RESEARCH ARTICLE

Targeting HSP90 Gene Expression with 17-DMAG Nanoparticles in Breast Cancer Cells

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Abstract

Background: Dysregulation of HSP90 gene expression is known to take place in breast cancer. Here we used D,L-lactic-co-glycolic acid-polyethylene glycol-17-dimethylaminoethylamino-17-demethoxy geldanamycin (PLGA-PEG-17DMAG) complexes and free 17-DMAG to inhibit the expression of HSP90 gene in the T47D breast cancer cell line. The purpose was to determine whether nanoencapsulating 17DMAG improves the anti-cancer effects as compared to free 17DMAG. Materials and Methods: The T47D breast cancer cell line was grown in RPMI 1640 supplemented with 10% FBS. Encapsulation of 17DMAG was conducted through a double emulsion method and properties of copolymers were characterized by Fourier transform infrared spectroscopy and H nuclear magnetic resonance spectroscopy. Assessment of drug cytotoxicity was by MTT assay. After treatment of T47D cells with a given amount of drug, RNA was extracted and cDNA was synthesized. In order to assess HSP90 gene expression, real-time PCR was performed. Results: Taking into account drug load, IC50 was significant decreased in nanocapsulated 17DMAG in comparison with free 17DMAG. This finding was associated with decrease of HSP90 gene expression. Conclusions: PLGA-PEG-17DMAG complexes can be more effective than free 17DMAG in down-regulating of HSP90 expression, at the saesm time exerting more potent cytotoxic effects. Therefore, PLGA-PEG could be a superior carrier for this type of hydrophobic agent.

Keywords: Breast cancer - HSP90 - 17DMAG-PLGA-PEG - T47D cell line

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Introduction

Breast cancer is a the most common form of cancer among women and the second most common cause of cancer related mortality (Tanaka et al. 2009, Goldhirsch et al. 2013). A enormous number of risk factors are associated with breast cancer: obesity, age, prior breast cancer, high risk pre-malignant lesion LCIS, ADH, excess endogenous or exogenous hormones, nulliparity or age >35 at birth of first child, history of breast biopsies, family history, e.g. BRCA ½, radiation exposure before age 40, mammographic density, energy balance, lifestyle factors (alcohol). (Goldhirsch et al. 2013, Bojesen et al. 2013).

Chemotherapy and radiation therapy are used to reduce tumor mass and stop disease progression (Sharp and Workman 2006; Brannon-Peppas et al. 2012). Radiation therapy is targeted directly to the specific area and the side effects are limited to the area being treated (Early Breast Cancer Trialists' Collaborative Group (EBCTCG). 2011, Goldhirsch et al. 2013). Chemotherapy effects the whole body even if the cancer has not spread (Goldhirsch et al. 2013). The side effects include nausea and losing all your hair (Lindgren et al. 2006, Tanaka et al. 2009). Most tumors, including breast cancer, are treated with a combination chemotherapy strategy with the common addition of biological agents that demonstrate synergistic or additive effects by multiple mechanisms (Tanaka et al. 2009). Side effects of chemotherapy are caused by the cell killing effect of such agents. Therefore, the development of a novel treatment strategy including selective delivery of cytotoxic agents to tumor mass for the treatment of advanced breast cancer is critical to improving the therapeutic index and efficacy/toxicity balance (Brannon-Peppas et al. 2012, Tanaka et al. 2009).

As nanotechnological applications in the field of medical science have expanded rapidly towards multiple directions in the past 10 years, the definition of nanotechnology has been broadened (Tanaka et al. 2009). Breast cancer is often divided into ductal carcinoma in situ (DCIS): Most common type of non-invasive breast cancer, lobular carcinoma in situ(LCIS): this condition begins in the milk glands, but does not go through the walls of the lobules into the breast tissue, invasive lobular carcinoma (ILC): it can enter the lymphatics and spread to other

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parts of the body, invasive ductal carcinoma(IDC): the most common type of breast cancer, inflammatory breast cancer(IBC): the most aggressive form of breast cancer. (Virnig et al. 2010, Zhang et al. 2011, Toikkanen et al. 1997, Arpino et al. 2004) Heat shock proteins (HSPs) are chaperones which have raised expression in tissues that are exposed to proteotoxic stressors. (Whitesell and Lindquist 2005; Rohl et al. 2013; Makhnevych and Houry 2012; Mestril et al. 2014). Moreover, Westerheide and Morimoto in 2005 showed that there is a raised expression of HSPs when cells undergo mutations or are exposed to environmental stress (such as inflammation, tissue repair, cancer, and neurodegenerative diseases). HSP90 is one of the most numerous chaperones and expressed at high levels in cancer cells in comparison to normal tissues (Dobo et al. 2013; Wu et al. 2014). PLGA-PEG (poly (D, L-lactic-co-glycolic acid)-polyethyleneglycol) - 17DMAG is a kind of nanoparticle that may be to inhibit the expression of HSP90 gene in breast cancer cell line (Qu et al. 2013; Sun et al. 2013). In this study, we investigated whether nanocapsulating 17DMAG develops the anti-cancer effect of free 17DMAG in T47D breast cancer cell line.

