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Silver Nanoparticles Enhanced Doxorubicin treatment for Improving their Efficacy against Esophageal Cancer Cells

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Abstract

Silver nanoparticles (SNPs) have shown potential in various biomedical applications, including biological imaging, antimicrobial and anticancer therapy, and promotion of wound repair and bone healing. In this study, we demonstrate the feasible biomedical application of SNPs coated with the FDA-approved anti-cancer drug doxorubicin (DOX) for dual-chemotherapy and photo thermal treatment (PTT) on esophagus tumors in vitro. The synthesized doxorubicin/ silver nanoparticles (DOX-SNPs) exhibited high cytotoxicity against the tumor cells and strong light harvesting for temperature increase in vitro. PTT-assisted DOX-SNPs entailed a highly cytotoxic effect on the esophagus cell line for 15 min after laser treatment, indicating the potential of DOX-SNPs as a nano-theranostic material for treating esophagus tumors. Furthermore, the study investigated gene expression levels in the esophagus cell line after laser exposure and found that the Bax gene was overexpressed, while the BCL-2 gene was down-regulated post-laser irradiation compared to the control group, as determined by RT-PCR analysis. According to the findings of this thesis, the mRNA expression level of pro-apoptotic genes became unregulated, while the expression of anti-apoptotic genes was found to be down regulated, while a higher level of caspase-3 mRNA was observed in SNPs/DOX nanocomposites treated OE33 cells with laser compared to control cells.

Keywords: Silver nanoparticles; Doxorubicin; Laser; photo-thermal therapy; Bax; BCL-2 genes; RT-PCR.

1. Introduction

The esophagus is a muscular tube that connects the throat to the stomach. Some of the main diseases that affect the esophagus include, Gastro esophageal Reflux Disease (GERD): GERD occurs when stomach acid flows back into the esophagus, causing symptoms such as heartburn, regurgitation, and difficulty swallowing [1]. Another esophagus disease is Barrett's Esophagus. Barrett's esophagus is a condition where the cells that line the esophagus are replaced by cells that are more similar to those in the intestines. It is often caused by long-term acid reflux and can increase the risk of esophageal cancer [2]. Esophageal cancer occurs when malignant cells form in the tissues of the esophagus. The two main types of esophageal cancer are squamous cell carcinoma and adenocarcinoma. Risk factors include smoking, heavy alcohol consumption, and GERD [3].

Cancer treatment methods vary depending on the type and stage of cancer, as well as other individual factors. There are several treatment methods for cancer, including surgery, radiation therapy, chemotherapy, targeted therapy, immunotherapy, as well as hormone therapy. It's important to note that not all cancer patients will receive all of these treatments, and the specific treatment plan will depend on the individual's diagnosis and other factors. Surgery involves the removal of the tumor and surrounding tissue. It is typically the first treatment option for solid tumors that are localized to one area of the body [4]. Radiation therapy uses high-energy radiation to kill cancer cells or slow their growth. It can be delivered externally or internally and is often used in combination with other treatments. Radiation therapy is commonly used to treat localized cancers, such as breast cancer, lung cancer, and prostate cancer [5].

Chemotherapy uses drugs to kill cancer cells or stop them from dividing. It can be given orally or intravenously and can be used to treat cancers that have spread to other parts of the body [6]. Targeted therapy uses drugs that target specific proteins or genes that are involved in cancer growth and spread. These drugs can be given orally or intravenously and are often used in combination with other treatments. Targeted therapy is commonly used to treat breast cancer, lung cancer, and colon cancer [7, 8].

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Immunotherapy uses drugs that stimulate the immune system to attack cancer cells. These drugs can be given orally or intravenously and are often used in combination with other treatments. Immunotherapy is commonly used to treat melanoma, lung cancer, and kidney cancer [9].

Doxorubicin is a chemotherapy drug used in the treatment of a wide range of cancers, including breast cancer, bladder cancer, leukemia, and lymphoma. It works by interfering with the DNA replication process in cancer cells, ultimately causing their death. Doxorubicin is an anticancer drug that belongs to the class of anthracyclines. Its mechanism of action involves multiple modes of action that work together to cause cancer cell death [10]. DNA intercalation: Doxorubicin intercalates between DNA base pairs and prevents DNA replication and transcription, leading to cell cycle arrest and ultimately cell death [11]. DNA strand breaks: Doxorubicin can cause DNA double-strand breaks and single-strand breaks by interfering with topoisomerase II, an enzyme required for DNA replication and repair [12].

