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Original Article

Study of the Efficacy and Multidrug Resistance Using Gold Nanoparticles-Based Drug Delivery Versus Conventional Chemotherapy in Non-Small-Cell Lung Cancer Cell Line

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Abstract

Background: Cisplatin is the main chemotherapy in the treatment of non-small-cell lung cancer (NSCLC) combined with gemcitabine or other agents.

Aim: The aim of this study was to evaluate in vitro the anti-tumor efficacy and multidrug resistance of gold nanoparticles (GNPs) conjugated with cisplatin and /or gemcitabine for the treatment of NSCLC.

Methods: MTT cytotoxicity assay was carried out in NSCLC cell line (A549). Cell viabilities were determined after treatment with different concentrations of cisplatin, gemcitabine, GNPs-cisplatin or GNPs-gemcitabine. Combinations of variable ratios (1:1, 1:2, 2:1, 1:9, 9:1) of cisplatin + gemcitabine, GNPs-cisplatin + gemcitabine, cisplatin + GNPs-gemcitabine or GNPs-cisplatin + GNPs-gemcitabine were also tested. The Combination Index was computed for each combination concentration ratio and dose to know the best synergistic effect. Histological changes and multidrug resistance were studied.

Results: The 1:1 ratio combination concentration showed the highest synergistic effect among all concentration ratios ($p<0.001$). Multidrug resistance revealed that the percent efflux of the GNPs-cisplatin + GNPs-gemcitabine combination with a 1:1 ratio was the minimal in comparison to the other drug combinations.

Conclusion: The use of nanoparticles functionalized with cytotoxic drugs such as cisplatin or gemcitabine alone or in combination enhances their antitumor effect and decreases the multidrug resistance in NSCLC cell line.

Keywords: Cisplatin, Gemcitabine, Multidrug resistance, Nanoparticles, Non-small cell lung cancer.

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Introduction

One of the leading causes of cancer-related deaths worldwide is lung cancer. Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer and accounts for up to 85% of all cases [1]. The selection of the appropriate chemotherapy for NSCLC is still a challenge. However, platinum compounds are still the cornerstone of chemotherapy in NSCLC combined with other agents like gemcitabine [2].

Cisplatin or cis-diamminedichloroplatinum (II) was initially manufactured for its antitumor activity [3]. It is used for the treatment of several types of cancers such as NSCLC, ovarian cancer, testicular cancer, osteosarcomas, germ cell tumors and others [4]. After cellular uptake, cisplatin may react with the N7 atom of purine bases in DNA to form adducts which eventually trigger apoptosis [5]. However, resistance could be developed after chronic use of cisplatin. The deactivation of the drug by interactions with glutathione and metallothioneins, increased DNA repair and/or cisplatin efflux are

possible reported mechanisms for cisplatin resistance [6]. Increasing the dose of cisplatin leads to poor patient compliance and severe systemic undesirable effects including peripheral neuropathy, gastrointestinal toxicity, ototoxicity and nephrotoxicity; which is considered the dose limiting factor [6, 7]. Therefore, highly stable nanoparticles (NPs) loaded with cisplatin that would have longer circulation time, better efficiency, and lower toxicity has been developed [8-11].

The antimetabolite gemcitabine (2', 2'-difluoro 2'-deoxycytidine) is a nucleoside analogue [12]. It is a prodrug which needs to be phosphorylated to gemcitabine monophosphate then di and triphosphate by intracellular nucleoside kinase [12]. The phosphorylated gemcitabine will incorporate into the DNA leading to inhibition of DNA polymerase and termination of DNA synthesis [13]. Gemcitabine is used for various solid tumors. However, resistance to this drug develops shortly after the first chemotherapy regimens [14]. Gemcitabine NPs could provide a significant chance for new medical treatments due to their low toxicity, and their ability to combine with different biochemical molecules [15].

There is a growing interest in the biomedical applications of gold nanoparticles (AuNPs/GNPs) [16-18]. Due to their unique physicochemical properties, GNPs have evolved as a non-toxic carrier for drug delivery [19].

