

Impact of Docetaxel Conjugated Nanodiamonds on Gene Expression of Prostate Cancer (PC3) Cells

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ABSTRACT

Prostate cancer (PC) is the second leading cause of adult male death in the United States which is due to uncontrolled growth of the prostate gland cells provoking the development of malignant tumors that could lead to death. Docetaxel (Dox) drug is used to treat PC but it becomes toxic at high concentrations or builds resistance to cancer cells in patients. Possible treatments for PC suggested the use of drug loaded nanoparticles as therapeutics. In this study, nanodiamonds (ND's) were used as carriers to deliver Dox to PC cells. ND's were conjugated with Dox and Dox along with RGD peptide using EDC/NHS chemistry. ND's in PC3 cells were tracked using ND-fluorescein which showed ND's were located in the cell nucleus after 24 hours of incubation. Drug release assay showed 92.3% of drug release from NDs in 24 hr. Microculture Tetrazolium Assay (MTT) assay showed 75% PC3 cells viability at 50 μ g/mL with ND's after 48 hr incubation compared to 50% with drug conjugated NDs stating that it efficient in causing cancer cell death. Gene expression analysis was conducted on PC3 cells with ND's and conjugated ND's after 24 hr incubation. NDR showed upregulation in many genes of PC cells. The study showed NDs can be used for efficient drug delivery for PC3 cells.

Keywords: Prostate cancer cells (PC3), Docetaxel, Nanodiamonds, Gene expression.

1. INTRODUCTION

Prostate cancer (PC) is one of the most common cancers that affect men around the world and the second leading cancer among men in the United States¹. The prostate gland makes the fluid that makes semen and nourishes and protects sperm. PC results due to uncontrolled prostate cells growth leading to malignant tumors. The incidence of this disease has been linked to western diet as well as fat/cholesterol-enriched diet, characterized by the consumption of high levels of red meat, saturated fats and dairy products². PC does not present early

symptoms, therefore could become fatal if not treated early. Dox is a semi-synthetically drug produced from the needles of the Pacific yew tree (*Taxus brevifolia*) and is administrated to PC patients as a first option treatment, but is known to becomes toxic at high concentrations as well as it builds up drug resistance. Dox increases tubulin polymerization, promotes microtubules assembly and inhibits tubulin depolymerization. This drug also induces the phosphorylation of Bcl- XL/Bcl-2, inactivating their anti-apoptotic capacity.² Bcl-2 down-regulation and p53 up-regulation are important ways of apoptosis induction by Dox. Some side effects of Dox are neurotoxicity, myelosuppression, acute hyper sensitivity reactions, nasolacrimal duct stenosis, asthenia, febrile neutropenia, and myalgia³.

Nanotechnology is a field that has been developing in the last years for biomedical research⁴. Nanoparticles are being used as carriers of drugs for therapeutics. ND's are carbon-based nanoparticles with a diameter of approximately 2-8nm and have unique properties such as electrostatic properties, chemically inert core and tunable surface, which can be modified with different functional groups to improve efficacy in drug resistance tumor therapies.

To improve the efficacy of Dox for PC treatment and reduce toxicity levels in an organism, nanoparticles can be used as a carrier to deliver the drug directly to PC cells⁵. In the present study NDs were conjugated with Dox and DOX along with RGD peptide to test their efficacy against PC3 cells growth and gene expression.

2. METHODS

2.1 Materials

The ND's used were donated by Dr. Derrick Dean (University of Alabama in Birmingham, AL). Docetaxel and RGD peptide was purchased from Sigma-Aldrich (Allentown, Pennsylvania). The CellTiter 96 Non-Radioactive Proliferation Assay kit was purchased from PromegaTM (Madison,

Wisconsin). RT² Profiler™ PCR Array Human Prostate Cancer and RNeasy Plus Mini Kit was purchased from Qiagen (Valencia, California).

2.2 PC3 Cell Culture

PC3 cells were procured from Dr. Bedi, Tuskegee university. PC3 were grown in F12K media with 10% FBS. Cells were incubated at 37°C in a 5%CO₂ humidified atmosphere and split every three to four days.

