS AND DISCUSSION**

Phospholipid-GNRs of an aspect ratio of ∼4 were synthesized and fully characterized as described previously. Briefly, phospholipid-GNR demonstrated typical optical spectra of two distinguished peaks, the transverse and longitudinal peaks, with excellent colloidal stability. The phospholipid moiety was conjugated into the surface of the GNRs via the thiol terminal since gold has a well-known high affinity toward thiol. The average length and width of the modified GNRs were ∼69 and 17 nm, respectively. The effective surface charge of phospholipid-GNRs was ∼−15 mV, reflecting successful surface conjugation. The surface conjugation of the phospholipid into GNRs was also confirmed by proton nuclear magnetic resonance (1H NMR) and Fourier transformed infrared (FTIR) spectroscopies and thermogravimetric analysis (TGA) as described previously.

This study investigated the biological responses and cellular uptake of GNRs against three different breast cancer cell lines. Classification of breast cancer lines is based on the following measures: histological type, tumor grade, lymph node status, and the presence of predictive markers such as ERs and human epidermal growth factor receptor 2 (HER2). Molecular profiling using DNA microarrays proved this heterogeneity. Breast cancer is classified into at least five subtypes: luminal A (such as MCF-7 and T47D), luminal B, HER2-positive, basal-like, and normal-like subtypes.

The results in Figure 1 indicate that the prepared GNRs were cytotoxic against all cell lines under investigation in a dose-dependent manner. Interestingly, the MDA-MB-231 cells demonstrated drastic reduction in cell viability upon exposure to GNRs (cellular viability <20%) over the range from 48.0 to 6.0 μg/mL (Figure 1A) and a half maximal inhibitory concentration (IC_{50}) of 0.59 μg/mL (Figure 1B). The ZR-75-1 cells showed a similar cytotoxicity profile to MDA-MB-231 (Figure 1A), and IC_{50} of 0.97 μg/mL (Figure 1B). On the other hand, T47D revealed a low cytotoxicity towards the
GNRs over the range from 48.0 to 3.0 μg/mL compared to other cell lines (Figure 1A,B). In our recent work, the phospholipid-GNRs demonstrated a higher IC₅₀ (~2.3 μg/mL) towards MCF-7 cells than MDA-MB-231, ZR-75-1, and T47D. Variations in response of different types of breast cancer to cytotoxic chemotherapeutic agents is a known phenomenon. It has been reported that doxorubicin and 5-fluorouracil induce different mechanisms in cancer cell death, where luminal cells repressed a large number of cell cycle regulated genes, whereas basal cell lines repressed genes that were involved in differentiation.

We propose that phospholipid-decorated GNRs interact differently, and are more potent in basal-subtype cells than the luminal ones, which could be due to the interaction with other cell components that can modulate the MDA-MB-231 cells adhesion properties, thus enhancing their cytotoxicity.

Internalization of GNRs into the breast cancer cell lines was quantified and is demonstrated in Figure 2; all cell lines show a similar percentage of cellular uptake. However, MCF-7 cells previously demonstrated a slightly higher percentage of cellular internalization than these cell lines. Our results indicate that the extent of cellular internalization is not the only factor affecting the degree of cytotoxicity; the rate of nanoparticle uptake and their interaction with other cellular components may contribute to cytotoxicity. Davis et al. indicated that the difference in cytotoxicity of two platinum metallointercalators could be related to variations in their interactions with other cellular components. Besides, Swanner et al. also reported that silver nanoparticles were efficiently taken up by triple negative cell lines as well as the normal epithelial breast tissue; however, the toxicity in cancer cells was related to endoplasmic reticular stress without causing such a damage in normal cells. Confocal imaging demonstrated in Figure 3 may support our hypothesis; images of T47D and ZR-75-1 cells revealed the distribution of nanoparticles (red spots) into the cell’s cytoplasm; however, MDA-MB-231 cells demonstrated a high concentration of GNRs accumulated in the cell’s nucleus; this could explain the cytotoxicity of GNRs and its low IC₅₀ value towards the MDA-MB-231 cell line compared to other cell lines.

Furthermore, the cellular death modality of breast cancer cell lines was studied upon exposure to phospholipid-GNRs. Figure 4 reveals that control untreated cell lines demonstrated a high average percentage of healthy cells (90–96%). For T47D cells, necrosis was the major cellular death modality responsible for cytotoxicity (average; ~30%) in addition to late apoptosis (average; ~18%). On the other hand, early apoptosis (average; ~30%), and late apoptosis (average; ~27%) were the cellular death modalities of MDA-MB-231 cells upon treatment with GNRs. Interestingly, ZR-75-1 cells revealed the highest percentage of healthy cells compared to other cell lines (average; ~70%); necrosis and late apoptosis were the cellular death modalities of these cells upon exposure to GNRs. The current flow cytometry results support the previous findings; MDA-MB-231 cells showed the highest cytotoxicity percentage upon exposure to phospholipid-GNRs. Our previous work demonstrated that the late apoptosis and necrosis were the dominant cellular death modalities of MCF-7 cells upon treatment with phospholipid-GNRs. A recent study demonstrated that the mechanism of cell death in leukemic cells toward chitosan GNP’s depends on the type of the leukemic cells; the nanoparticles induced apoptosis in the T-acute lymphocytic leukemia cell line (CEM) and necroptosis in the chronic myeloid leukemia cell line (K562).

