Gold Tripod Nanoparticles' Effectiveness for Killing PC-3 Prostate Cancer Cells *in Vitro*

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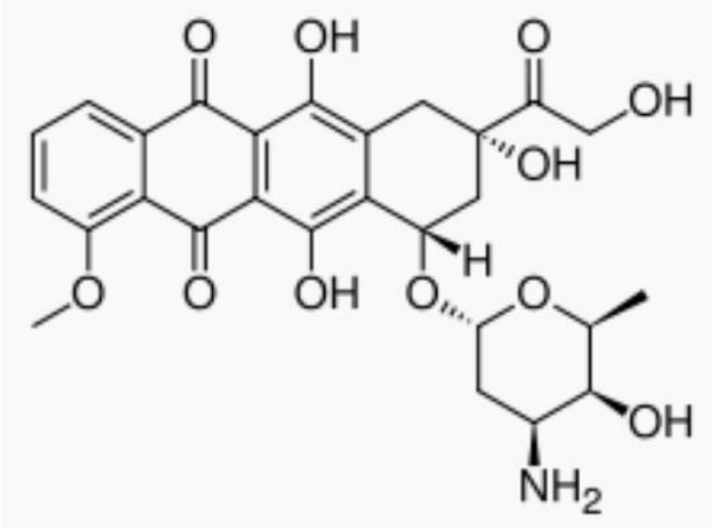
Abstract: Gold Tripod Nanoparticles Effectiveness of Killing PC-3 Prostate Cancer Cells in Vitro

In 2013 approximately 238,590 new cases of prostate cancer will be diagnosed, and around 29,720 men will die from it in the United States alone. Current methods of treatment, including chemotherapy and surgery have a number of detrimental side effects. Nanoparticles are currently being tested as alternative methods of drug delivery into the body, and studies have shown that they have fewer side effects. Nanoparticles are particles having one or more dimension being 100 nm or less. This study was done to see if a novel type of nanoparticle, the tripod, when conjugated with cancer treating drug doxorubicin, could effectively kill prostate cancer cells. It was found that gold tripods conjugated with doxorubicin killed around 90 % of the prostate cancer cells in vitro. This means that gold tripods could possibly kill tumors in vivo, and these studies should be conducted.

Introduction

In 2013, approximately 238,590 new cases of prostate cancer will be diagnosed, and roughly 29,720 men in the United States alone will die from it. Incredibly, approximately one in six men will be diagnosed with prostate cancer during his lifetime (Cancer.org). The treatment of prostate cancer can involve many different types of invasive surgeries and radiation therapy. Along with being astonishingly expensive, these treatment options can have terrible side effects, including impotence, incontinence, hormonal changes, nausea and pain; the treatments can also cause reduced resistance to diseases.

Doxorubicin is an anti-cancer drug commonly used to treat some leukemias and Hodgkin's lymphoma, as well as cancers of the bladder, breast, stomach, lung, ovaries, thyroid, soft tissue sarcoma, multiple myeloma, and others. Doxorubicin was chosen for this study because it is a fairly common anti-cancer drug, and has been shown to be effective at killing PC-3 prostate cancer cells. These cancer cells have high metastatic potential, or ability to spread throughout the body, compared to other prostate cancer cell lines.



It is an anthracycline antibiotic, closely related to the natural product daunomycin, and like all anthracyclines, it works by intercalating DNA, with the most serious adverse effect being life-threatening heart damage. It is commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, and soft tissue sarcomas. The drug is administered intravenously, as the hydrochloride salt. Acute adverse effects of doxorubicin can include heart arrhythmias, nausea, and vomiting. It can also cause neutropenia (a decrease in white blood cells), as well as complete alopecia (hair loss). Thus, it is important to find ways to make doxorubicin less toxic, such as improved targeting to tumors.

Drug delivery in medical treatment has evolved from conventional pills to modified dosage forms offering a variety of release profiles fit for the need, such as immediate, sustained, pulsatile, and delayed release. Modified drug release through different routes has been made possible through advancements in biodegradable implants, intravenous pumps, transmucosal delivery systems, and inhalers. The new advancements in drug delivery technology are making the old drugs work better, and able to deliver novel classes of drugs, which would otherwise be difficult to deliver.

Targeted delivery approach is the long sought after "Holy Grail" of drug delivery. The introduction of nanotechnology has revolutionized the science and technology of drug delivery (Devadasu et al. , 2013). For example, nanoparticles conjugated with cancer treatment drugs can selectively deliver the drugs to the cancer tumor. Such nanoparticle-based methods could also be used in conjunction with some current treatments to enhance the latters' effects.

