RESEARCH ARTICLE

Comparison of Inhibitory Effect of 17-DMAG Nanoparticles and Free 17-DMAG in HSP90 Gene Expression in Lung Cancer

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Abstract

Background: Up-regulation of hsp90 gene expression occurs in numerous cancers such as lung cancer. D,L-lactic-co-glycolic acid-poly ethylene glycol-17-dimethylaminoethylamino-17-demethoxy geldanamycin (PLGA-PEG-17DMAG) complexes and free 17-DMAG may inhibit the expression. The purpose of this study was to examine whether nanocapsulating 17DMAG improves the anti cancer effect over free 17DMAG in the A549 lung cancer cell line. Materials and Methods: Cells were grown in RPMI 1640 supplemented with 10% FBS. Capsulation of 17DMAG is conducted through double emulsion, then the amount of loaded drug was calculated. Other properties of this copolymer were characterized by Fourier transform infrared spectroscopy and H nuclear magnetic resonance spectroscopy. Assessment of drug cytotoxicity on the grown of lung cancer cell line was carried out through MTT assay. After treatment, RNA was extracted and cDNA was synthesized. In order to assess the amount of hsp90 gene expression, real-time PCR was performed. Results: In regard to the amount of the drug load, IC50 was significant decreased in nanocapsulated(NC) 17DMAG in comparison with free 17DMAG. This was confirmed through decrease of HSP90 gene expression by real-time PCR. Conclusions: The results demonstrated that PLGA-PEG-17DMAG complexes can be more effective than free 17DMAG in down-regulating of hsp90 expression by enhancing uptake by cells. Therefore, PLGA-PEG could be a superior carrier for this kind of hydrophobic agent.

Keywords: Lung cancer - HSP90 - 17DMAG-PLGA-PEG - real-time PCR

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Introduction

Cancer is a primary cause of death in the world, and lung cancer is the second-leading cause of cancer death in women and men (Parkin et al., 2005; Jemal et al., 2008; Benzo et al., 2011; Crandall et al., 2014). A enormous number of lung cancers are associated with cigarette smoke, however other factors such as environmental influences may be also observed (Jemal et al., 2008).

Chemotherapy and radiation therapy are used to reduce tumor mass and stop disease progression. However, such therapies are usually ineffective for lung cancer. Therefore, development of effective prevention and therapy systems against lung cancer is essential for reducing mortality rate (Sharp and Workman, 2006; Tsuda, 2010).

Lung cancer is often divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), (includes squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC)) (Hussain et al., 2001).

Oncogene activation or loss of tumor suppressor gene function take places in probably all lung cancers (Hussain et al., 2001; Breuer et al., 2005).

Heat shock proteins (HSPs) are chaperones which have considerable expression in tissues that are exposed to proteotoxic stressors (such as elevated temperatures, heavy metals, hypoxia and acidosis) (Whitesell and Lindquist, 2005; Makhnevych and Houry, 2012; Mestril et al., 2014).

HSP90 is a 90 kDa protein which is one of the most plentiful chaperones and is widely distributed in prokaryotes and eukaryotes (Richardson et al., 2011; Sakthivel et al., 2012; Dobo et al., 2013; Wu et al., 2014).

There is increased expression of HSP90 in cancer cells in comparison to normal tissues, (as much as 4-6% of the total protein in cancer cells in comparison to 1-2% in normal cells) (Bagatell and Whitesell, 2004; Chiosis and Neckers, 2006; Pick et al., 2007; Shirinbayan and Roshan, 2011), HSP90 leading to increased degradation of client proteins by the proteasome pathway (Fukuyo et al., 2010).

PLGA-PEG (poly (DL-lactic-co-glycolic acid)-

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polyethyleneglycol)-17DMAG is a kind of nanoparticle that may be to inhibit the expression of HSP90 gene in lung cancer cell line (Pearl and Toft, 2008; Banerji, 2009; Qu et al., 2013; Sun et al., 2013). In this study, we investigated whether nanocapsulating 17DMAG improve the anti cancer effect of free 17DMAG in the A549 lung cancer cell line.

Materials and Methods

Cell culture and cell line

Fetal Bovine Serum (FBS), RPMI 1640, Tripsin-EDTA Antibiotics, and TRIzol reagent were purchased from Invitrogen (Germany). Syber Green Real Time PCR Master Mix kit was purchased from Roche (Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PLGA-PEG and 17DMAG were purchased from Sigma (USA). A549 lung cancer cell line, prepared from Pasteur Institute cell bank of Iran, code: C203.