The aim of this study was to evaluate in vitro the anti-tumor efficacy and multidrug resistance of GNPs conjugated with conventional chemotherapeutic drugs cisplatin and /or gemcitabine for the treatment of NSCLC.

Methods

Preparation of gold nanoparticles [20]

Preparation of L- aspartate gold nanoparticles

Trihydrated tetrachloroauric acid ($HAuCl_4.3H_2O$, 99.9%) and L-Aspartate were purchased from Sigma-Aldrich. All glass-wares were cleaned in aqua regia (3 parts HCl, 1 part HNO_3), rinsed with deionized water and then oven dried.

For synthesis of gold nanostructures, HAuCl4 and L-Aspartic acid solutions were prepared in concentrations (C) of $0.5x10^{-3}$M and $1.5x10^{-3}$M, respectively. Fifty mL of aqueous solution of $HAuCl_4$ ($C=0.5x10^{-3}$M) was brought to boiling condition while stirring. Fifteen ml aspartic acid ($C=1.5x10^{-3}$ M) was added to the chloroauric solution. The color of the solution changed within several minutes from yellow to red or purple depending on the size of nanostructures. After resting for 24 hours at room temperature, the solution was twice centrifuged at 15,000 rpm for 30 minutes then washed with double distilled water and finally redispersed in phosphate buffered saline (PBS) to a concentration of 3 nM.

Preparation of GNPs-L-Aspartate conjugate with cisplatin or gemcitabine

The aspartic nanostructures were functionalized with cisplatin (cisplatin [Unistin] 10mg/10ml, Hikma pharmaceutical company) and gemcitabine (gemcitabine hydrochloride [Gemzar] vial 1gm, Lilly pharmaceutical company). In an ice bath and vigorous mixing conditions, over 10 ml gold nanostructures solution were added to 500 µl of the drug (C = 50 µg/ml). The solution was mixed for 1 hour then the resulting compound was centrifuged three times at 15,000 rpm for 1 hour and washed with PBS. After centrifugation, the compound was redispersed in PBS to a final concentration of 5 µg/ml.

Cell culture [21]

The NSCLC cell line "A549" preserved in liquid nitrogen (-180°C) was provided by VACSERA, Giza, Egypt. T25 culture flasks (CELLSTAR®, Greiner Bio-One) were used for cultivation of cells in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 2mM L-glutamine (Lonza, Switzerland), supplemented with a 10% fetal bovine serum (Seralab, UK), and 100 units/ml penicillin / 2 mg/ml streptomycin (Invitrogen Corporation, NY, USA) at 37 °C in 5% CO_2 and 95% humidified air incubator.

Exponentially growing cells were trypsinized (Trypsin-EDTA; Lonza, Switzerland) then resuspended in antibiotic-containing medium (100 units penicillin G and 0.1 mg streptomycin / ml). Cells demonstrating 70-90% confluency were harvested and then counted. Following counting, dilutions were prepared to give the suitable cell densities, 1 x 10^6 cells / ml for inoculation onto 96-well microtiter plates (final cell number/well was 1×10^5 cells in 100 µl culture media) and were incubated for 24 hours before adding the test drugs (gemcitabine, cisplatin, GNPs-cisplatin, GNPs-gemcitabine or GNPs alone) in different concentrations.

Test formulations were sterilized using 0.22 µm sterile syringe filters. The dispersions were serially

diluted in 96-well plate to reach the desired concentrations (500-0.195 µg / ml) and for L-aspartate GNPs (5-0.002 µg / ml).

Solutions of test drugs at the desired dilutions were added in triplicates to the wells containing the cells and incubated for 48 hours before applying the MTT cytotoxicity assay.