Nanoparticles can deliver drugs to tumors either passively or actively. Passive delivery refers to nanoparticle transport through natural processes. After injection, nanoparticles are

transported through leaky tumor capillary openings into the tumor by passive diffusion. Passive nanoparticle delivery allows nanoparticles to function as a carrier of drugs through small openings in tumor vasculature, allowing direct access to the cell. Active nanoparticle delivery involves drug delivery to a specific site based on molecular recognition, the specific interaction between two or more molecules. In addition to this, nanoparticles are used because larger particles will get flushed out of the body faster, and when pegylated nanoparticles are invisible to macrophages, thus increasing their ability to deliver drugs for increased amounts of times (Haley, Frenkel 2008).

A major limitation inherent to most conventional anticancer chemotherapeutic agents is their lack of tumor selectivity. One way to achieve selective drug targeting to solid tumors is to exploit abnormalities found in tumor vasculature, namely hypervascularization, vascular permeability factors which stimulate extravasation (fluid leakage) within tumor tissues, and lack of lymphatic drainage. In order for tumor cells to grow quickly, they must stimulate the production of blood vessels and other growth factors involved in cancer angiogenesis. The newly-formed tumor vessels are usually abnormal in form and lack a smooth muscle layer, and generally lack effective lymphatic drainage. All of these factors lead to exploitable abnormal molecular and fluid transport dynamics, especially for macromolecular drugs, and particles of nano dimensions. Because of these factors, nanoparticles and large molecules, e.g., lyposomes and macromolecular drugs, tend to accumulate in tumor tissue much more than they do in normal tissue (Greish, 2010).

The phenomenon of enhanced transport of macromolecules and nanoparticles into cancer tumor tissue is referred to as the “enhanced permeability and retention (EPR)” effect. The EPR effect is further enhanced by many pathophysiological factors involved in enhancement of the

extravasation of macromolecules in solid tumor tissues. One phenomenon which lends to increased macromolecule/nanoparticle uptake is the lack of lymphatics around the tumor region which would filter out such particles under normal conditions.

The reason for the enormous amount of interest in the nanoscale level materials in all areas of science is fact that many materials at this scale show different properties, such as optical, electrical and chemical, in comparison to their bulk counterparts. There are intrinsic nanoscale properties that result from the confinement of atoms and electrons within the boundaries of a few nanometers; these effects become especially dominant at dimensions of less than about 30 nm. It is probably reasonable to conclude that the transition from classical bulk material properties to non-classical, or quantum mechanical, properties should be the best gauge of the scale length at which a particular material should be classified as a nanomaterial. This transition will be different for different materials (Devadasu et al. 2013)

Besides their non-classical properties, nanoparticles also have an incredibly large surface area to volume ratio, and since many drugs adsorb readily on surfaces, their large surface area makes them excellent vehicles for delivery of drugs. For example, the surface area to volume ratio of a 10 nm nanoparticle is about 7,500,000 times that of a baseball. While high surface area to volume ratios are important for applications such as catalysis, the actual properties of gold are different at the nanoscale. For example, the plasmon resonance of spherical gold nanoparticles results in the particle's exceptional ability to scatter visible light; thus, solutions with gold particles of less than 100 nm in diameter exhibit an intense red color. This property also allows the gold to be excellent at enhancing magnetic resonance imaging images.

At the macroscale, gold is an inert element, meaning it does not react with many chemicals. At the nanoscale, the surface reactivity of golf nanoparticles causes them to be good catalysts for reactions and also makes them able to adsorb drug molecules. This combined with their facile synthesis and preparation has ensured that gold nanoparticles are among the most studied nanoparticles in the field of nanomedicine.

