RESEARCH ARTICLE

Comparison of Inhibitory Effects of 17-AAG Nanoparticles and Free 17-AAG on HSP90 Gene Expression in Breast Cancer

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Abstract

Background: HSP90 may be overexpressed in cancer cells which are greatly dependent on Hsp90 function. Geldanamycin derivative 17 allylamino-17-demethoxygeldanamycin (17-AAG) inhibits the function and expression of HSP90. 17-AAG has poor water-solubility which is a potential problem for clinical practice. In this study for improving the stability and solubility of molecules in drug delivery systems we used a β-cyclodextrin-17AAG complex. Materials and Methods: To assess cytotoxic effects of β-cyclodextrin-17AAG complexes and free 17AAG, colorimetric cell viability (MTT) assays were performed. Cells were treated with equal concentrations of β-cyclodextrin- 17AAG complex and free 17AAG and Hsp90 gene expression levels in the two groups was compared by real-time PCR. Results: MTT assay confirmed that β-cyclodextrin- 17AAG complex enhanced 17AAG cytotoxicity and drug delivery in T47D breast cancer cells. The level of Hsp90 gene expression in cells treated with β-cyclodextrin- 17AAG complex was lower than that of cells treated with free 17AAG (P=0.001). Conclusions: The results demonstrated that β-cyclodextrin- 17AAG complexes are more effective than free 17AAG in down-regulating HSP90 expression due to enhanced β-cyclodextrin-17AAG uptake by cells. Therefore, β-cyclodextrin could be superior carrier for this kind of hydrophobic agent.

Keywords: β-cyclodextrin - geldanamycin - cytotoxic effects - MTT assay - uptake

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Introduction

Breast tissue malignancy is the most common cause of cancer with a high mortality rate in women (Najafi et al., 2013). While the progress has decreased death rates of breast cancer, the complexity of breast cancer and several genetic abnormalities has made it difficult. therefore targeting a single pathway for inhibiting the activity of one element is improbable to be effective (Zajac et al., 2010; Ge et al., 2012; Shawky et al., 2014). Identification of a molecular target that will modulate mechanisms of several signaling pathways would be suitable for anticancer therap (Zhang et al., 2012; Cihan 2014). Heat shock protein 90 (HSP90) is believed to be an excellent molecular target in cancer treatment. HSP90 overexpression has been found in cancer cells and showed these cells are vastly dependent on the Hsp90 function(Shirinbayan and Roshan 2011). HSP90 is a molecular chaperone that is induced in response to cellular stress and stabilizes client proteins involved in cell cycle control and proliferative/anti apoptotic pathways (Richardson et al., 2011; Sakthivel et al., 2012; Dobo et al., 2013; Wu et al., 2014).

This molecule is necessary for stability of several oncogenic client proteins (ERBB2, B-RAF, CDK4, AKT, mutant p53, among others) involved in transcriptional regulation, signal transduction, and cell cycle control as well as in other critical processes leading to malignant phenotype (Zajac et al., 2010). Therefore, Hsp90 plays a unique role in cellular homeostasis, thus has been an encouraging anticancer target. *In vivo*, HSP90 itself exists as a homodimer and each monomer chain contains of 732 amino acids, and comprises an N-terminal domain, a flexible linker region, a middle domain, and a C-terminal domain. The N-terminal domain includes a nucleotide-binding site and also involved in interactions with co-chaperones (Richardson et al., 2011). It is assumed that hydrolysis of ATP to ADP, at the nucleotide-binding site energizes the functions of the HSP90 chaperone complex (Bagatell and Whitesell 2004). Several inhibitors of HSP90 have been recognized; the most well categorized ones are derivatives of the benzoquinone ansamycin antibiotic geldanamycin (GA) and the macrolide