Materials and Methods

Cell culture and cell line

Fetal Bovine Serum (FBS), RPMI1640, 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). T47D breast cancer cell line, prepared from Pasteur Institute cell bank of Iran, code: C203. T47D breast 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 12 h 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 inscribed at RT with Fourier transform infrared spectroscopy (FTIR) Perkin Elmer Series. The 1H NMR spectra was inscribed 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. D, L-lactide (14.4 g), glycolide (3.86 g) 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 4 h. The polymerization was performed under vacuum. The co-polymer was recuperated 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 coupled to the PLGA-PEG in submission with Dilnawaz et al. protocol. For coupling, 240mg of nanoparticle was dissolved in 20ml chloroform. In the next step, 20mg of 17DMAG was added to mixture, and then these two solutions were homogenized by homogenizer. 20ml 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 h to dry, and crushed into powder by mortar. Productive loading of 17DMAG was approved by FTIR measurement (Shimadzu FTIR 8400S).

Cytotoxicity Assay and Cell Treatment

Cytotoxicity of 17DMAG-loaded PLGA-PEG was measured at 24, 48 and 72 h using the MTT (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide) (MTT; Sigma-Aldrich) assay. First, 2 × 104 cells per well were seeded and kept for 24 h in the incubator to advance cell attachment. Then, cells were treated with different concentrations of free 17DMAG and 17DMAG-loaded PLGA-PEG (20-120 μM) in replicates of four. T47D 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 assessment of nanoparticle effect; and the third one was cells alone. After 24, 48 and 72 h exposure times, the cell culture medium was replaced with 200μl fresh medium for 24 h, after that the cells were incubated with MTT solution for 4 h. Then, contents of all wells were dismissed and 200μl of pure DMSO were added to the wells followed by adding 25μl Sorensen's glycine buffer to each well. The absorbance was recorded at 570nm ELISA-reader and IC50 was calculated at most within 1 h. Their Cytotoxic effects were measured by 24, 48 and 72 h MTT assay in the triplicate model. Generally

2000 cell per well were cultured in a 96 well plate and after 24 h incubation, cells were treated with different concentrations of PLGA-PEG-17DMAG complex (0 nM-320 nM) and free 17DMAG (0 nM-320 nM). The control and treated cells were incubated at 37˚C and 5% CO2 for 24 h.

Quantitative Real-Time PCR assay

After 24 h, 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 Prime¬Script RT Enzyme Mix1, 0.5µl of Oligo dT Primer and 0.5µl of Random 6 mers accompanied by 500ng of total RNA were used that reached to 10µl by adding RNase Free dH2O. The reaction mixture was incubated under the following conditions: 37˚C, 15 minutes (Reverse Transcription); 85˚C, 5 seconds (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 β-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 β-actin a 131bp amplicon were generated in a 25µl reaction mixture that contained: 5pmole of the forward and reverse PCR primers of β-actin or for HSP90, 2X PCR Master Mix Syber Green I and 2µl of the cDNA. The β-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 β-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 dH2O 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, 25 seconds, 45 cycles (Extension); 75-99 ˚C, 1 cycle (Melting).

Statistical analysis

Statistical analyses were performed with Graph Pad 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

1H NMR spectrum of PLGA- PEG co-polymer

The chemical structure of PEG-PLGA co-polymer was confirmed by 1H NMR spectra which were recorded at RT with a Brucker DRX 300 spectrometer acting at 400 MHz. Chemical shift (δ) was allocated 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 ascribed 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 CH group of the lactic acid and glycolic acid, respectively, with the high complexity of the 2 peaks resulting from different D-lactic, L-lactic and glycolic acid sequences in the polymer backbone.

FTIR spectroscopy

The FTIR spectrum is compatible with the structure of supposed copolymer. 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. 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 peak at