There are many different types of nanoparticles, each with different advantages. Dendrimers are branched three-dimensional nanoparticles with a specific core molecule. Drugs can be attached to the functional groups on the surface. Micelles are spherical structures that form as a result of hydrophobic and hydrophilic molecular reactions. These are useful for the delivery of hydrophobic drugs because they can be carried in the hydrophobic core. Nanospheres are spherical nanoparticles where the drug can either be entrapped in the core, or adsorbed onto their surface. These spheres have surfaces which can be modified by polymer addition and ligands for specific targeting. Nanocapsules are similar to nanospheres, however their core can be either solid, liquid, gaseous, or aqueous. Nanotubes are ellipsoid tubes with hollow cage insides to entrap drugs for delivery. Liposomes are a commonly used nanoparticle for drug delivery. They are composed of lipid layers and are classified according to their number of bilayers. Unimellar liposomes have an aqueous core for the encapsulation of water soluble drugs. Multimellar liposomes are used for the entrapment of lipid soluble drugs. When injected intravenously, these are cleared by the kidneys quickly and also disintegrate quickly, however they can also be PEGylated. PEGylation is the process of covalent attachment of polyethylene glycol (PEG) polymer chains to another molecule; it makes nanoparticles undetectable to macrophages.

Courvreur et. al. found that doxorubicin-loaded nanospheres are effective at killing prostate cancer cells in mice, while decreasing their mortality rate and weight loss. Those treated with inhalable effervescent gold nanoparticles had a survival rate more than three times that of those treated with doxorubicin alone. Unlike chemotherapy, nanoparticles provide treatment with reduced toxicity to healthy tissue, thus decreasing harmful side-effects (Haley, Frenkel,2008).

Tripod nanoparticles are a relatively recent type of nanoparticle. Tripod nanoparticles are shaped like tripods, and are most commonly made of gold because of the enhanced computed tomography (CT) imaging sensitivity and increased MRI capabilities. Tripods have less of a tendency to agglomerate into larger, undesirable particles with relatively lower surface area to volume ratios, than typical spherical nanoparticles, which makes them more efficient for adsorbing and transporting drugs (Chen, 2003). They are also excellent contrast agents in cellular and biological imaging, making them excellent for cancer treatment as they will help image the tumor and kill it (Jain et. al., 2005). Gold tripod nanoparticles can be functionalized with certain hormones, or "signals", depending on the cancer type desired for targeting. This procedure enables the tripod-type nanoparticles to be better at selectively concentrating around the tumor area than other types of gold nanoparticles. Gold-tripods are a highly promising type of nanoparticles for both imaging to track the cancer progression, and for targeted drug delivery to kill the cancer tumor (Cheng, 2011). Tripods were studied because they are a novel type of nanoparticle, with not much information known about them. We proposed that the use of doxorubicin-loaded gold tripod nanoparticles for targeted drug delivery to the cancer site would be efficient at killing prostate cancer cells. In an earlier study, Chen radio-labeled gold nanoparticles with copper 64 isotope, and was able to show a three-dimensional image distribution of the tumor using the positron emission tomography (PET) scan technique (2003).

Methodology

PC-3 prostate cancer cells were cultured for the study and split into different containers as needed. Gold tripod nanoparticles were prepared from previously synthesized solutions to make them biocompatible so we could test them. The tripods were then conjugated with doxorubicin to test the uptake by adsorption on their surface. Tripods alone, tripods conjugated with doxorubicin, tripods in solution with free doxorubicin, and doxorubicin alone were introduced into wells containing PC-3 prostate cancer cells to test their ability to kill the prostate cancer cells. Cell viability was tested with a flourometer, comparing the flourometer results from each solution to the flourometer results of the prostate cancer cells alone. Two trials were done to compare results.

Preparation of solutions

Pure gold tripods were prepared by taking 0.50 mL out of the previously synthesized tripod solution, evaporating to dryness, adding chloroform, evaporating the chloroform, and then adding 0.10 mL of water. Tripod nanoparticles conjugated with doxorubicin were prepared by taking 0.50 mL out of the tripod solution, evaporating the solution, adding chloroform while stirring, evaporating the chloroform, and adding 0.50 mL of doxorubicin with 1.50 mL of water. The solution was then stirred for one hour, and was then purified using a purification column to separate the free doxorubicin from the tripods conjugated with doxorubicin. The tripods conjugated with doxorubicin were then collected.

Cell Culture

PC-3 Prostate cancer cells were cultured from a frozen sample. They were suspended in phosphate-buffered saline (PBS) solution, and split into two 75 mL flasks with 15 mL of F-12

medium. The cells were routinely split every few days for around three weeks and new medium was added. Later, these cells were split into 96 well plates, and 12 well plates as needed.

Cell Viability

A cell viability test was done in order to measure the percent of living cells after the different solutions were added to the PC-3 cells. Solutions with doxorubicin alone, nanoparticles alone, nanoparticles conjugated with doxorubicin, and free doxorubicin mixed with nanoparticles were each added to 36 wells, for a total of 144 wells, which were then incubated for 24 hours and analyzed using a spectrophotometer.