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antibiotic radicicol that either bind to the N-terminal domain, nucleotide binding site, inhibit the ATPase activity, ADP-ATP exchange activity, increase the usage of ubiquitin ligases to the HSP90 chaperone complex and consequently leading to increased degradation of client proteins by the proteasome pathway (Fukuyo et al., 2010).Geldanamycin derivative17-allylamino-17-demethoxy-geldanamycin (17-AAG) exhibited lower toxicity and improved stability and demonstrated that 17-AAG induces reduction of key regulators of signal transduction in many human tumors, including colon and breast cancer (Usmani and Chiosis 2011; Schulz et al., 2012). In cancer cells, 17AAG binds more strongly to Hsp90, because it is in the form of a heteroprotein complex and in normal cells is mainly homodimeric and this could result in the selective accumulation of it in cancer cells (Usmani and Chiosis 2011). 17AAG has poor water-solubility which is a potential problem for clinical construction and obstacle for its clinical application (Guo et al., 2008). Despite all of these, Cyclodextrin is commonly used for improving the stability and solubility of molecules in drug delivery systems. There are three common types of Cyclodextrin including α-Cyclodextrin, β-Cyclodextrin and γ-Cyclodextrin. The difference between these three types is in their internal cavity. The inner cavity of α-Cyclodextrin is very tiny for 17AAG loading while the inner cavity of γ-Cyclodextrin is too large, but inner cavity of β-Cyclodextrin is suitable. β-Cyclodextrin is a semi-natural compound with low toxicity, which could enhance drug bioavailability (Challa et al., 2005; Yadav et al., 2014). β-Cyclodextrin was used for encapsulation of 17AAG (Sakthivel et al., 2012). In this study, we investigate that β-Cyclodextrin- 17AAG complex has inhibitory effect on Hsp90 gene expression in T47D breast cancer cell line, as well inhibitory function on hsp90 activity. Anticancer effect of free 17AAG and β-Cyclodextrin- 17AAG was compared. The level of Hsp90 gene expression after 24 h exposure was measured by Real-time PCR.

Materials and Methods

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), B-Cyclodextrin and 17AAG were purchased from Sigma (USA). T47D epithelial like breast cancer, prepared from Pasteur Institute cell bank of Iran, code: C203.

Cell culture and cell line

T47D epithelial like 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.

Preparation of β-cyclodextrin-17AAG complex

40mg of β-Cyclodextrin was dissolved in 8 mL deionized water; and 12mg of 17AAG was dissolved in 500µl acetone. These two solutions were mixed together and were sited on the stirrer at 400 rpm for 24h to evaporate the acetone without a cap. Then, it was centrifuged at 1000 rpm for 5 min and supernatant were collected by freeze drying. (Yallapu et al., 2012; Yin et al., 2013)

Size Distribution (SEM)

The surface morphology of the nanospheres during the incubation time was observed by SEM. The nanographs of β-CD-17AAG in nanoparticles are shown in Figure (3). As it is demonstrated the size of the particles is about 30- 65nm and dispersion of the particles was significantly improved. Also, the samples were layered with gold particles.

Determination of 17AAG loading

2mg of β-Cyclodextrin-17AAG complex was dissolved in 100mL dimethylsulfoxide (DMSO) and the solution was placed on shaker for 24h at room temperature. Then, the solution was Centrifuged at 12,000 rpm and supernatant collected for the calculations. The standard 17AAGve of 17AAG concentration in DMSO was drowned using UV-Vis spectrophotometer by absorbance rate of 17AAG.

In vitro cytotoxicity (MTT assay)

After growing, sufficient amount of cells in exponential phase of growth, exposed to free 17-AAG and β-Cyclodextrin-17AAG complex. Their Cytotoxic effects were measured by 24, 48 and 72h MTT assay in the triplicate model. Generally 2000 cell per well were cultured in a 96 well plate and after 24h incubation, cells were treated with different concentrations of β Cyclodextrin-17AAG complex (0 µg/ml-320µg/ml) and free 17-AAG (0 µg/ml-320 µg/ml). In addition, β-Cyclodextrin in PBS or DMSO was used as the control. After this different exposure duration, medium was removed and then the cells were fed with 200µl fresh medium. Cells waited for 24h, next 50µl of 2mg/ml MTT was dissolved in PBS and was added to each well and plate was covered with aluminum foil and incubated for 4h in dark place. In the next step, wells content were removed and 200µl pure DMSO and 25µl Sorensen's glycine buffer were added to wells. Finally, total of formazan was determined by measuring the absorbance at 570 nm by ELISA plate reader (Bio Tech Instruments, USA) with a reference wavelength of 630nm.