The gene expression of CDH1 was determined in the breast cancer cell lines treated with GNRs. It was found to be overexpressed by 22 folds in MDA-MB-231, while it was downregulated in ZR-75-1 and T47D cell lines (Figure 5). CDH1 encodes E-cadherin, an important tumor suppressor gene involved in cell-to-cell adhesion, and acts as an inhibitor of metastasis. Several mechanisms of CDH1 downregulation were reported in metastatic breast cancer cells, promotor hypermethylation, and transcriptional suppression that activates mesenchymal genes leading to epithelial to mesenchymal transition. CDH1 overexpression by GNRs might indicate promotor hypomethylation in MDA-MB-231, while it did not promote gene expression in ZR-75-1 and T47D cell lines. This is another aspect where the response of luminal and basal cells shows significant differences. Similarly, Stapf et al. demonstrated that the response of five breast cancer cell lines toward methotrexate-coupled magnetic nanoparticles was very heterogenous and cell-line-dependent; further, the cell-line-specific uptake of the nanoparticles agreed with the observed cytotoxicity effect. Furthermore, Caco-2 cells showed a higher sensitivity and lower gene expression than MCF-7 upon exposure to silver nanoparticles.

The overall results indicate that different breast cancer cell lines demonstrated different biological responses and different cellular death modalities upon exposure to surface-decorated GNRs. In the current study, surface-decorated GNRs has a promising future as a potential candidate for breast cancer treatment, particularly triple-negative breast cancers, the most aggressive subtype with poor clinical outcomes.

**CONCLUSIONS**

This study highlights the different biological responses and cellular death modalities of three breast cancer cell lines. Although the cell lines demonstrated similar cellular uptake of nanoparticles, their biological responses and cellular death modalities revealed some differences. MDA-MB-231 cells demonstrated the highest cytotoxicity and the highest nanoparticle internalization into the cell’s nucleus. MDA-MB-231 exhibited a dramatic increase in the gene expression of CDH1, which indicates the possible anti-invasion and anti-metastasis effect of GNRs. Early apoptosis and late apoptosis are the dominant cellular death modalities of treated MDA-
MB-231 cells; however, necrosis also contributes to the cellular death modality of other cell lines upon treatment with the nanoparticles.

**MATERIALS AND METHODS**

**Materials and Instruments.** 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-PEG-SH (DSPE-PEG-SH, MW ~2000 g/mol) was purchased from Nanosoft Polymers, USA. Chloroauric acid, HAuCl₄·3H₂O (99.9%); silver nitrate, AgNO₃ (99%); sodium borohydride, NaBH₄ (99%); ascorbic acid (99%); sodium oleate (NaOL); methoxy-polyethylene glycol-thiol; m-PEG-SH (MW ~2000 g/mole); cetyltrimethylammonium bromide, CTAB (99%); polyethylene glycol thiol; m-PEG-SH (MW ~2000 g/mole); cetyltrimethylammonium bromide, CTAB (99%); the gold standard for inductively coupled plasma-optical emission spectrometry (ICP-OES) (1000 ppm); and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye were from Sigma-Aldrich Chemicals, USA. Dimethyl sulfoxide (DMSO) was from Euroclone, Italy. Potassium bromide was from Acros, Belgium. MDA-MB-231, T47D and ZR-75-1 breast cancer cell lines were from American Type Culture Collection (ATCC), USA. The Roswell Park Memorial Institute tissue culture medium (RPMI 1640); fetal bovine serum (FBS); gentamycin, penicillin, and streptomycin (Penstrep) (50 μg/mL); and trypsin ethylenediaminetetraacetic acid (EDTA) 0.2% in phosphate buffer saline (PBS) were from Euroclone, Europe. Iscove’s modified Dulbecco’s medium (IMDM) was from Eurobio, France. 96-well plates were from Greiner Bio-One, Germany. Acetic acid, 97%, and ethanol, 70%, were from Tedia Company Inc., USA. 4,6-Diamidino-2-phenylindole (DAPI) stain and the Annexin V/PI apoptosis kit were from Molecular Probes, USA. Hydrochloric acid (HCl) was from Scharlau, Spain. Isopropanol was from Gainland Chemical Company, U.K. Nitric acid was from Vickers, U.K. Paraformaldehyde was from Fluka, Switzerland. The RNeasy Plus Mini Kit was from Qiagen, USA. The RT2 First Strand Kit was from Qiagen, USA.