Fluorescent Staining

Dapi nucleus staining was added to each of the wells of treated PC-3 cells with doxorubicin. A confocal imaging machine was then used to produce an image to determine if the tripod nanoparticles adsorbed the doxorubicin, and if where it was in the cells.

Statistics

One way anova statistics were done to calculate the significance of the cell viability results at a concentration of 4 mL dox/ mL medium. Tukeys honestly significant difference was calculated to test the significance between groups.

Results

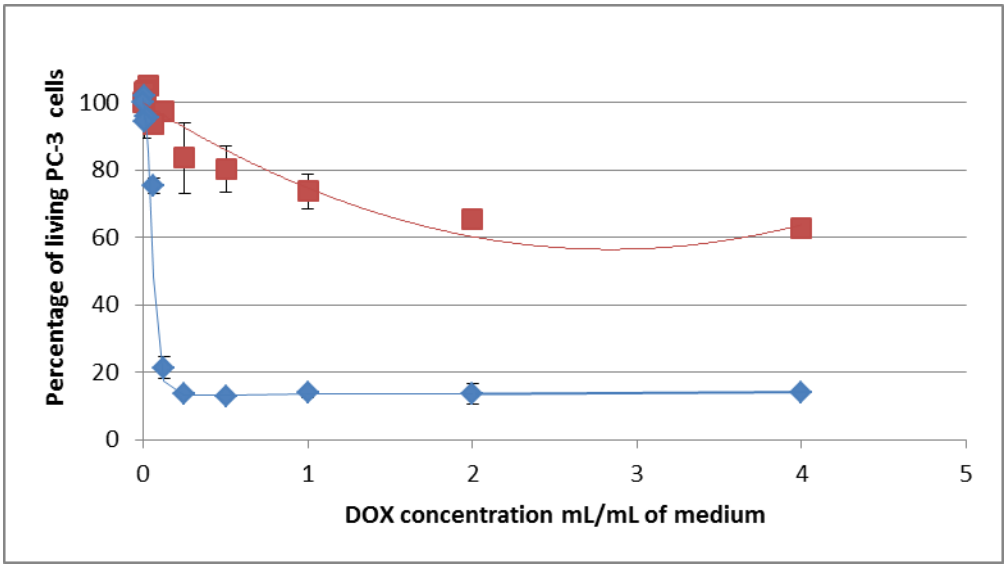
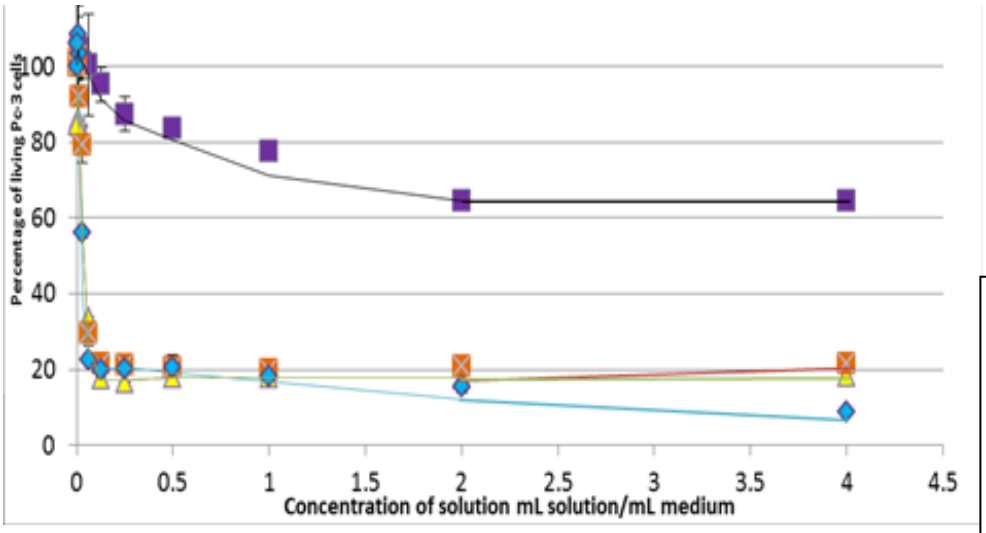


Figure 1. **Cell Viability Test**

Figure 1 shows the percentage of PC-3 Cells living 24 hours after the addition of doxorubicin conjugated nanoparticles and doxorubicin alone. As shown, both treatments resulted in a much lower percentage of living cells when the concentration went up and the doxorubicin conjugated tripods were more effective at killing the cells than doxorubicin alone. The lowest concentration tested was 0.00390625mL dox/ mL medium. The results suggest that there is a limit to how well the doxorubicin works at killing the prostate cancer cells, and there is little change between 0.015625mL dox/ mL medium and 4 mL dox/mL for both tests.