Cell treatment

After appointment of IC50, a counted (106) cell was treated in a 6-well plate with different concentrations of free 17-AAG and β-Cyclodextrin-17AAG complex (0, 10, 20, 40, 80, 160 and 320 µg/ml). For control cells, the same volume of 10% DMSO without drugs was added to plate containing the control cells. An equivalent amount of β-Cyclodextrin in PBS was used as another control. Next, plates were incubated for 24, 48 and 72h exposure

time in an incubator 5%CO_2 and 37°C.

RNA extraction and reverse transcription

Total RNA was extracted with Trizol in a clean RNase-free tube, according to the manufacturer's protocol for cell lines. Efficiency of our extraction tested with nanodrop analysis. Also purity of our extracted RNA was calculated with spectrophotometer at 260/280 nm ratio and the firmness of extracted RNA was defined by electrophoresis in 0.5µg/ml ethidium bromide contained agarose gels. Complementary DNA (cDNA) was synthesized using random hexamer primers with purchased reverse transcriptase kit from fermentas, K1622.

Quantitative real-time PCR assay

Quantity of hsp90 gene expression was determined by quantitative Real-time PCR technique using Syber Green-I by means of the Rotor-GeneTM 6000 machinery (Corbett research, Australia) according to the manufacturer's protocols. For real-time PCR, hsp90 primers (Genbank accession: NM_005348, bp60-79) 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. The program for real-time PCR reaction contained of an Inhibition of hsp90 Gene Expression by β-Cyclodextrin-17AAG initial denaturation step at 95°C for 5 min and 45 cycles initial denaturation step at 95°C for 5 min and 45 cycles of denaturation (95°C for 10 seconds), annealing (60°C for 10 seconds), and extension (72°C for 25 seconds). Finally, amplicons were experienced with melting 17AAGve analysis of 95-65°C.Changes that oc17AAGred in hsp90 expression amounts between the control and T47D cells that treated with β-Cyclodextrin 17AAG complex and free 17-AAG, normalized to β-Actin mRNA amounts, calculated with the $2^{-\Delta\Delta CT}$ method. Each DNA sample was divided so that hsp90 and beta actin could be amplified, in parallel, the reactions were done in duplicate with equal amounts of starting material.

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 17AAG loading

Standard curve of 17AAG concentration in DMSO was prepared via UV-Vis spectrophotometer at 450 nm. One mg of β-Cyclodextrin-17AAG complex contained 575.69 µg17AAG.