MTT Cytotoxicity assay and drug synergism

The cell viability was assessed by the colorimetric 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl- 2H-tetrazolium bromide (MTT) metabolic activity assay (Sigma-Aldrich) which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells producing purple-colored precipitate of formazan after incubation of cells with the different test formulations [22]. The medium was then removed following centrifugation at 1800 rpm for 15 min. Each pellet was resuspended in MTT solution 5mg/10 mL DMEM. After incubation for 3 hours at 37°C in the dark, centrifugation at 1800 rpm was done and the supernatant in each tube discarded. Dimethyl sulfoxide (DMSO) (100 µL) was used to dissolve the precipitated formazan crystals produced from MTT conversion by mitochondrial enzymes in viable cells using a shaker for 10 min. The absorbance of the resulting solution was measured at 570 nm by a Bio-Rad microplate reader. All experiments were performed in triplicate. The IC_{50} value of the test formulations was then calculated.

The relative cell viability was expressed as the mean percentage of viable cells compared to control non-treated cells and the half maximal growth inhibitory concentrations (IC_{50}) of the studied formulations were calculated by the trend line equation.

Viability % = Average absorbance (OD) test / Average Absorbance (OD) control x100

Combinations of cisplatin + gemcitabine, GNPs-cisplatin + gemcitabine, cisplatin + GNPs-gemcitabine and GNPs-cisplatin + GNPs-gemcitabine were tested. The combination index (CI) values were calculated using the CompuSyn 3.0.1 program. Based on the dose–response curves, using the MTT assay for cells treated with inhibitors, alone or in combination at a constant ratio, a series of CI values were generated over a range of levels of growth inhibition (GI) from 5% to 95% of the fraction affected. Synergism, additive effect and antagonism were defined as CI < 1, CI = 1, and CI >1, respectively [23].

Multidrug Resistance (MDR) assay

Non-small-cell lung cancer cell line was seeded overnight in growth medium at 40,000–80,000 cells/well/90 mL in a 96 well plate and allowed to grow for 36 hours, including 3 wells of medium only to serve as blanks and then subjected to multidrug resistance (MDR) assay using MDR assay kit (Cat. number M1580, Marker Gene™ Technologies, Eugene, Oregon, USA) according to the manufacturer's instructions. The mean fluorescence intensity values were analyzed using fluorescence multi-well microtiter plate reader.

The fluorescence intensity was monitored at Ex/Em = 504/538 nm straight away to give a zero-time reading then read every thirty minutes for three hours. The percentage of efflux in each well was then calculated using the following formula:

[(n-time well - n-time blank)/ (0-time well - 0-time blank)] x 100

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY: IBM Corp). One way analysis of variance (ANOVA) and Wilcoxon tests were used. Differences were considered significant when the *p* value was <0.05. The CompuSyn 3.0.1 software (ComboSyn Inc, NJ, USA) was used for CI values calculation.

Results

Characterization of the prepared L- aspartate gold nanoparticles

In this work, size-sorted L-aspartate GNPs were prepared by reduction of trihydrated tetrachloroauric acid ($HAuCl_4.4H2O$) by different concentrations of trisodium citrate in order to get GNPs different sizes of 20nm and 50nm. The color change is slower for larger NPs than for smaller ones. Size-sorted L-aspartate GNPs were prepared by reduction of trihydrated tetrachloroauric acid ($HAuCl_4.4H2O$) by trisodium citrate in order to get GNPs with a particle size of 50nm.

The Transmission Electron Microscopy (TEM) examination revealed a sample spherical in shape and quite uniform in size in the range of 50nm (Figure 1 A). The solution of 50nm average size GNPs was also characterized by UV-Vis spectroscopy (Figure 1 B). The sample showed a sharp and single absorption band. Moreover, the spectrum of 50 nm GNPs was located at around 530 nm [24].

Cell viability

The MTT assay was carried out after treatment with different concentrations of cisplatin, gemcitabine, GNPs-cisplatin and GNPs-gemcitabine. The viability % was graphed as shown in Figure 2. Combination of variable ratio (1:1, 1:2, 2:1, 1:9, 9:1) of cisplatin + gemcitabine or GNPs cisplatin+ gemcitabine or cisplatin + GNPs-gemcitabine or GNPs-cisplatin + GNPs-gemcitabine were also established

Using the ANOVA test, there was a highly significant difference ($p < 0.001$) in the synergistic effect of each combination by changing the combination concentration ratio, where 1:1 concentration ratio showed the highest synergistic effect among all concentration ratios as represented in Table 1. A significant difference ($p < 0.05$) was observed regarding the effect of drug combinations on the cell viability using the Wilcoxon test (Table 2).