Dox= doxorubicin alone

Dox + Nps = tripods with free doxorubicin

Dox conjugated nps = tripods conjugated with doxorubicin

Nps = tripods alone

Fig 2. Cell Viability Test, Trial 2

Figure 2 shows the percentage of PC-3 cells living after the addition of doxorubicin conjugated nanoparticles, doxorubicin alone, tripods alone, and free doxorubicin with free tripods. Again it was confirmed that the doxorubicin conjugated tripods were the most effective at killing the prostate cancer cells. The doxorubicin conjugated nanoparticles killed around 90 % of the prostate cancer cells.

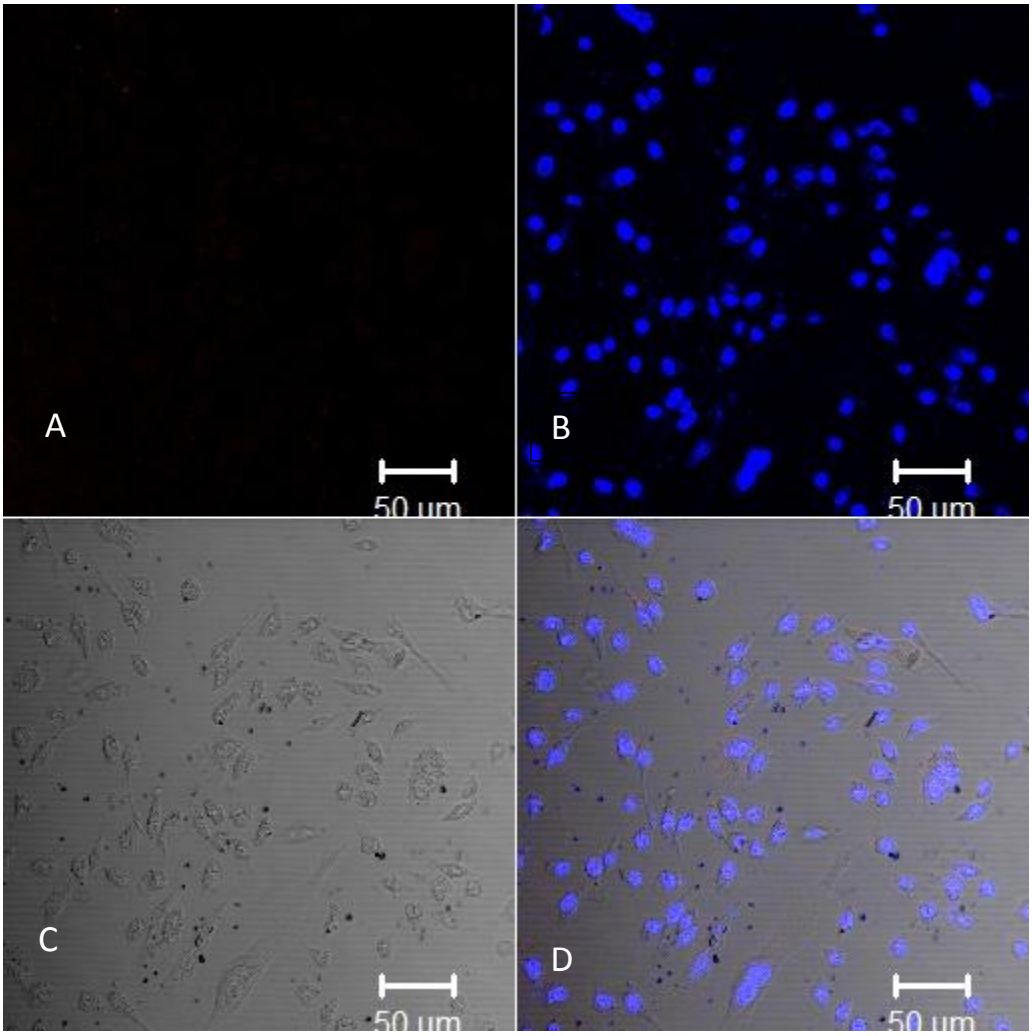


Figure 3. Confocal Imaging of nanoparticles alone with Dapi Nuclear staining

Figure 3 shows the fluorescent imaging of the PC-3 cells after adding tripod nanoparticles without doxorubicin, leaving for an hour, and washing with PBS and replacing with new medium. The blue shows the nuclei of the prostate cancer cells. Panel A shows the testing for the doxorubicin fluorescing. Panel B shows the fluorescing of the nuclei. Panel C shows both A and B overlapped in black and white. Panel D shows the fluorescing overlapped panels. This image was created as a baseline to illuminate the nuclei for comparison with the other

images.