The following instruments and equipment were used in this study: ultraviolet–visible (UV–vis) spectrophotometer, UV-1800, Shimadzu, Japan; Nano UV-spectrophotometer, Quwell, USA; size/zeta potential analyzer, Nicomp Nano Z3000 particle, USA; Hettich EBA 12 Centrifuge, Gemini BV, Netherlands; confocal laser scanning microscope, LSM 780, Carl Zeiss, Germany; inductively coupled plasma-optical emission microscope, Optima 2000 DV, PerkinElmer, USA; centrifuge, Labofuge I, Heraeus Christ, Germany; thermogravimetric analyzer (Mettler-Toledo, Columbus, OH, TA3000 System); inverted microscope, Meiji, Japan; ELISA plate reader, BioTek Instruments, USA; laminar air flow cabinet,
Figure 4. Flow cytometry assay of cellular death modality (presented as an average percentage of the total cells) of the treated T47D, MDA-MB-231, and ZR-75-1 breast cancer cell lines and the control untreated cells stained with FITC-conjugated annexin V and PI-stained. The dot plot for each sample was divided into four quadrants to indicate viable cells (lower left quadrant, Q3), early apoptotic cells (lower right quadrant, Q4), late apoptotic cells (upper right quadrant, Q2) and necrotic cells (upper left quadrant, Q1).
Cell Lines. Quantiﬁcation of untreated cells. was calculated relative to the cellular viability of the control absorbance was recorded at 570 nm using an ELISA plate. DMSO was added to solubilize the formed blue formazan

three cancer cell lines was seeded per 25 cm² tissue culture media and allowed to attach for 48 h (in three replicates). Then, phospholipid-GNRs suspensions in the cell culture medium without FBS were immediately applied to the cells to obtain a concentration of 75 μg/mL and incubated for 3 h for T47D and ZR-75-1 cells, and 2.30 h for MDA-MB-231 cells. The cells were trypsinized after two washing steps with PBS and centrifuged at 1400 rpm for 30 min at 4 °C, and the obtained cell pellets were mixed with aqua regia (HNO₃ and HCl; 1:3) in a water bath (70 °C) for 3 h. The digested samples were diluted with Milli-Q water up to 3.0 mL and ﬁltered using a 0.22 μm Teflon syringe ﬁlter. Untreated cells presented the control.

Using a validated ICP-OES analytical method, the concentration (mg/L) and the percentage of the internalized gold into cells were quantiﬁed at a wavelength of 242.795 nm using a gold standard calibration curve ICP (0.2–10.0 ppm). The experiment was done in triplicate.

Imaging of Breast Cancer Cell Lines Treated with Phospholipid-GNRs by Confocal Laser Scanning Microscopy. A total of 3 cancer cell lines were seeded onto round coverslips in a 12-well plate at a density of 2 × 10⁶ cells/well in the RPMI tissue culture medium and allowed to attach for 24 h. Phospholipid-GNRs in tissue culture media with FBS (20.0 μg/mL) were immediately applied to the cells and incubated for 24 h, after which the media and treatments were removed, and the wells were washed with PBS. The cells were ﬁxed for 30 min in paraformaldehyde (4%) at 4 °C and washed with PBS three times. After that, the coverslips were gently removed and slowly ﬂipped over clean slides covered with 50.0 μL of DAPI stain. Cells not exposed to the GNRs suspension are considered as the control in the experiment. The cells were imaged at excitation/emission wavelengths of 532 nm/750 nm for gold and to 360 nm/460 nm for DAPI.

Cellular Death Modality of T47D, MDA-MB-231, and ZR-75-1 Breast Cancer Cell Lines after Treatment with Phospholipid-GNRs. Cell Death Analysis by Flow Cytometry Analysis. To investigate the growth inhibition of cancer cell lines treated with phospholipid-GNRs, the cell death mechanism was determined by Annexin V/PI stain using ﬂow cytometry. The cells (4 × 10⁶ cells) were seeded into 6-well plates, and they were exposed to phospholipid-GNRs [1.0 μg/mL for MDA-MB-231, and 1.5 μg/mL for ZR-75-1 and T47D]. After 24 h, cells were trypsinized utilizing trypsin EDTA (0.25%). Then, the collected cells were washed with PBS. After that, the Annexin V/PI apoptosis kit was used to stain the cell pellets following the kit’s instructions. Doxorubicin (1.0 μM) was used as a positive control. A ﬂuorescein-activated sorter FACS Canto II was used to analyze the samples.

Estimation of Gene Expression of CDH1 in T47D, MDA-MB-231, and ZR-75-1 Breast Cancer Cell Lines after Treatment with Phospholipid-GNRs. Total mRNA was extracted from breast cancer cell lines treated with their respective IC₅₀ concentrations of GNRs, and untreated cells were used as their control using the RNeasy Plus Mini Kit following the kit’s instructions and stored at −80 °C until cDNA synthesis.

cDNA prepared from mRNA extracted using the RT2 First Strand Kit (Qiagen, USA) according to the manufacturer’s protocol was applied in real-time PCR ampliﬁcation using CFX96 qRT-PCR to determine gene expression levels of CDH1.

Statistical Analysis. Statistical analysis was conducted by one-way ANOVA by GraphPad Prism version 7.0. The results are considered signiﬁcant when p < 0.05.
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Notes
The authors declare no competing financial interest.

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