Histological study

Histological study revealed that the combination of cisplatin and gemcitabine ± GNPs exhibit a deleterious effect on the growth of the NSCLC cell line with a change in the shape of cells, increase in cytoplasmic vacuolization, smudge nuclei and karyorrhectic cell debris. Different effects after treatment for 48 hours are presented in Figure 3.

Multidrug Resistance

The 1:1 combination of GNPs-cisplatin and GNPs-gemcitabine had the least percent efflux in comparison to other drug combinations as shown in Figure 4.

Discussion

Cancer is a universal leading cause of death and poses enormous financial costs. Nanotechnology offers novel processes for therapy in different types of cancers [25]. The current study highlighted the efficacy of GNPs in lung cancer. It showed that combinations of GNPs-gemcitabine + GNPs-cisplatin, or GNPs-gemcitabine + cisplatin or GNPs-cisplatin + gemcitabine in a concentration ratio of 1:1 are more potent and efficacious than non-GNPs chemotherapies. The maximum growth inhibition of the NSCLC cell line was reported with these combinations indicating that it has a significant antiproliferative effect. van Moorsel et al found that there is a synergistic effect between gemcitabine and cisplatin that may be due to an increase in the formation of platinum–DNA adducts in ovarian and NSCLC cell lines [26]. Clinically, the usefulness of gemcitabine and cisplatin combination was demonstrated in the postoperative treatment of stage II and III NSCLC in terms of safety and efficacy [27].

Kai et al. reported that the use of cisplatin NPs increases tumor concentration of the drug, prolongs its circulation in the blood and protects the kidney from tubular necrosis [10]. Also, Comenge et al. proved that the use of nanotechnology modifies the pharmacokinetic properties of cisplatin leading to better direction of the drug to the tumor cell mass in mice models and less cisplatin-induced nephrotoxicity [28]. Therefore, platinum NPs were used for the treatment of lung cancer patients and other malignancies such as malignant pleural mesothelioma with good to modest response [29].