Generation of reactive oxygen species (ROS): Doxorubicin can also generate ROS, which can damage cellular structures such as proteins, lipids, and DNA, leading to cell death[13]. Inhibition of RNA and protein synthesis: Doxorubicin can inhibit RNA and protein synthesis by interfering with RNA polymerase and ribosomes [14]. Overall, doxorubicin's multiple mechanisms of action make it a potent anticancer drug that can kill cancer cells through multiple pathways, but its use is associated with significant side effects due to its toxicity to normal cells.

However, there are several drawbacks associated with the use of Doxorubicin in cancer treatment. One of these drawbacks is Cardiotoxicity, Doxorubicin can cause damage to the heart muscles, leading to heart failure or cardiomyopathy, a condition where the heart muscle becomes enlarged, thick, and rigid [15]. Another drawback is Suppression of immune system, Doxorubicin can suppress the immune system, making the patient more vulnerable to infections [16]. Doxorubicin also can have Risk of secondary cancers, Long-term use of Doxorubicin increases the risk of developing secondary cancers, such as leukemia or lymphoma [17]. It was noticed that Doxorubicin can cause nausea and vomiting, which can be severe and debilitating for patients [18]. Despite its widespread use, Doxorubicin has Limited efficacy in some cancer types, and patients may not respond well to the treatment [19]. Overall, while Doxorubicin can be effective in treating certain types of cancer, its potential side effects and limitations make it necessary to weigh the benefits and risks carefully before using it in cancer treatment.

The aim of this study was to enhance the efficiency of doxorubicin via conjugation with silver nanocomposites for active targeting and Laser treatment for esophageal cancer.

2. Materials and methods

2.1. Materials

Doxorubicin was obtained from Sigma Chemical Co., St. Louis, MO. The following reagents were also used: silver nitrate ($AgNO_3$), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), absolute methanol, trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$), and phosphate-buffered saline (PBS, pH 7.2), all of which were provided by Sigma-Aldrich (Darmstadt, Germany). The RPMI-1640 cell culture medium supplemented with L-glutamine and fetal bovine serum (FBS) was purchased from Gibco® (Thermo Fisher Scientific, UK). Penicillin-streptomycin (1×) and trypsin/EDTA (1×) were obtained from Biowest® (South Africa).

2.2. The cell lines

Two cell lines were used: one is a BHK normal fibroblast cell line and the other is an esophageal cancer (OE33) cell line (ATCC) acquired from the American Type Culture Collection (ATCC). The cells were grown in the United States and kept in the VACSERA-Cell Culture Unit in Cairo, Egypt. Cells were cultivated in a humidified 5% CO_2 incubator at 37^{o}C in RPMI-1640 L-glutamine medium enhanced with 10% FBS and penicillin-streptomycin antibiotic. Sub-culturing was done twice a week on a regular basis with trypsin/EDTA.

2.3. Methods

2.3.1. Preparation of silver nanoparticles

Preparation of $5x10^{-3}$ mol/l of Ag nanoparticle was done by citrate-reduction route [20]. A stock solution of silver nitrate was prepared by dissolving 0.0850g of ($AgNO_3$) (puress, Fluka) in 100 ml of double distilled water. Then, 25 ml of the stock solution was added to 100 ml of double distilled water. The solution was heated until it begins to boil where 5ml of 1 % of sodium citrate was added with vigorous magnetic stirring. Heating was continued until the color of the solution gradually changed to yellow. Then, stirring continued for another 15 minutes after that the solution was removed from the heater.

2.3.2. Anticancer activity method

The two cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics were added (100 units/mL penicillin and 100 µg/mL streptomycin) at 37°C in a 5% CO_2 incubator. The cytotoxic effect of DOX, SNPs, and

DOX-SNPs nanocomposite derivatives against OE33 cells and BHK were estimated by MTT (3-[4, 5-Dimethylthiazol]-2, 5-Diphenyltetrazolium bromide) assay [21]. The data were expressed as the mean percentage of viable cells as compared to the respective control cultures treated with the solvent. The half maximal growth inhibitory concentration (IC50 values) was calculated from the line equation of the dose-dependent curve of each compound.

