RESEARCH ARTICLE

Inhibitory Effects of β-Cyclodextrin-Helenalin Complexes on H-TERT Gene Expression in the T47D Breast Cancer Cell Line - Results of Real Time Quantitative PCR

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Abstract

  <u>Background</u>: Nowadays, the encapsulation of cytotoxic chemotherapeutic agents is attracting interest as a method for drug delivery. We hypothesized that the efficiency of helenalin might be maximized by encapsulation in β-cyclodextrin nanoparticles. Helenalin, with a hydrophobic structure obtained from flowers of Arnica chamissonis and Arnica Montana, has anti-cancer and anti-inflammatory activity but low water solubility and bioavailability. β-Cyclodextrin (β-CD) is a cyclic oligosaccharide comprising seven D-glucopyranoside units, linked through 1,4-glycosidic bonds. <u>Materials and Methods</u>: To test our hypothesis, we prepared β-cyclodextrin-helenalin complexes to determine their inhibitory effects on telomerase gene expression by real-time polymerase chain reaction (q-PCR) and cytotoxic effects by colorimetric cell viability (MTT) assay. <u>Results</u>: MTT assay showed that not only β-cyclodextrin has no cytotoxic effect on its own but also it demonstrated that β-cyclodextrin-helenalin complexes inhibited the growth of the T47D breast cancer cell line in a time and dose-dependent manner. Our q-PCR results showed that the expression of telomerase gene was effectively reduced as the concentration of β-cyclodextrin-helenalin complexes increased. <u>Conclusions</u>: β-Cyclodextrin-helenalin complexes exerted cytotoxic effects on T47D cells through down-regulation of telomerase expression and by enhancing Helenalin uptake by cells. Therefore, β-cyclodextrin could be superior carrier for this kind of hydrophobic agent.

Keywords: Helenalin - Telomerase - real time quantitative PCR - β-cyclodextrin

Introduction

Breast cancer starts usually occurs in the inner lining of the lobules or milk ducts in breast. There are different types of breast cancer, with different stages, aggressiveness, and genetic frame. It is categorized as a prevalent disease which is the second most common cancer after lung cancer and the fifth most common cause of death among women around the world. Studies have shown that white women are more probable to be diagnosed with the breast cancer in the USA and black women are more possible to die from breast cancer. Patients with the same stage of illness can have very different treatment responses and outcome (Schramm et al., 2010).

Treatment includes surgery, drugs (hormone therapy and chemotherapy), and radiation. Studies showed that telomerase is over expressed in about 85-90% of human breast cancer cells (Herbet et al., 2001; Kirkpatrick et al., 2003; Torkani and Aradi, 2008). Telomerase is a

RNA dependent DNA polymerase that synthesize the repetitive nucleotide sequence (TTAGGG in human beings) at telomeres which are the regions of repetitive nucleotide sequences at each end of a chromosome with protective effect on the end of linear chromosomes and avoid decline or fusion with adjacent chromosomes (Mokbel, 2000; Nasiri, 2013). During cell division, DNA polymerase is not capable to fully replicate the ends of linear DNA and consequently genetic material is lost from chromosomal ends (Mokbel, 2000; Kirkpatrick et al., 2004). Consequently, inhibition of telomerase action is an effective aim for the treatment of breast cancer (Tian et al., 2010; Kazemi et al., 2011).

The important function of telomerase in replicative senescence and the particular over expression of this enzyme in the majority of cancers raise the potential use of telomerase inhibition for cancer therapy (Saretzki, 2003). According to the telomere hypothesis for replicative senescence, inhibition of telomerase in the malignant

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cells is expected to result in the erosion of telomeres and lastly leading to growth arrest, senescence or cell death (Feng et al., 1995; Kondo et al., 1998; Hahn et al., 1999; Zhang et al., 1999; Huang et al., 2005). Helenalin (Figure 1A) is a sesquiterpene lactone found in Arnica Montana and Arnica chamissonis foliosa flowers. Studies on the molecular mechanism by which Helenalin applies its anti-inflammatory effects, have discovered that Helenalin inhibits the transcription factor NF-kB, a central arbitrator of the human immune response, by targeting the subunit of NF-kB (Lyss et al., 1998; Huang et al., 2005). Cyclodextrin commonly is used for improving the stability and solubility of molecules in drug delivery systems. Cyclodextrins are ordinary, broadly studied and cheaply accessible supramolecular hosts (Atwood et al., 1996) with a multiplicity of applications in the food, cosmetics and pharmaceutical industries (Del Valle, 2004). The crystal structure of β-Cyclodextrin, $C42H70O35\_10.41H_2O$, consists of truncated cone-shaped