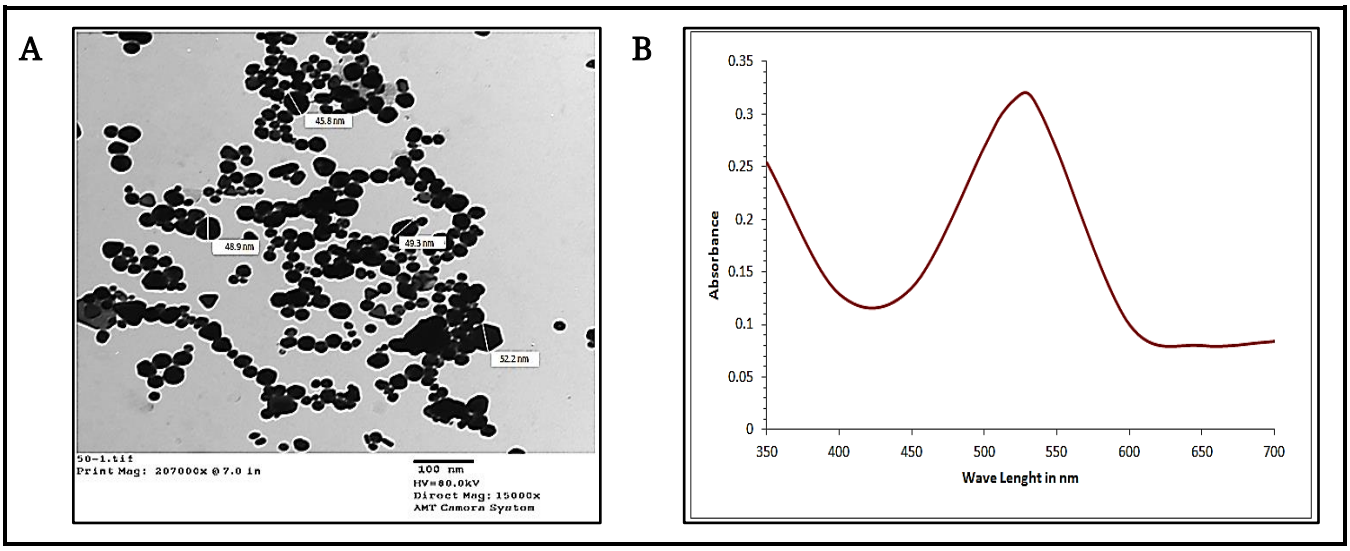

Figure 1: L-aspartate gold nanoparticles with an average size of 50nm. A) Transmission Electron Microscopy (TEM) image, **B)** UV-Vis spectrum of L-aspartate gold nanoparticles solution