2.3.3. Molecular studies

The extraction of RNA from the OE33 cell line, which was treated with nanoparticle compounds, was performed using a commercially available RN easy kit, following the manufacturer's protocol. For the analysis of mRNA expression levels in both the control and treated OE33 cells, the real-time PCR method was used. The expression of the β-actin gene was used as a reference gene for normalization. The real-time PCR reaction was conducted in triplicate, using the Power master mix 2x (ABI reagent in the Bio systems Applied, USA), according to the manufacturer's instructions. The amplification conditions were set as follows: an initial denaturation step of 5 min at 95°C, followed by 40 cycles of a two-step cycle at 95°C for 15 s and 64°C for 40 s. The changes in gene expression levels between the control and treated OE33 cells were determined using the 2-ΔΔCT method [22, 23].

2.3.4. Laser irradiation source

The nanoparticles were subjected to continuous-wave (CW) laser irradiation using a low power diode laser (532 nm DPSS laser-LSR-PS-1, Lasever Inc., China) with a 1 cm^2 spot size. The samples were exposed to 80 mW laser power, resulting in an energy density of 75 J/cm^2 for each sample. [24-25].

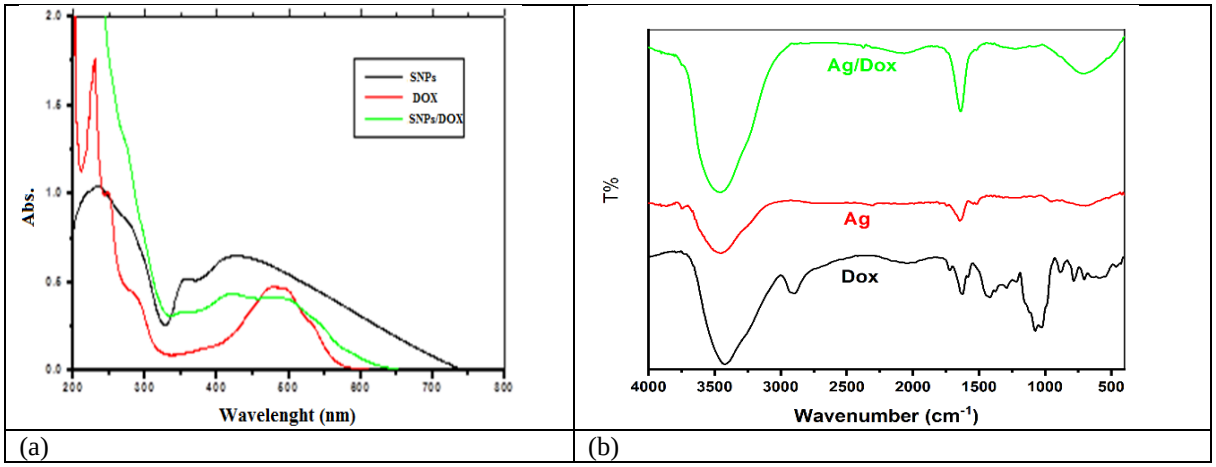
3. Results

3.1. Characterization of silver nanoparticles and SNPs-DOX

Figure 1(a) illustrates the UV-Vis spectra of silver nanoparticles and SNPs-DOX, both exhibiting a peak with the highest absorption at approximately 415 nm, which corresponds to silver nanospheres (SNPs). The absorption peak of SNPs-DOX composites are broader than that of Ag nanoparticles, centered at approximately 430 nm.