Table 1. Primer Sequences

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

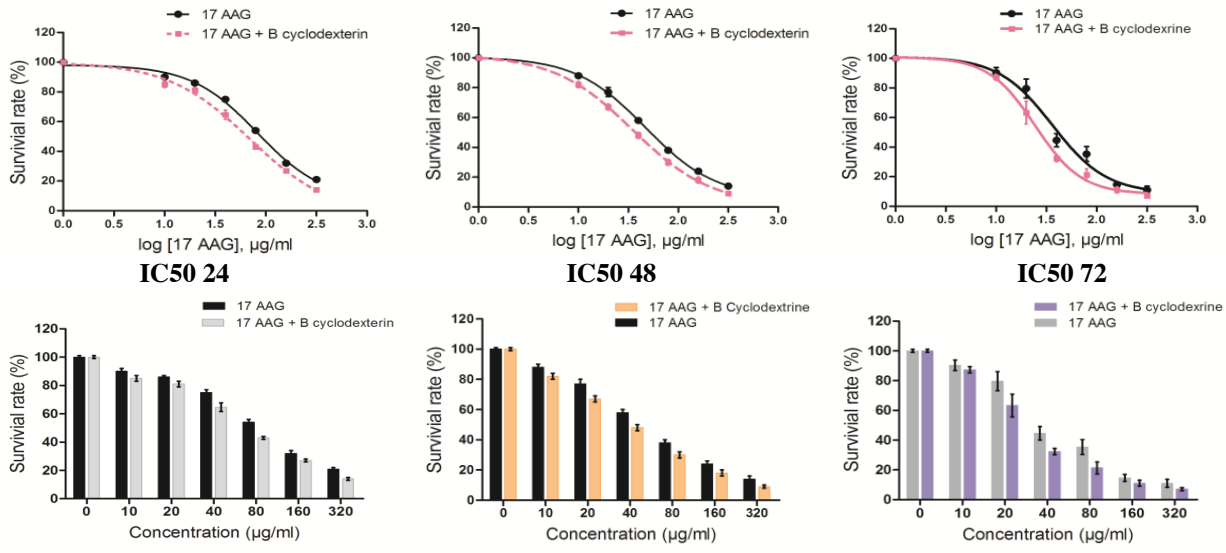

Figure 1. Cytotoxicity Effect of β-Cyclodextrin- 17AAGComplex and free 17AAG on T47D for A) 24h ; B) 48h ; C) 72h Exposure

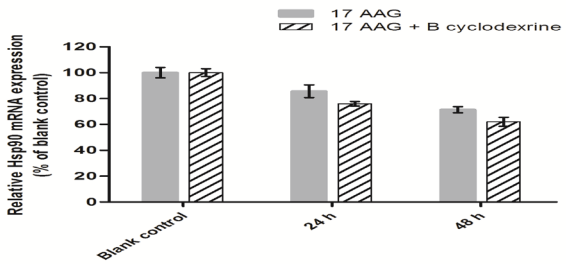

Figure 2. Level of HSP90 mRNA Expression in Cells Treated with CD-17AAG or Free 17AAG

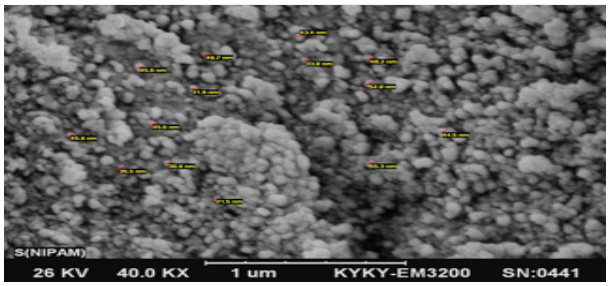

Figure 3. The Surface Morphology of the Nanospheres During the Incubation Time

Effects on cell viability

In this study to evaluate the cytotoxic effect (MTT assay) of β-CD-17AAG complex and free 17AAG, T47D breast cancer cell lines were treated with different concentration mentioned above. The obtained IC50 of free 17AAG for 24, 48 and 72h was 82, 46 and 35 μg/m and β-CD-17AAG complex for 24, 48 and 72h was 69, 35 and 24 μg/m respectively. Our Data analysis of cytotoxicity assay showed that IC50 of β-Cyclodextrin-17AAG complex on T47D breast cancer cell line was time and dose-dependent (Figure 1).

Effect on gene expression

Cells were treated with β-CD-17AAG complex and free 17AAG for 24 and 48h. The expression of hsp90 mRNA levels was calculated via q-RT PCR. The level of hsp90 mRNA was standardized to mRNA level of the uniformly expressed housekeeping gene, beta actin, within each sample. Increasing $2^{-\Delta\Delta Ct}$ amount resulted in enhanced expression of mRNA levels. Data analysis of q-RT PCR exhibited that with increasing concentration of β-Cyclodextrin17AAG complex, a decreasing trend appeared in mRNA level of hsp90. Each sample was repeated two 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 17AAG, in the same concentration, β-CD-17AAG resulted in a lower level and expression of hsp90 mRNA. When we treated T47D cells with 61 and 41μg/m concentrations of β-Cyclodextrin-17AAG complex for 24 and 48 hours, expression of hsp90 was significantly reduced (Figure2).