A549 lung cancer cells were cultured in RPMI1640 complemented with 10% heat-inactivated fetal bovine serum (FBS), 0.05mg/ml penicillin G, 0.08mg/ ml streptomycin (Merck co, Germany), 2mg/ml sodium bicarbonate and Cells were grown at 37°C in an incubator with 55% humidity and 5%CO_2.

Materials and experiment for preparation of 17DMAG-loaded PLGA-PEG

D, L-lactide and glycolide were purchased from Sigma and recrystallized with ethyl acetate. Stannous octoate (Sn (Oct) 2: stannous 2-ethylhexanoate), polyethylene glycol (PEG) (molecular weight, 4000) and DMSO were purchased from Sigma (St Louis, USA). PEGs were dehydrated under vacuum at 70°C for 12h and were used without further purification. The drug loading capacity and release behavior were determined using a UV-Vis 2550 spectrometer (Shimadzu).

IR spectra were recorded at RT with fourier transform infrared spectroscopy (FTIR) perkin elmer series.

The 1H NMR spectra was recorded at RT with a Brucker DRX 300 spectrometer operating at 300.13 MHz. The samples were homogenized using a homogenizer (Heidolph Instruments GmbHand Co. KG, SilentCrusher M). The organic phase was evaporated by rotary (Rotary Evaporators, Heidolph Instruments, Hei-VAP Series).

Preparation of PLGA-PEG tri-block co-polymer

Poly(lactide-co-glycolide) poly(polyethylene glycol), PLGA-PEG co-polymers with molecular weight of polyethylene glycol (PEG4000) as an initiator was prepared by a melt polymerization process under vacuum using stannous octoate (Sn(Oct)2: stannous 2-ethylhexanoate) as catalyst.13 D, L-lactide (14.4g), glycolide (3.86g) and PEG4000 8g (45% w/w) in a bottle-neck flask were heated to 140˚C under nitrogen atmosphere for complete melting. The molar ratio of D, L-lactide and glycolide was 3:1. Then, 0.05% (w/w) stannous octoate was added and the temperature of the reaction mixture was raised to 180˚C. The temperature was maintained for 4h. The polymerization was performed under vacuum. The co-polymer was regained by dissolution in methylene chloride followed by precipitation in ice-cold diethyl ether. A tri-block co-polymer of PLGA-PEG was prepared by ring opening polymerization of D, L-lactide and glycolide in the presence of PEG4000.

Preparation of 17DMAG-loaded PLGA-PEG

Polymer was mixed through the double emulsion method and then the mixture encapsulated the drug physically. To prepare 17DMAG-loaded PLGA-PEG, it was physically conjugated to the PLGA-PEG in submission with Dilnawaz et al. (2010) protocol. For conjugation, 240 mg of nanoparticle was dissolved in 20 ml chloroform. In the next step, 20 mg of 17DMAG was added to mixture, and then these two solutions were homogenized by homogenizer. 20 ml 1% PVA (poly vinyl alcohol) was added as a stabilizer and was mixed for 8 min. It was then centrifuged for 30 min at 11000 rpm to become sediment. After that the mixture was strained, put in room temperature for 5 hours to dry, and crushed into powder by mortar. Successful loading of 17DMAG was confirmed by FTIR measurement (Shimadzu FTIR 8400S).

Cytotoxicity assay and cell treatment

Cytotoxicity of 17DMAG-loaded PLGA-PEG was measured at 24, 48 and 72h using the MTT (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide) (MTT; Sigma-Aldrich) assay. First, 2×10^4 cells per well were seeded and kept for 24h in the incubator to promote cell attachment. Then, cells were treated with different concentrations of free 17DMAG and 17DMAG-loaded PLGA-PEG (20-120 μM) in replicates of four. A549 cells were exposed to free 17DMAG and the 17DMAG-loaded PLGA-PEG in the logarithmic phase of growth. Three controls were used; the first was 1% DMSO; the second was PLGA-PEG control for estimation of nanoparticle effect; and the third one was cells alone. After 24, 48 and 72h exposure times, the cell culture medium was replaced with 200μl fresh medium for 24h, after that the cells were incubated with MTT solution for 4h. Then, contents of all wells were removed and 200μl of pure DMSO were added to the wells followed by adding 25μl Sorensen's glycine buffer to each well. The absorbance was record at 570 nm ELISA-reader and IC_{50} was calculated at most within 1h. A concentration of 70Mm of free 17DMAG and 3 concentrations of 20, 40 and 60Mm of 17DMAG-loaded PLGA-PEG were applied. Control and treated cells were incubated at 37˚C under 5% CO_2 for 24h.