2.3 ND-Fluorescein Synthesis and Cell Tracking

ND's were functionalized with 5mg/mL 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) / N-Hydroxysuccinimide (NHS) fluorescein. For cell tracking, PC3 cells (2.7 x 10⁴ cells) were plated in an 8-chamber slide and incubated with functionalized ND's (fND's) and tracked at different time intervals (4, 6 and 24hr). Cells were fixed with 10% Tricarboxylic acid (TCA) and dehydrated using 70, 90 and 100% ethanol. Cell nucleus was stained with DAPI and cells were examined under Immunofluorescence microscope (Nikon Ti Eclipse) to localize ND's in PC3 cells.

2.4 Nanodiamonds Conjugation with Docetaxel and RGD

NDs were conjugated using EDC /NHS chemistry. For ND's conjugation with Dox alone, a 10mg/mL solution of the drug was mixed with a 2mg/mL solution of ND's and 1mg/mL solution for both EDC and NHS. The supernatant was saved to estimate unconjugated drug and conjugated ND's were re-suspended in 100µL of nuclease free water. ND's conjugation with Dox along with RGD was performed with a 5mg/mL RGD solution mixed with 2mg/mL ND's solution, and 1mg/mL solution for both EDC and NHS and a 2mg/mL solution of Dox. The supernatant was saved and conjugated ND's were re-suspended in 500µL of nuclease free water.

2.5 Drug Release Assay

The drug release assay was performed using a 1mg/mL solution of Dox at different time intervals and analyzed by a UV-Vis Spectrophotometer at a wavelength of 290nm.

2.6 Cell Viability Assay

The Cell Titer 96-well proliferation assay (MTT) was performed on PC3 cells after 24 and 48hr of incubation. Cell (20,000) were plated and incubated with ND's, ND's+Dox, ND's+Dox+RGD and Dox at different concentrations (5-100 µg/mL) using the

protocol provided by Promega™. The plates were read in a 96-well plate reader at 570 nm.

2.7 RNA Extraction and qPCR

RNA was extracted using RNeasy Plus Mini Kit from PC3 cells incubated with NDs, NDs +Dox, NDs+Dox+RGD and Dox. cDNA was synthesized using RT² First Strand kit and gene expression analysis was done using RT² Prolifer™ Human Prostate Cancer PCR Array. qPCR was performed using Applied Biosystems® ViiA™ 7 Real Time PCR Systems. Data was analyzed using Qiagen PCR Array Data Analysis Tool.

3. RESULTS AND DISCUSSION

3.1 ND's Tracking

ND's tracking in PC3 cells was performed using Immunofluorescent Microscopy (Fig. 1).

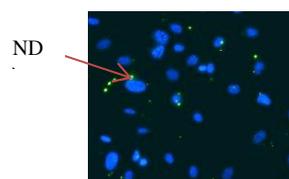


Fig.1. ND's (10mg/ml) tracked using immunofluorescent microscope after 24hr incubation

ND uptake was increased with incubation time and concentration. We observed NDs inside the cells from 4 hr of incubation and they reached the cell nucleus in 24 hr (Fig.1). The data indicates that cells must be incubated for at least 6hr or more with drug encapsulated nanoparticle to deliver drug to the cells.

3.2 Drug Release Assay

A drug release assay was performed to estimate the amount of Dox released from conjugated ND's (Fig.2). The amount of released drug from

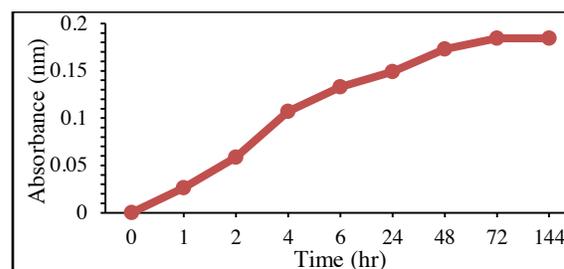


Fig. 2. Drug release profile of Docetaxel

conjugated ND's was analyzed at 290nm upto 144 hr. Drug release assay showed a 92.3% Dox release from

conjugated ND's after 24hr indicating it can be successfully used to deliver drug to the cells..