Discussion

While Chemotherapy has toxic side effects in healthy tissues in treatment of human cancers, Nanotechnology attempts to resolve these problems by encapsulating or loading drugs to nonmaterial which are resistant to drug efflux (Tsuda, 2010; Ghasemali et al., 2013). In recent times, Cyclodextrin nanoparticles are used mostly as recyclable transporters for drug delivery and several studies have shown that encapsulating drugs to Cyclodextrin polymers decreases adverse side effects of the drugs leading to use lower dosages of drug (Challa et al., 2005). In recent years, targeting Hsp90 has appeared as an exciting therapeutic intervention for a wide variety of human cancers. Hsp90 play a critical role in maturation and function of client proteins which related to oncogenic pathways, and expression levels of it seem to be upregulated in cancer (Challa et al., 2005). A variety of Hsp90 inhibitors have emerged as promising anticancer agents. 17AAG decreases hsp90 activity by inhibition of nucleotide binding site in breast cancer cells (Karkoulis et al., 2010). Through its ability to control the activity and stability of many client proteins involved in the oncogenic process, targeting Hsp90 has the potential to affect all the hallmarks of cancer (Zajac et al., 2010). It is unclear whether cell stress is important for efficiency of HSP90 inhibitors (Pick et al., 2007). 17AAG has poor water-solubility which is a potential problem for clinical construction and obstacle for its clinical application (Guo et al., 2008). However, Cyclodextrin is commonly used for improving the stability and solubility of molecules in drug delivery systems. In the present study, we have comparatively studied the antiproliferative effects of free 17-AAG and β-CD-17AAG complex in breast cancer cells as shown in figure (1 and 2). we see Free 17-AAG compared with β-CD-17AAG complex induced Hsp90 downregulation in breast cancer cell lines showing the efficiency of our nanoparticle by comparison between our results obtained from T47D cell lines treated with β-Cyclodextrin-17AAG-complex and free 17AAG over a 24 ,48 and 72 hours treatment period. 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; Zajac et al., 2010) and it maybe a result of the fact that β-Cyclodextrin-17AAG 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 hp90 is in the form of a heteroprotein complex unlike in normal cells that is mainly in homodimeric 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). Our experiments demonstrated that when we treat cell lines with the same values of β-Cyclodextrin-17AAG complex and 17AAG-free, under the same conditions in the three exposure times 24, 48 and 72h, β-Cyclodextrin-17AAG complex is more effective and kill some more breast cancer cells. For evidence, IC50s are different in the stated exposure times and decrease with time as shown in the Figure (1). In conclusion, our data shows that β-Cyclodextrin- 17AAG complex had inhibitory

effect on breast cancer T47D cell line. This inhibition was time and dose-dependent(Bhattacharjee et al., 2012). Cytotoxic effect of β-Cyclodextrin- 17AAG complex in the cells was increased with increasing concentration of β-Cyclodextrin-17AAG complex. Data analysis showed that by increasing concentration of β-Cyclodextrin-17AAG complex, decreasing trend of hsp90 expression was observed. In summary, our results showed that low dosage of β-Cyclodextrin-17AAG complex has more inhibitory effect on expression of hsp90 mRNA than 17AAG free. Besides, β-Cyclodextrin-17AAG complex has fewer side effects than 17AAG free and has more inhibitory effect on hsp90 expression and function; consequently we can use this complex (β-Cyclodextrin-17AAG) as a new anticancer compound in breast cancer treatment (Valizadeh et al., 2012; Mirakabad et al., 2013; Mollazade et al., 2013; Nejati-Koshki et al., 2013; Pourhassan-Moghaddam et al., 2013; Davaran et al., 2014; Kouhi et al., 2014; Wang et al., 2014)

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