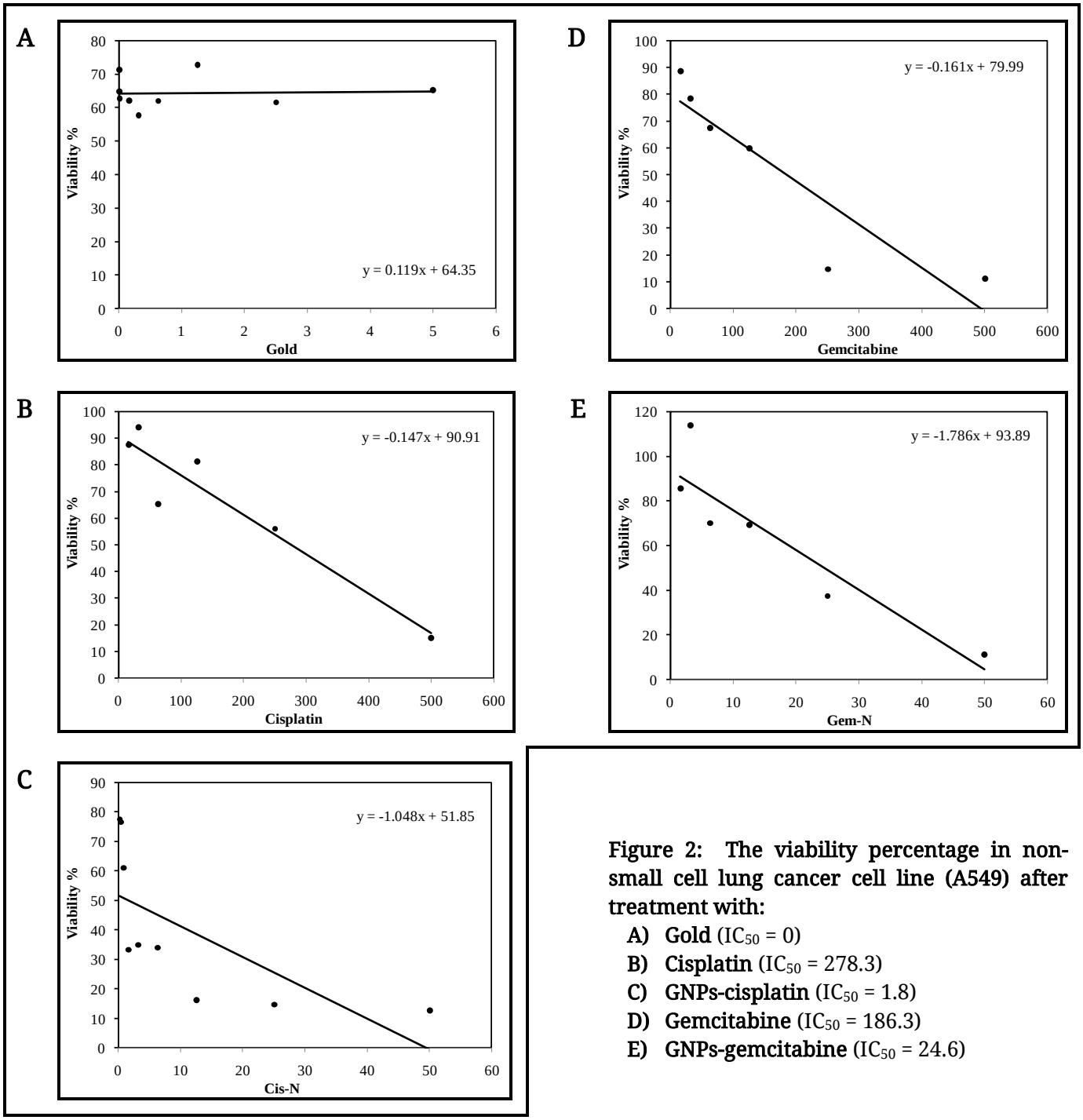

Figure 2: The viability percentage in non-small cell lung cancer cell line (A549) after treatment with:

- **A) Gold** (IC_{50} = 0)
- **B) Cisplatin** (IC_{50} = 278.3)
- **C) GNPs-cisplatin** (IC_{50} = 1.8)
- **D) Gemcitabine** (IC_{50} = 186.3)
- **E) GNPs-gemcitabine** (IC_{50} = 24.6)

Table 1: Combination Index of different drug combinations in correlation to concentration ratio showing strength of synergistic effect

Concentration Ratio	Combinations	Synergistic Effect	*p* value
1:1	Cisplatin + GNPs-gemcitabine	0.018	0.001
1:1	Gemcitabine + GNPs-cisplatin	0.02	0.001
1:1	GNPs-gemcitabine + GNPs-cisplatin	0.03	0.001
9:1	Cisplatin + GNPs-gemcitabine	0.039	0.002
2:1	Cisplatin + GNPs-gemcitabine	0.04	0.002
2:1	Gemcitabine + GNPs-cisplatin	0.15	0.002