Quantitative real-time PCR assay

After 24h, cells were washed with PBS and their total RNA was extracted from each sample using TRIZOL reagent (Invitrogen, USA). Complementary DNA (cDNA) was reverse-transcribed using the First Strand cDNA Synthesis Kit (Fermentase). To synthesize cDNA, the reaction of mixture was prepared on ice as for each reaction, 2μl of 5 X PrimeScript Buffer, 0.5μl of PrimeScript RT Enzyme Mix1, 0.5μl of Oligo dT Primer and 0.5μl of Random 6 mers accompanied by 500 ng of total RNA were used that reached to 10μl by adding

RNase Free Dh20. The reaction mixture was incubated under the following conditions: 37˚C, 15 minutes (Reverse Transcription); 85˚C, 5 sec (inactivation of reverse transcriptase with heat treatment); 4˚C.

Levels of HSP90 expression were determined by real-time PCR (RT-PCR). For real-time PCR, hsp90 primers (Genbank accession: NM_005348, bp 60-79) and beta actin primers (Genbank accession: NM-001101, bp 787-917) were used. These primers were blasted by primer- blast site on NCBI website. The forward (F) and reverse (R) primer sequences of hsp90 and β-actin used in real-time PCR were shown in Table 1. For hsp90, a 162bp amplicon and for beta actin a 131bp amplicon were generated in a 25µl reaction mixture that contained: 5pmole of the forward and reverse PCR primers of beta actin or for hsp90, 2X PCR Master Mix Syber Green I and 2µl of the cDNA. The Beta-Actin mRNA was calculated as the internal standard control gene by specific primers.

Each RNA sample was divided into equal amounts and then, HSP90 and beta-actin in parallel with each other were amplified by real-time PCR in triplicate. Dh2O water per reaction. Negative controls were prepared each time with 2µl DdH2O instead of the cDNA template. Real time PCR amplification was performed using a Corbett (Rortor Gene 6000) system with the following setting as manufacture protocol. The reaction mixture was incubated under the following conditions: 95˚C, 2 minutes, 1 cycle (Holding step); 65˚C, 20 seconds, 45 cycles (Annealing); 72˚C, 20 seconds, 45 cycles (Extension); 75-99 ˚C, 1 cycle (Melting).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.01 software. Results were expressed as the mean±standard deviation (SD). Statistical differences were assessed by unpaired student t-test; and a value of P less than 0.05 was considered significant.

Results

Determination of 17DMAG loading

Standard curve of 17DMAG concentration in DMSO was prepared via UV-Vis spectrophotometer at 450nm. One mg of PLGA-PEG-17DMAG complex contained 570.69 µg17DMAG.

1H NMR spectrum of PLGA- PEG co-polymer

The basic chemical structure of PEG-PLGA co-polymer was confirmed by 1H NMR spectra that were recorded at RT with a Brucker DRX 300 spectrometer operating at 400 MHz. Chemical shift (δ) was measured in ppm using tetramethylsilane (TMS) as an internal reference (Figure 1). One of the noticeable features was a large peak at 3.65 ppm, corresponding to the methylene groups of the PEG. Overlapping doublets at 1.55 ppm were attributed to the methyl groups of the D-lactic acid and L-lactic acid repeat units. The multiples at 5.2 and 4.8 ppm corresponded to the lactic acid CH and the glycolic acid CH, respectively, with the high complexity of the 2 peaks resulting from different D-lactic, L-lactic and glycolic acid sequences in the polymer backbone.

Table 1. Forward (F) and Reverse (R) Primer Sequences of β-actin and Hsp90 α Used in Real-Time PCR

Oligonucleotide	Location	Sequence	PCR product size
Hsp90 α			
Forward primer	60	5'AGGCTTCTGGAAAAAGCGCC3'	
Reverse primer	221	5'GTTGGTCTTGGGTCTGGGTT3'	162bp
Beta-actin			
Forward primer	787	5'TCCCTGGAGAAGAGCTACG3'	
Reverse primer	917	5'GTAGTTTCGTGGATGCCACA3	131bp

FTIR spectroscopy

The FTIR spectrum is consistent with the structure of assumed copolymer. FTIR spectroscopy was used to show the structure of PLGA-PEG copolymer nanoparticle. From the infrared spectra shown in Figure 2. The absorption band at 3509.9 cm^{-1} is assigned to terminal hydroxyl groups in the copolymer which PEG homopolymer has been removed from. The bands at 3010 cm^{-1} and 2955 cm^{-1} are due to C-H stretch of CH, and 2885 cm^{-1} due to C-H stretch of CH. A strong band at 1630 cm^{-1} is assigned to C=O stretch. Absorption at 1186-1089.6 cm^{-1} is due to C-O stretch. FTIR spectroscopy was done by Shimadzu spectrophotometer .