Fourier transform infrared (FTIR) spectroscopy was employed to investigate various interactions and functional groups of the compounds formed. The study examined the binding of SNPs, DOX, and SNPs-DOX surfaces using FTIR analysis. Additionally, FTIR absorption spectra can reveal information about the chemical changes in the functional groups involved in the mixture formation. The FT-IR spectra of the fabricated SNPs and SNPs-DOX were shown in figure 1(b). The most intense and broad peak was come in spectra at 3345 cm^{-1} corresponds to OH stretching vibrations of phenol and –COOH. We can notice the shift of the peak to 3365 cm^{-1} in the SNPs-DOX spectra. The peak located at 1635 cm^{-1} in all spectra, indicating C=O stretching and amide binding. At 2060 cm^{-1} peak observed by the alkanes present due to stretching. Moreover, at 1240 cm^{-1} that peak corresponding to C-O-C stretching aromatic ring of the Doxorubicin compound. Furthermore, the peaks at wavenumbers 667 cm^{-1} and 985 cm^{-1} are corresponding to -C≡C-H pending and aromatic C-H. Correspondingly, in FTIR spectra displaying the absence of DOX peaks like the peaks of carboxyl, carbonyl, amide, phenol group which may be used in the loading of the DOX on silver nanoparticles.

Figure 1: (a) UV-Vis absorption spectra of DOX, SNPs and SNPs/DOX, (b) FT-IR spectra of SNPs, DOX, and SNPs-DOX.



(a) (b)

Furthermore, the transmission electron microscopy (TEM) of the silver nanoparticles displays that the size was about 15 ± 1.5 nm. Instead, TEM image of the fabricated SNPs-DOX nanocomposites shows approximately spherical shapes with different sizes ranging from 15 to 35 nm with a spherical shape (Figure 2 a, and b).

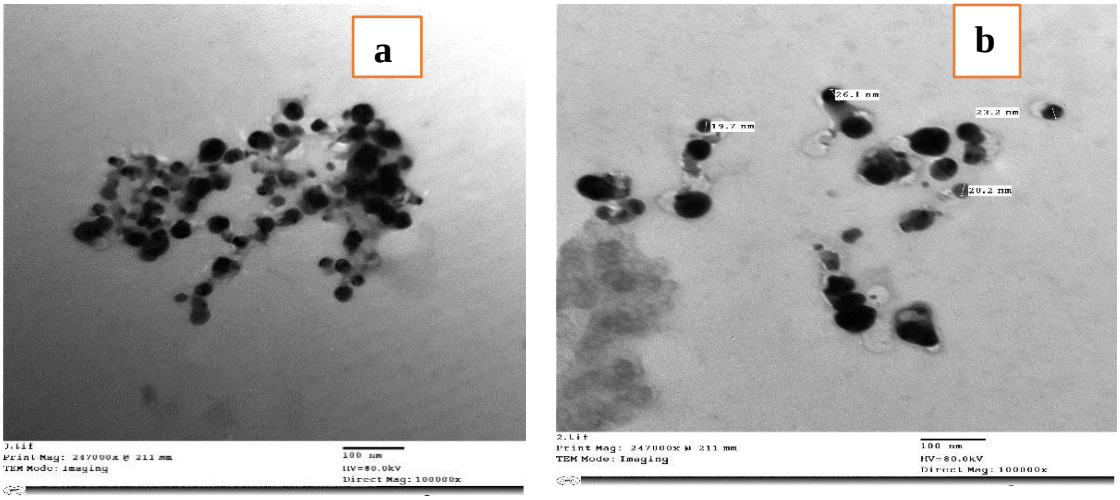


Figure 2: TEM image of (a) SNPs, (b) SNPs/DOX.

X-ray crystallography revealed the crystalline nature of nanoparticles. Figure 3 depicts the XRD pattern of the synthesized *SNPs*. Four significant reflections at 38.45°, 46.35°, 64.75°, and 78.05° correspond to planes of (1 1 1), (2 0 0), (2 2 0), and (3 1 1), which may be categorized according to the facets of silver's face-centered cubic crystal structure. The Debye-Scherrer formula is used to compute the average crystalline size. The *SNPs* have an estimated average crystallite size of 25 nm.