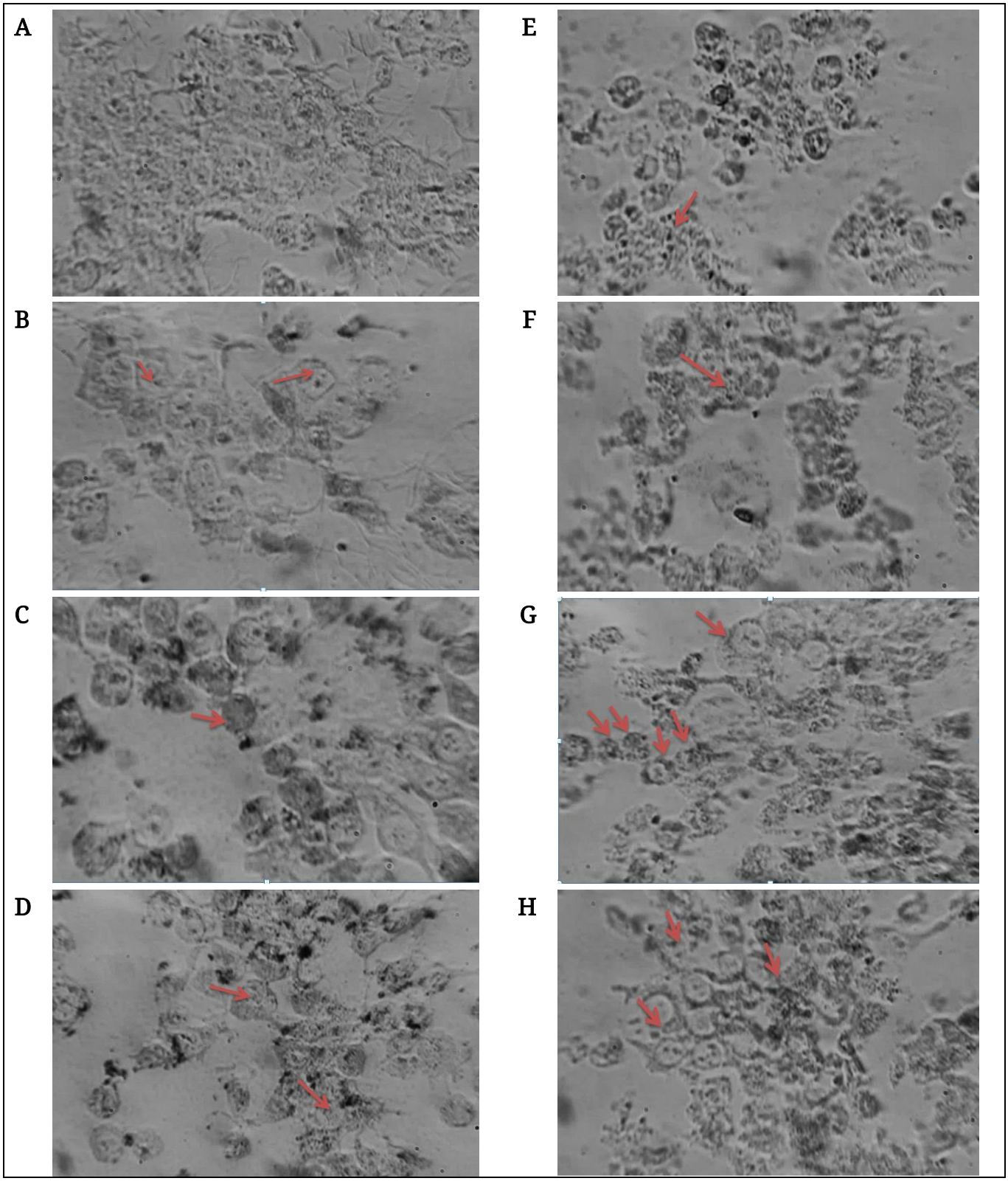

Figure 3: Effect of different drug combinations on A549 cell line (20X). A) Gemcitabine [adherent monolayered sheets of cells, 50% confluency], **B)** Cisplatin [some round cells with smudged nuclei], **C)** GNPs-gemcitabine [few adherent round cells], **D)** GNPs-cisplatin [very few attached cells with increased cytoplasmic vacuolization and smudged nuclear chromatin, confluence 30%], **E)** Cisplatin + gemcitabine (1:1) [Floating detached dying cells with rounding up and few karryorhectic cell debris], **F)** GNPs-gemcitabine + cisplatin (1:1) [few cells show karyolysis with extensive cytoplasmic vacuolization], **G)** GNPs-cisplatin + gemcitabine (1:1) [detached floating cells with pyknotic nucleus, and cytoplasmic vacuolization], **H)** GNPs-gemcitabine + GNPs-cisplatin (1:1) [increased cytoplasmic vacuolization and pyknotic nucleus]

Table 2: Cell viability of drugs combinations in correlation to concentration ratio

Concentration Ratio	Drugs Combinations	% Cell Viability	*p* value
1:1	GNPs-cisplatin + GNPs-gemcitabine	13.59	0.004
1:1	Gemcitabine + GNPs-cisplatin	14.13	0.006
1:1	Cisplatin + GNPs-gemcitabine	20.83	0.007
9:1	Cisplatin + GNPs-gemcitabine	27.9	0.010
2:1	Gemcitabine + GNPs-cisplatin	32.97	0.012
2:1	Cisplatin + GNPs-gemcitabine	35.87	0.012