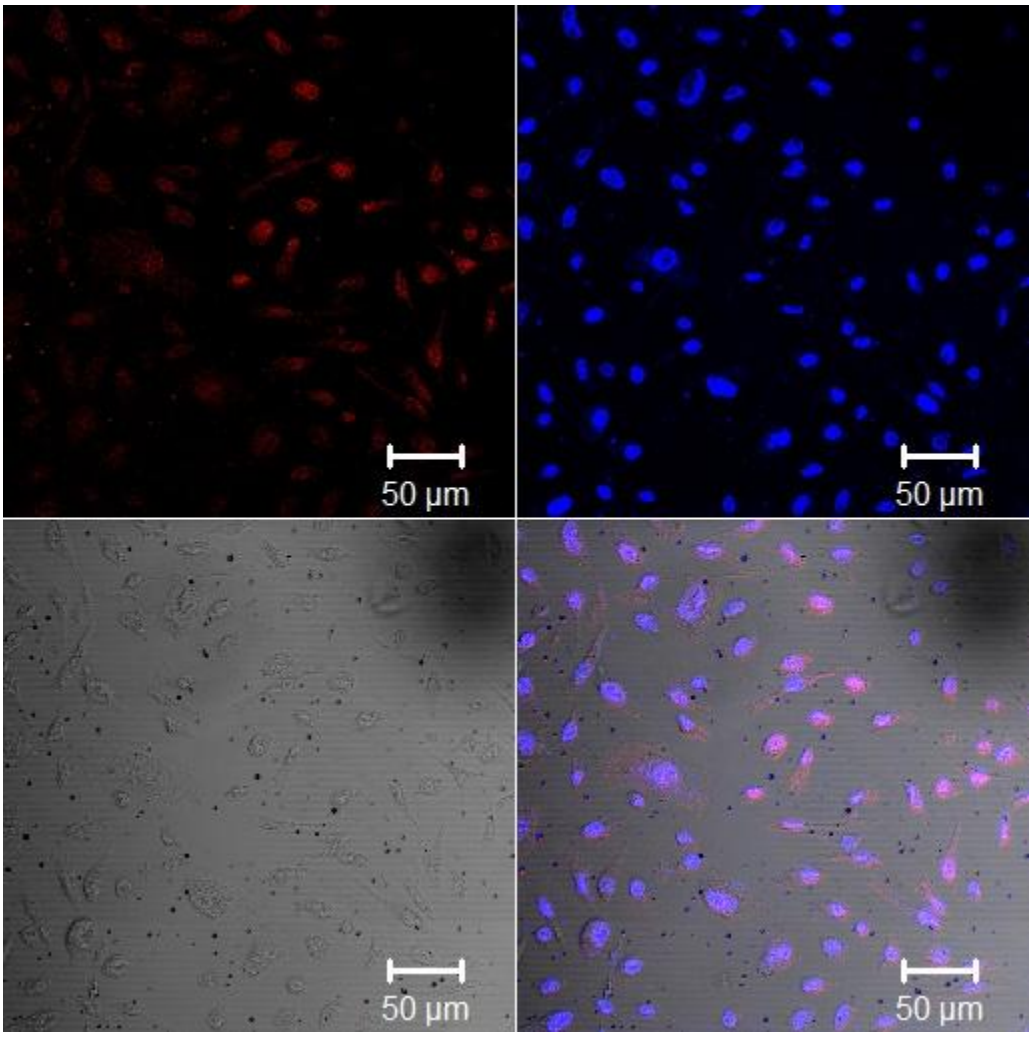


Figure 4 Confocal Imaging of nanoparticles and free dox with Dapi staining

Figure four shows the imaging of PC-3 cells after being treated with a solution of free doxorubicin mixed with gold tripod nanoparticles without adsorbed doxorubicin. Like the previous sample, the nanoparticle and dox solution was then washed with PBS and replaced with medium for testing after one hour. In these images, red indicates the presence of doxorubicin This image shows that there is some, but not much, doxorubicin within the cells themselves. This can be seen in panel D, because of the low presence of red (doxorubicin).