Effects on cell viability

Cell viability was estimated by MTT assay through exposing A549 cell line to different concentrations of free 17DMAG and 17DMAG-loaded PLGA-PEG during 24, 48, 72h. The results in all cases show that the toxicity effect was dose-dependent and time-dependent. This is

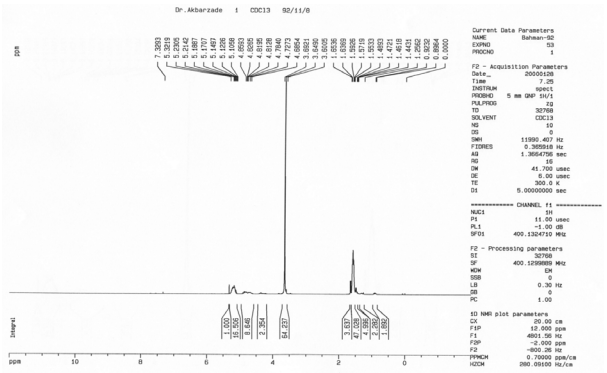

Figure 1. 1H NMR Spectrum of the PLGA- PEG Co-Polymer

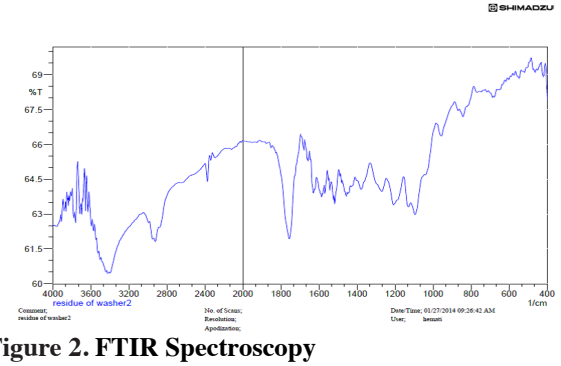

Figure 2. FTIR Spectroscopy

completely opposite for the viability factor.

The free 17DMAG had cytotoxic effect on A549 cell line with inhibitory concentration at 50% (IC50), 99.37nM for 24h, 72.70nM for 48h and 56.03nM for 72h and 17DMAG loaded on PLGA-PEG had cytotoxic effect of 70.02nM for 24h, 49.22nM for 48h and 32.25nM for 72h respectively. Our Data analysis of cytotoxicity assay showed that IC_{50} of PLGA-PEG-17DMAG complex on A549 lung cancer cell line was time and dose-dependent (Figure 3).

Effect on gene expression

The levels of HSP90 gene expression were measured by Real-Time PCR. Changes in HSP90 expression levels between the Control and treated A549 cells were normalized to beta-actin mRNA levels and then calculated by the $2^{-\Delta\Delta ct}$ method. As amount of nanodrug increased, levels of Hsp90 gene expression decreased accordingly. Real-time PCR data analysis indicated that by increasing amount of 17DMAG-loaded PLGA-PEG, HSP90 mRNA level expression would be decreased .Each experiment was repeated three times. Q-RT PCR results showed a considerable decrease in hsp90 gene expression in the treated cells in comparison with the control cells. Compared to 17DMAG, in the same concentration, PLGA-PEG-17DMAG resulted in a lower level and expression of Hsp90 mRNA. When we treated A549 cells with 70.02 and 49.22nM concentrations of PLGA-PEG-17DMAG complex for 24 and 48 hours, expression of hsp90 was significantly reduced (Figure 4).