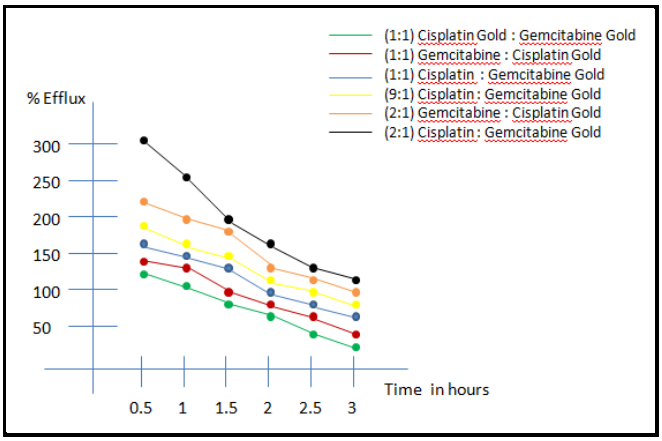

Figure 4: Percent Efflux of drug combinations in correlation to time intervals

In line with the results of the current study, previous work showed that the use of gemcitabine NPs enhances the antitumor activity in mice models with increased blood circulation time and augmentation of the drug concentration in the tumor cells in different cell cultures [30]. In this study, it is was demonstrated that using GNPs-gemcitabine with cisplatin or with GNPs-cisplatin increases the synergistic effect of both chemotherapeutic drugs in NSCLC cell lines. Zhang et al findings coincides with ours [31]. They reported that NPs co-loading gemcitabine and platinum could significantly increase in vitro the cytotoxicity against human NSCLC cells (NCI- H460) and enhance in vivo the antitumor efficacy in mice bearing NCI- H460 cells xenografts. The efficacy of this combination had been studied in other tumor types. In one study, the gemcitabine–cisplatin combination in NPs increased the synergistic interaction by overcoming the transporter requirement for the delivery of gemcitabine intra-cellularly in ovarian cancer subtypes [32]. In another study, the combination of gemcitabine and cisplatin NPs improved the anti-tumor effect of both in stroma-rich xenograft bladder cancer mouse model [33].

The use of nanoparticles cytotoxic drugs alters the chemo-resistance of cancer cells that is characterized by overexpression of p-glycoprotein which performs an energy-dependent drug efflux pump that reduces the accumulation of chemotherapeutic drugs intracellularly and it is the major cause of refractory cancerous disease and disseminated metastasis. It was demonstrated in this research that the use of combinations of gemcitabine and cisplatin either both as GNPs or one of them only and the other in the conventional form, decreases the chemo-resistance of NSCLC cell line. Tomuleasa et al. agreed with our results as they found that the use of conjugated nanoparticles with cytotoxic drugs had a great potential in diminishing the resistance in hepatocellular carcinoma [20]. Also, Barabadi et al. found that biosynthesized GNPs had a significant anticancer activity against hepatic cancer cells in-vitro models as well as prostate cancer cell lines [25, 34].

It was found that the use of cisplatin nanoparticles overcomes the overexpression of cisplatin efflux in NSCLC cell line [28]. and the use of gemcitabine-cisplatin combination in nanoparticles increased the chemosensitivity of ovarian cancer cells [32]. Barabadi et al. reported the importance of using nanoparticles against colorectal and cervical cancers [25, 35]. Yao et al. proved that the utilization of nano-carriers for the delivery of cancer chemotherapy reduces their systemic toxicity and leads to overcoming drug resistance [36]. Also, Shi et al. discussed the importance of using nanoparticles to overcome the multidrug resistance to prevent lung metastasis in breast cancer patients [37].

Although several studies have emphasized the significant biomedical potential of biogenic metallic nanoparticles such as GNPs, it is of great importance to take into consideration the hazards associated with the use of such technology [38].

Conclusions

Nanotechnology employs novel approaches to develop nanoparticulate platforms to conquer different types of cancer. The current study demonstrated that the use of functionalized NPs with the cytotoxic drugs gemcitabine and cisplatin, alone or in combination, enhance their antitumor effect and increase the chemosensitivity of NSCLC cell line.

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Authors' contribution
Conception or design: AAG; Acquisition, analysis or interpretation of data: ED, TIS, SKA; Drafting or revising the manuscript: AAG, RN; Approval of the manuscript version to be published: All authors; Agreement to be accountable for all aspects of the work: All authors.

Conflict of interest
The authors declare that they have no conflict of interest to disclose.

Data availability
Data used to produce the results of this study are available from the corresponding author (AAG) upon request.

Ethical considerations
The study was approved by the Research Center of the Faculty of Medicine – Alexandria University.

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Study registration
None.

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