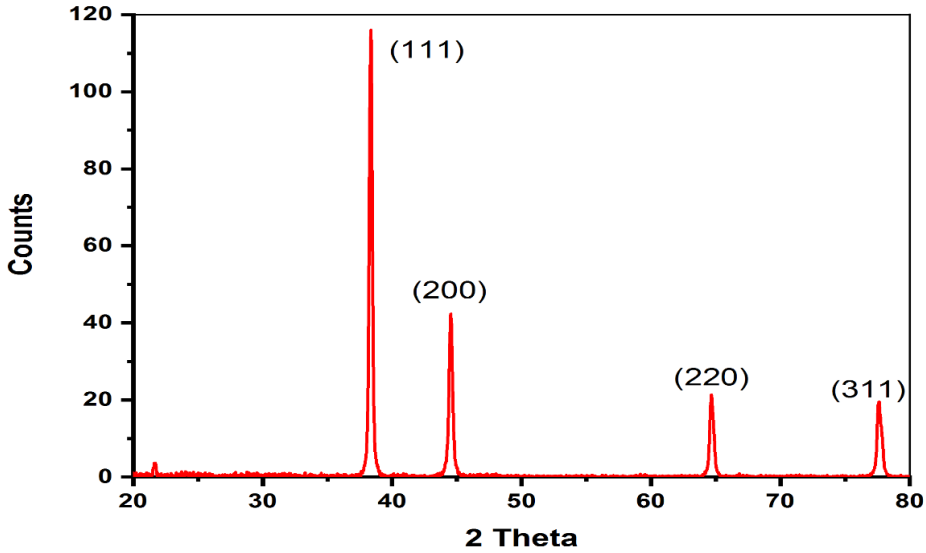


Fig. 3. XRD examination of the prepared SNPs

3.2. Cytotoxicity assay (MTT)

The applications of DOX, SNPs and SNPs/DOX nanocomposites with laser or without laser treated revealed cytotoxicity on OE33 cell line as shown in (Figure 4). The results indicated that in OE33 cells, derivative SNPs revealed no cytotoxicity at low concentrations but had a moderate toxicity at high concentrations against OE33 as shown in (Fig. 4). The derivative SNPs was the best cytotoxic compound with IC50 6.79 ±0.61 µM compared to doxorubicin with IC50 8.0 ± 0.03 µM against OE33 cells.

The derivative SNPs/DOX nanocomposites without laser treatment exhibited a moderate cytotoxicity,

while derivative SNPs/DOX nanocomposites with laser treatment displayed mild effect on normal cell viability against OE33 cells. The derivative SNPs/DOX nanocomposites with laser treatment showed mild cytotoxicity on normal cell, whereas it exhibited a moderate cytotoxicity against OE33 cancer cells. Results revealed that derivatives SNPs/DOX nanocomposites with laser treatment had the most potent cytotoxic effect with IC50 2.399 ±1.39 µM, compared to SNPs/DOX nanocomposites without laser treatment with IC50 2.788±3.3 µM against OE33 cells.

3.3. q RT- PCR results

Relative expression of mRNA of bax was significantly up regulated in OE33 laser treated or untreated cell lines when compared to control cell line. On the contrary, alterations in bcl2 genes level expression in OE33 laser treated or untreated cell lines were minimal in comparison to control cell lines (Figure 5).

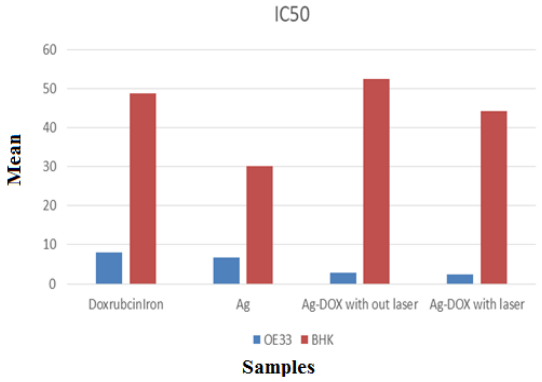


Figure 4: Cytotoxicity of silver nanoparticles and SNPs/DOX nanocomposites against human cell lines

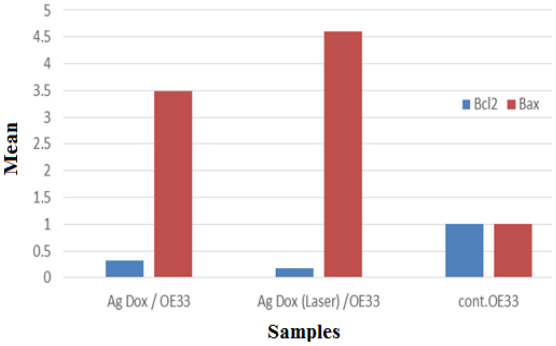


Figure 5: Gene Expression in SNPs/DOX nanocomposites with and without laser treated groups.