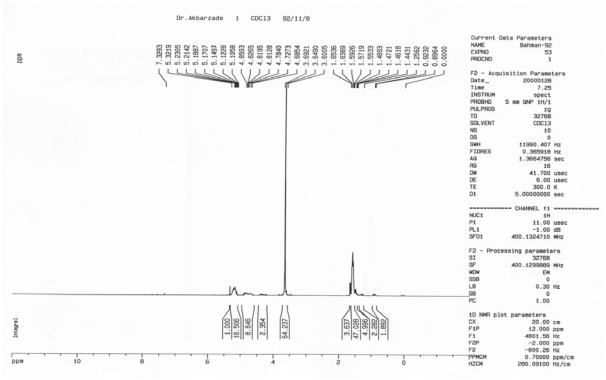

Figure 1. 1H NMR spectrum of PLGA- PEG co-polymer

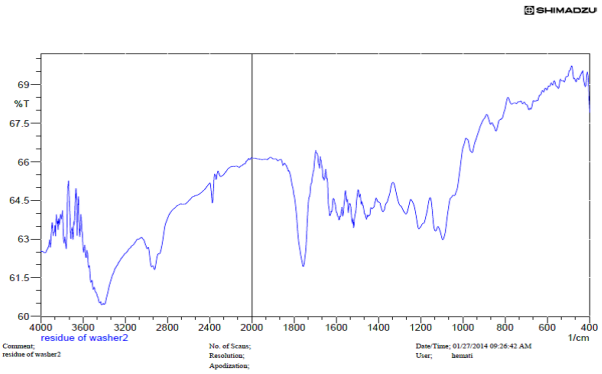

Figure 2. FTIR spectroscopy

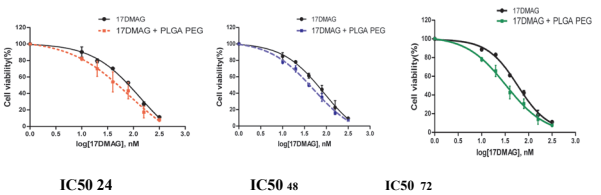

Figure 3. Cytotoxicity Effect of PLGA-PEG-17DMAGComplex and free 17DMAG on T47D for A) 24h; B) 48h; C) 72h Exposure

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

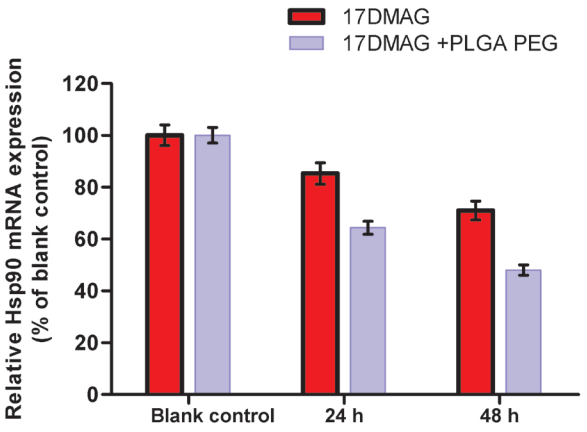

Figure 4. Level of HSP90 mRNA expression in cells treated with PLGA-PEG-17DMAG or free 17DMAG.

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

MTT assay was used to show the cell viability through exposing T47D cell line to different concentrations of free 17DMAG and 17DMAG-loaded PLGA-PEG during 24, 48, 72 h. The results show that the toxicity effect was dose-dependent and time-dependent. This is completely contrary for the viability factor.

The free 17DMAG had cytotoxic effect on T47D cell line with inhibitory concentration at 50% (IC50), 130.3 nM for 24 h, 86.48 nM for 48 h and 58.75 nM for 72 h and 17DMAG loaded on PLGA-PEG had cytotoxic effect of 81.75 nM for 24 h, 50.79 nM for 48 h and 32.82 nM for 72 h respectively. Our Data analysis of cytotoxicity assay showed that IC50 of PLGA-PEG-17DMAG complex on T47D breast cancer cell line was time and dose-dependent (Figure 3).

Effect on gene expression

Real-Time PCR was used to show the levels of HSP90 gene expression. Changes in HSP90 expression levels between the control and treated T47D cells were normalized to β-actin mRNA levels and then calculated by the 2-ΔΔct method. As amount of nanodrug increased, levels of HSP90 gene expression decreased accordingly. 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 T47D cells with 81.75 and 50.79 nM concentrations of PLGA-PEG-17DMAG complex for 24 and 48 hours, expression of HSP90 was significantly reduced (Figure 4).

Discussion

In this study, we tested anti-proliferation effects of 17DMAG-loaded PLGA-PEG and 17DMAG free on breast cancer cell line T47D. Result of the MTT assay showed that

17DMAG-loaded PLGA-PEG has more cell death effect than free 17DMAG on breast cancer cell line T47D in same condition.

Studies have indicated that encapsulating drugs to PLGA-PEG reduces dosage of drug and causes low discordant side effects of the drug (Mirakabad et al. 2013). 17DMAG is a hydrophilic gaeldamaycin derivative that can benefit bioavailability and better activity in vitro and in vivo and has important 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 addition to, 17DMAG by inhibition of HSP90 gene expression reduces HSP90 activity in cancer cells. In this work, used PLGA-PEG-17DMAG complex nanoparticles and free 17DMAG to inhibit T47D breast cancer cell lines. Our study indicated 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 destroy some more breast cancer cells. Our experiments showed that PLGA-PEG-17DMAG complex nanoparticles meaningfully inhibit HSP90 mRNA gene expression .In conclusion, our data showed that PLGA-PEG-17DMAG complex had inhibitory effect on breast cancer T47D cell line and this inhibition was time and dose-dependent. Cytotoxic effect of PLGA-PEG-17DMAG complex was increased with increasing concentration of PLGA-PEG-17DMAG complex in the cells. Analysis of data recognized that

decreasing HSP90 expression level is straight associated with increasing PLGA-PEG-17DMAG concentration. Briefly, our data indicated that low dosage of PLGA-PEG-17DMAG has more inhibitory effect on expression of HSP90 mRNA than 17DMAG free. In addition to, PLGA-PEG-17DMAG has fewer side effects on T47D cell lines than 17DMAG free and we will use this complex (PLGA-PEG-17DMAG) as a new anti-cancer drug in breast cancer treatment.

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