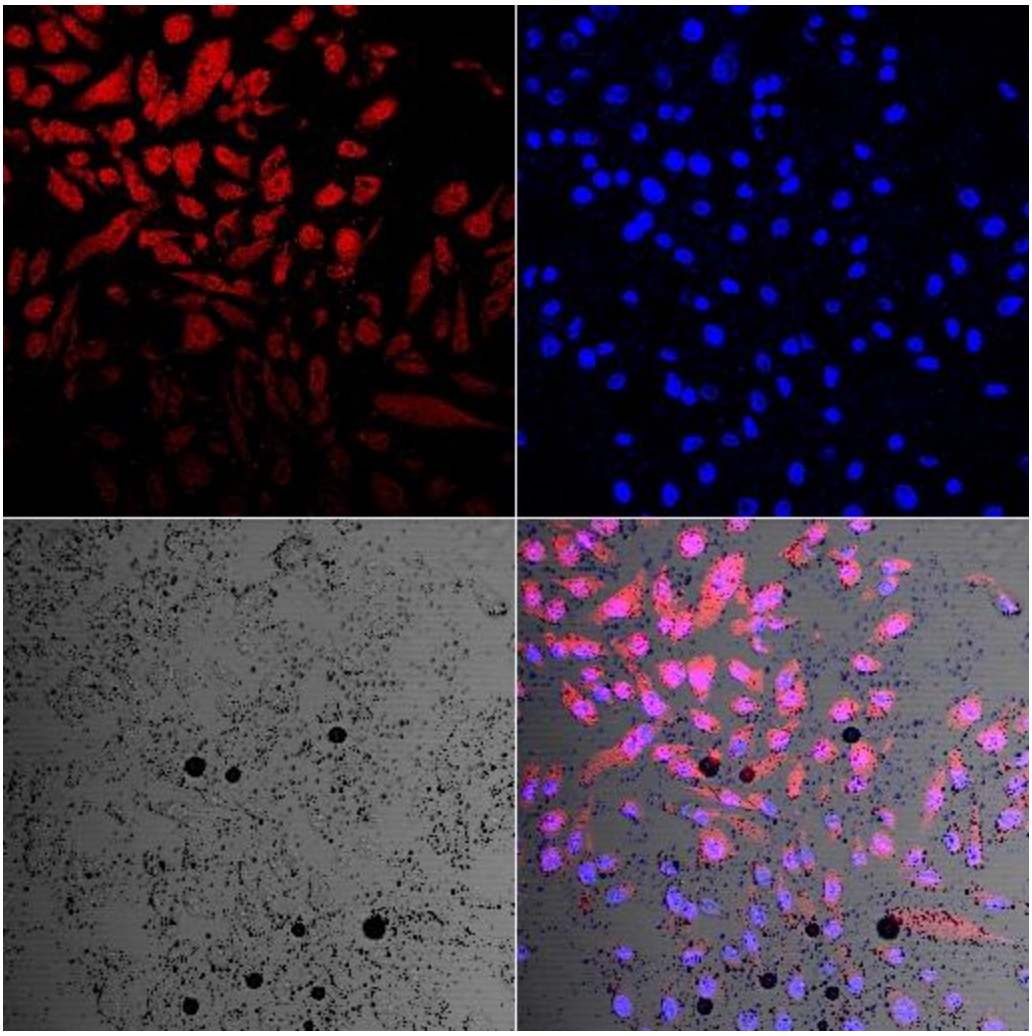


Fig. 5 Confocal Imaging of Nanoparticles conjugated with Dox and stained with Dapi Nuclear staining

These images show the fluorescence of PC-3 after being treated with tripods conjugated with doxorubicin for an hour. The overlap of blue and red panel D cells shows how the doxorubicin conjugated with nanoparticles was more effective at infiltrating the cells than in the previous slide, thus delivering more doxorubicin.

Statistics

Table 1) Cell Viability at Concentration 4mL doxorubicin /mL medium

Solution	Doxorubicin conjugated tripods	Doxorubicin alone	Free doxorubicin and free tripods	Tripods alone
Average percent of living cells	8.890	21.52	18.58	64.74

This table shows the percentage of living cells in the different solutions at the concentration of 4mL doxorubicin/ mL of medium. A statistical analysis was done on the three trials done for each solution to see if the results were significant.