3.5. Discussion

In recent years, there has been extensive research on developing and analyzing silver nanoparticles with various sizes and properties for biomedical purposes. The stabilizing molecules play a crucial role in preventing aggregation and controlling chemical reactions, which in turn determine the size and uniformity of the nanoparticles.

The current study pointed to investigate the toxicity of silver nanoparticles and SNPs/DOX nanocomposites on human esophagus (OE33) cells. The findings indicated that exposure to these nanoparticles caused cytotoxicity in the cells. Laser treatment dependent MTT assays revealed that silver nanoparticles and silver/doxorubicin nanocomposites also exerted significant cytotoxicity to OE33 cells. To assess the pathways involved in apoptosis, the mRNA expression levels of three genes, bax, bcl-2, and B-actin as a control, were analyzed in response to exposure to silver nanoparticles and silver/doxorubicin nano composites in OE33 cells.

The UV-vis absorbance spectra of these nanoparticles dispersed in aqueous solutions were demonstrated. It could be found that there was no obvious absorbance for the blank but DOX; SNPs/DOX nanocomposites and SNPs demonstrated maximum absorption peaks at approximately 480; 430 and 415 nm, respectively. These results were in line with the results by Shao et al.[26].

The present investigation validates the potential of FTIR spectroscopy in detecting DOX, SNPs and SNPs/DOX nanocomposites along with their functional groups. The SNPs, DOX, and DOX-SNPs samples were analyzed using FTIR spectroscopy, which revealed two peak absorptions at 3437.26 cm^{-1} and 1619.69 cm^{-1}. The peak at 1619.69 cm^{-1} could be attributed to the aromatic ring carbonyl group stretch vibration of doxycycline. Furthermore, the peak at 3437.26 cm^{-1} corresponds to the OH stretch vibration in alcohol and phenol, which is consistent with the findings reported by Haddada et al.; [27].

The micromorphology of the nanoparticles was determined by transmission electron microscopy (TEM), and the results revealed that all of the obtained nanoparticles exhibited a spherical structure. The silver nanoparticles synthesized in the present study were spherical and range from 15 to 35 nm in size, this result agreement with Lijun Yang et al.; [28].

In the present study, we assessed the cytotoxicity of doxorubicin and doxorubicin conjugated with SNPs in OE33 cell line depression by PTT procedure. The cell viability after exposure to the mentioned SNPs/DOX nanocomposites was

significantly different in the mentioned three OE33 human cell lines. Laser wavelength and duration of exposure time to the laser play an important role in toxicity evaluation as well. This was in agreement with results reported by Behnam et al., [29]
Quantitative real-time PCR was used to analyze the mRNA levels of apoptotic genes bax and bcl-2 in cancer cells exposed of SNPs/DOX nanocomposites without laser treated and with laser treated for 15min. Results showed that SNPs/DOX nanocomposites rubicund significantly altered the expression levels of mRNA of these genes in OE33 cell lines. The mRNA expression level pro-apoptotic gene bax was found to be significantly unregulated while the expression of ant apoptotic gene bcl-2 was found to be significantly down regulated in SNPs/DOX nanocomposites treated OE33 cells with laser compared to control cells. We also observed higher level of caspase-3, mRNA in SNPs/DOX nanocomposites laser treated cells than those of control cells similar findings were reported by Sakuragi et al., [30-31]

Conclusion

Nanotechnology has been used to enhance the photophysical, chemical, and optical characteristics of chemotherapy medications, hence increasing therapeutic efficacy. Silver nanoparticles have distinct physical, chemical, optical, and biological features that distinguish them from other biomedical materials and, as a result, may be used as therapeutic platforms in a variety of biomedical applications. In this study, silver nanoparticles were connected with doxorubicin. Doxorubicin with silver nanoparticles enhanced photo-thermal therapy efficacy and significantly decreased the amount of doxorubicin required for killing cancer cells. Using silver nanoparticles with other chemotherapeutics is expected to improve the efficacy of killing cancer cells in vivo, particularly metastatic tumor cells that have moved to bones and cancer stem cells (metastasis) that repopulate the tumor following therapy.

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