3.3 Cell Viability Assay

MTT assay was performed on PC3 cells and HEP-2 cells to test cell viability with ND's and conjugated ND's (0- 50µg/mL) treatment (Fig. 3)

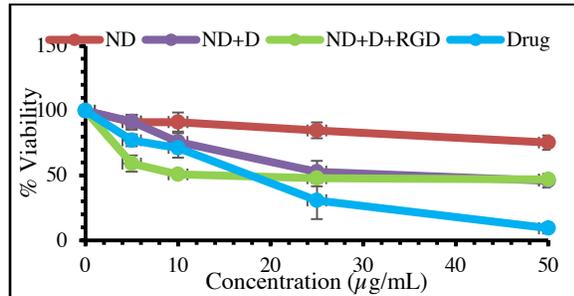


Fig. 3. Viability (%) of PC3 cells with ND's, ND's + Dox, ND's+Dox+RGD and Dox after 48hr incubation

ND's showed less than 30% PC3 cell death even at 50 µg/ml compared to 50% cell viability with ND's+Dox and ND's+Dox+RGD. Dox alone on the other hand show high (90%) PC3 cell death at 50 µg/ml. Dox is defiantly able to kill more PC3 cells, however, it is known to develop resistance at high concentration because of which ND+Dox and ND+Dox+RGD can be a good alternate for therapeutics purposes as they provide slow release of drug along with cell death.

3.4. Gene Expression Studies

PCR array was performed to analyze gene expression in PC3 cells when treated with 5µg/ml ND's and conjugated ND's (Fig. 4-7). Scatter plots show more

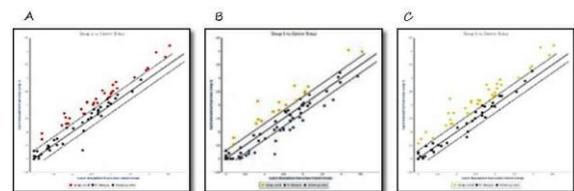


Fig. 4. Scatter Plot showing gene expression in PC3 cells after 24hrs incubation with A) ND's B) ND's + Dox C) ND's + Dox + RGD

genes were up-regulated with ND's alone (Fig. 4A) whereas with ND's+Dox and ND's+Dox +RGD treatment (Fig. 4B-C), genes were both up- and down-regulated compared to control group.

The bar charts (Fig. 5) indicate the gene expression changes with treatments. After ND treatment

TMPRSS2 gene, a transmembrane serine protease 2 membrane serine protein, was down-regulated by 10 fold (Fig. 5A). This gene was demonstrated to be up-regulated by androgenic hormones in prostate cancer cells and down-regulated in androgen-independent prostate cancer tissue⁶. TFPI2 (tissue factor pathway inhibitor 2) gene encodes a member of the Kunitz-type serine proteinase inhibitor family and has been identified as a tumor suppressor gene in several types of cancer. We observed up-regulation of this gene after treatments (Fig. 5A-C). SUPT7L gene is up-regulated in prostate cancer; however in our study it was down-regulated after ND+Dox treatment (Fig. 5B).