Table 2) Cell Viability Statistics

Source	Degrees of Freedom	Sums of squares	Mean square score	F ratio
Between Groups	3	5536.14	1845	319.5
Within Groups	8	46.21	5.780	
Total	11	5582		

The F value must be greater than 1 in order to show that there is any variance between groups not related to random error. In this study the critical value F, was 319.5, thus showing that these results are statistically significant, and the variance between groups was not due to random error.. The p value was <.01, meaning that these results were extremely significant. The effect size was calculated to be .992, which means that 99 % of the variance between the groups, doxorubicin conjugated tripods, doxorubicin alone, tripods alone, and free tripods with free doxorubicin, can be attributed to the different solutions.

Because the anova statistics showed that the results were in fact significant, a post hoc test was performed to compare each of the groups to each other. Tukeys honestly significant difference was calculated (HSD) and showed that the difference between the percentages had to be greater than 3.62 for the results to be significant. As seen in table one, all the percentages except doxorubicin alone and doxorubicin with tripods free in solution varied by more than 3.62, which was expected. The main comparison is between doxorubicin conjugated tripods and doxorubicin alone, and the difference between these two groups was significantly larger than 3.62.

Discussion

The results of the first trial show that gold tripod nanoparticles, when conjugated with doxorubicin, destroy prostate cancer cells *in vitro* with an efficiency of 87% (Figure 2), compared to doxorubicin alone. The cell viability tests showed the relative effectiveness of the different solutions of nanoparticles for killing prostate cancer (Figure 1). These results were similar to those of the study by Wilson H. Roa et. al. (2010), Roa found the doxorubicin conjugated with spherical nanoparticles were the most effective at reducing tumor size, compared to doxorubicin alone and nanoparticles alone.

The second trial of cell viability tests produced unexpected results. It was predicted that the doxorubicin conjugated with the nanoparticles would be significantly more effective at killing prostate cancer cells, and this was verified as shown in Figure 1. However as shown in figure two, the results of the second trial did not show much difference between the tripods conjugated with doxorubicin, free doxorubicin and nanoparticles, and free doxorubicin alone. This could have caused by a contamination of the solution which could have caused an increased cell death, a not uncommon occurrence in such studies, or a malfunction in the flourometer. A repeat of this cell viability test should be done to see if similar results are obtained. Perhaps cell viability tests should be repeated using a different method to verify the results. In vivo studies would also clarify this result.

Cell viability tests should also be done over a longer time interval to see if a longer time in solution increases the effectiveness of the doxorubicin-conjugated nanoparticles for prostate cancer cell killing. It is reasonable to suggest that gold tripods conjugated with appropriate cancer treating drugs should destroy other types of cancer, such as lung and breast forms. In addition to that, cell viability should be tested using a different method.

The fluorescent staining, Figures 3 through 5, showed that the gold tripods were an efficient vehicle for transporting doxorubicin into the cell body, and worked better than using doxorubicin alone. This suggests that the tripod nanoparticles should be explored as drug transporting vehicles to cancer cells *in vivo*, and should be able to do specific cancer cell targeting when conjugated with the appropriate ligand targeter. As shown in the confocal fluorescent imaging studies, gold tripods conjugated with doxorubicin were more effective at delivering doxorubicin into the cancer cells than free doxorubicin. Thus, this study has demonstrated the ability of doxorubicin-conjugated gold tripods to efficiently deliver

doxorubicin into prostate cancer cells *in vitro*, and thus to promote the destruction of the cancer cells.

The results of the statistics show that the impact of the drug solution on the cell viability was significant; meaning that the higher death rate in the doxorubicin conjugated tripod solution was caused by the solution itself, it was not random error.

Conclusion

The purpose of this study was to test the ability of gold tripods conjugated with doxorubicin to kill prostate cancer cells. It was demonstrated that gold tripods were efficient adsorbers of the cancer treating drug doxorubicin , and increased the effectiveness of doxorubicin transport into human prostrate cells over doxorubicin alone. When in a concentration of between 0.015625mL dox/ mL medium and 4.0 mL dox/mL medium, tripod nanoparticles conjugated with doxorubicin were able to efficiently kill around 90 % of the prostate cancer cells. Much research still needs to be done to confirm and expand these results; however, they show much promise for the use of gold tripod nanoparticles for the enhancement of drug delivery to cancerous cells.

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