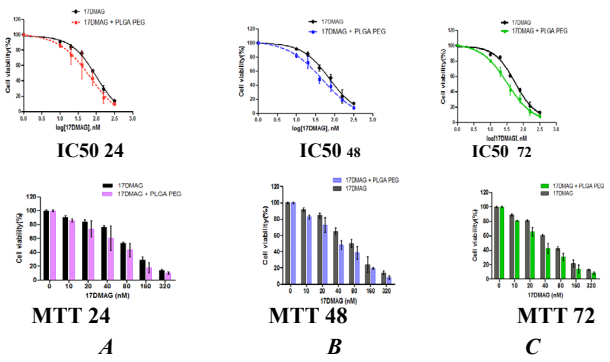

Figure 3. Cytotoxicity Effect of PLGA-PEG-17DMAGComplex and Free 17DMAG on A549 for A) 24h ; B) 48h ; C) 72h Exposure

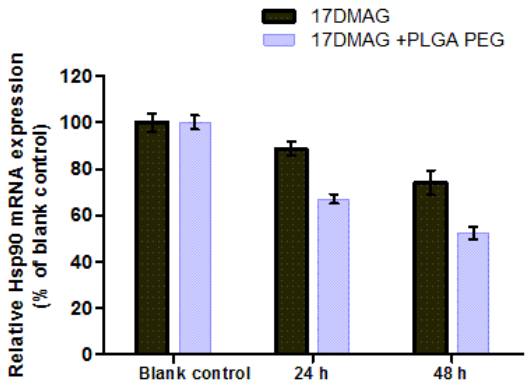

Figure 4. Level of HSP90 mRNA Expression in Cells Treated with PLGA-PEG-17DMAG or Free 17DMAG

Discussion

While Chemotherapy has toxic side effects in healthy tissues in treatment of human cancers, Nanotechnology attempts to resolved these problems by encapsulating or loading drugs to nonmaterial which are resistant to drug efflux (Tsuda, 2010; Ghasemali et al., 2013).

In these studies, we assayed anti-proliferation effects of 17DMAG-loaded PLGA-PEG and 17DMAG free on lung cancer cell line A549. MTT assay showed that 17DMAG-loaded PLGA-PEG has more cell death effect than free 17DMAG on lung cancer cell line A549 in same condition.

Studies have showed that encapsulating drugs to PLGA-PEG reduces dosage of drug and causes low adverse side effects of the drug (Mirakabad et al., 2013). 17DMAG is a hydrophilic gaeldamaycin derivative that can good bioavailability and better activity *in vitro* and *in vivo* and has significant anticancer activity (Sharp and Workman, 2006; Qu et al., 2013; Sun et al., 2013).

As shown in pervious study , treatment with 17-AAG declined the levels of the growth promoting client protein kinases, transcription factors (Karkoulis et al., 2010) and it maybe a result of the fact that PLGA-PEG-17DMAG complex nano-particles reduce Hsp90 mRNA gene expression especially when its concentration is increased. It should be noted that exposure dose also plays a key role in the inhibition of expression levels (a time-and dose-dependent manner similar to that of the cell growth inhibition).

Hsp90 inhibitors are being actively considered as potential anti-tumor agents, because Hsp90 is in the form of a heteroprotein complex unlike in normal cells that is mainly inhomodimeric shape. This could cause the selective accumulation of these molecules in cancer cells which results in a highly specific treatment with fewer side effects (Guo et al., 2008).

In this study, we used PLGA-PEG-17DMAG complex nanoparticles and free 17DMAG to inhibit A549 lung cancer cell lines. Our study demonstrated that when we treat cell lines with the same amounts of PLGA-PEG-17DMAG complex and 17DMAG-free, under the same conditions, PLGA-PEG-17DMAG complex is more effective and kill some more lung cancer cells. Our experiments showed that PLGA-PEG-17DMAG complex nanoparticles significantly inhibit hsp90 mRNA gene expression .In conclusion, we data show that PLGA-PEG17DMAG complex had inhibitory effect on lung cancer A549 cell line and this inhibition were time and dose-dependent. Cytotoxic effect of PLGA-PEG-17DMAG complex in the cells was increased with increasing concentration of PLGA-PEG-17DMAG complex. Data analysis showed that with increasing concentration of PLGA-PEG-17DMAG complex, decreasing trends of hsp90 expression was observed. Briefly, our data showed that low dosage of PLGA-PEG-17DMAG complex has more inhibitory mRNA on expression of Hsp90 mRNA than 17DMAG free. Besides, PLGA-PEG-17DMAG complex has fewer side effects on A549 cell lines than 17DMAG free and we will use this complex (PLGA-PEG-17DMAG) as a new anti cancer drug in lung cancer treatment.

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