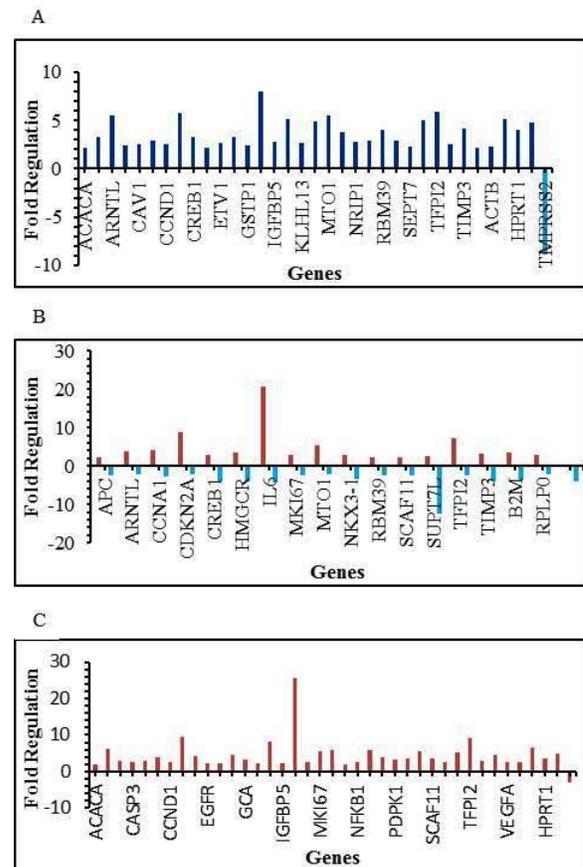


Fig. 5. Gene expression of PC3 cells after 24hrs incubation with A) ND's B) ND's + Dox C) ND's+ Dox+RGD

Cluster gram shows the hierarchical clustering of PC3 genes to display a heat map with dendrograms indicating co-regulated genes across different treatments or individual samples (Fig. 6). Red color indicates maximum gene expression and green color indicates minimum gene expression.

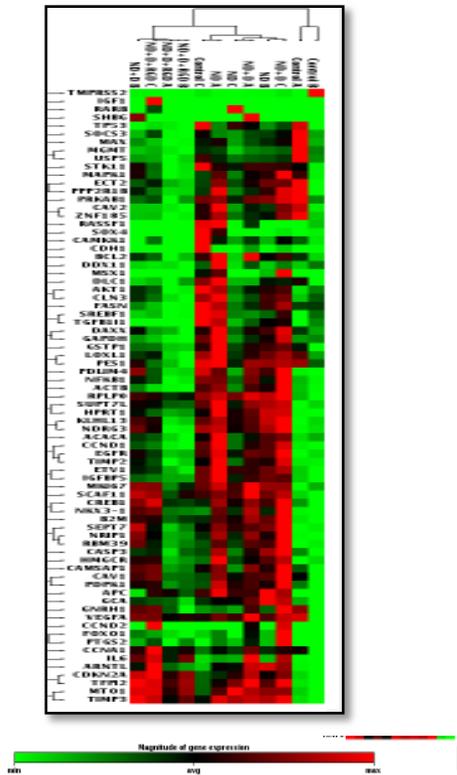


Fig. 6: Cluster gram for the gene expression of PC3 cells after 24hr treatment with ND's, ND's+Dox, ND's+Dox+RGD. Red color is the maximum gene expression and green color is the minimum expression of genes.

Heat map showing gene fold regulation expression between different groups' shows varied PC3 gene expression (Fig. 7). An increase in PC3 gene expression is shown when exposed to ND's alone and ND's+Dox. However, with ND's+Dox+RGD treatment, both increase and decrease in PC3 gene expression was observed. Our results illustrate that

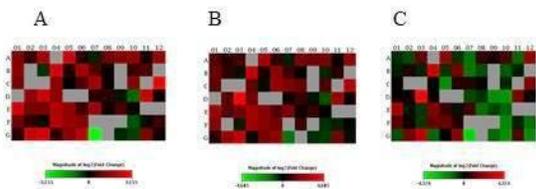


Fig. 7. Heat map showing gene expression in PC3 cells after 24hrs incubation with A) ND's B) ND's + Dox C) ND's+Dox+RGD. Red color is the maximum gene expression and green color is the minimum expression of genes.

ND's, ND's + Dox and ND's + Dox + RGD induces gene expression variation in PC3 cells after 24hr of incubation even at nontoxic concentration of 5µg/ml ND's and conjugated ND's.

CONCLUSION

The present study indicates that ND's and conjugated ND's induce PC3 cells death. Gene expression analysis shows mainly gene up-regulation by NDs whereas ND's+Dox and ND's+Dox+RGD induced both up- and downregulation of gene expression. The present study indicated these nanoparticles can be used as drug delivery agents.

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ACKNOWLEDGEMENT

This work was supported by NSF-REU (DBI-1358923) to Dr. Komal Vig (PI) and by NSFCREST (HRD-1241701) to Dr. Shree S. Singh (PI).