Cyclodextrin molecules that are herringbone packed. The primary hydroxy groups form an intramolecular hydrogen-bonded array. The semi polar cavity of the Cyclodextrin host is filled with water molecules, which show incomplete occupancy and disorder (Rudiger et al., 2009). There are three common types of Cyclodextrin including α-Cyclodextrin, β-Cyclodextrin and γ-Cyclodextrin (Vivek et al., 2009). The difference between thesethree types is in their inner cavity. The inner cavity of α-Cyclodextrin is very tiny for Helenalin loading while the inner cavity of γ-Cyclodextrin is too large, but inner cavity of β-Cyclodextrin (Figure 1B) is suitable (Vivek et al., 2009; Kazemi et al., 2011). B-Cyclodextrin (β-CD) is a cyclic oligosaccharide comprising seven D-glucopyranoside units, linked through 1,4-glycosidic bonds. The first room temperature crystal structure determination of β-CD dodecahydrate was shown about 40 years ago. A number of room temperature determinations have been reported so far (Budai, 1997; Rudiger et al., 2009). In other nanoparticle, encapsulation technique such as poly lactic-co-glycolic acid (PLGA), only 5-10% of drug loading is probable , but in Cyclodextrin more than 30% of drug loading is possible (Murali et al., 2010). The inhibitory effect ofHelenalin on telomerase in cancer cell lines and preparation of β-Cyclodextrin-Helenalin complex for improving Helenalin stability and solubility has been studied (Cui, 2006; Jurenka, 2009; Hsina et al., 2010; Murali et al., 2010). In this study, we investigate the inhibitory effect of β-Cyclodextrin- Helenalin complex on telomerase gene expression in T47D breast cancer cell line by q-PCR.

Materials and Methods

Fetal Bovine Serum (FBS), RPMI 1640, TRIzol reagent, Tripsin-EDTA and Antibiotics were Purchased from Invitrogen (Germany). Syber Green Real Time PCR Master Mix kit wasPurchased from Roche (Germany).B-Cyclodextrin, and (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma (USA). Helenalin extract also was purchased from Enzobio life science (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 were cultured in RPMI1640 supplemented 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-helenalin complex

B-Cyclodextrin-Helenalin complex was prepared according to the method of Murali (Murali et al., 2010). 40mg of β-Cyclodextrin was dissolved in 8 mL deionized water; and 12mg of Helenalin was dissolved in 500μl acetone. These two solutions were mixed together and were placed on the stirrer at 400rpm for 24h to evaporate the acetone without a cap. After that, it was centrifuged at 1000rpm for 5 min and supernatant were collected by freeze drying.

Size Distribution (SEM)

The surface morphology of the nanospheres during the incubation period was observed by SEM. The nanographs of β-CD-Helenalin in nanoparticles are shown in Figure 2. As it is demonstrated the size of the particles is about 25-75nm and dispersion of the particles was greatly improved. Also, the samples were coated with gold particles.

Determination of helenalin loading

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

In vitro cytotoxicity (MTT assay)
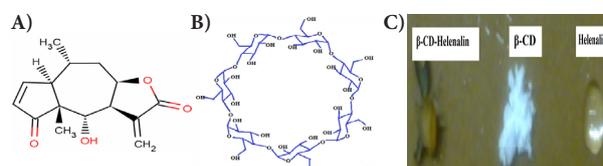
Cells in the exponential phase of growth were exposed

Figure 1. A) Schematic Illustration of Helenalin; B) β-Cyclodextrin; and C) Solid Powder Samples of Helenalin, β-Cyclodextrin and β-Cyclodextrin-Helenalin Complex
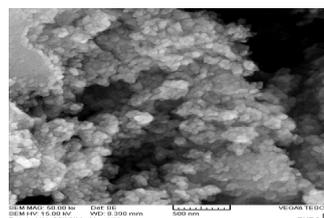

Figure 2. The SEM Micrographs of β-CD-Helenalin Nanoparticles

to β-Cyclodextrin-Helenalin complex. Cytotoxic effect of β-Cyclodextrin-Helenalin complex was studied by 24, 48 and 72h MTT assay in the triplicate model. Generally 2000 cell in per well were cultivated in a 96 well plate and after 24h incubation, cells were treated with different concentrations of β-Cyclodextrin-Helenalin complex (0 nM/ml^{-2} nM/ml). In addition, β-Cyclodextrin in PBS or DMSO was used as the control. After this different exposure duration, medium was removed and after that the cells were fed with 200μl fresh medium. Cells were kept standing 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. 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 with a reference wavelength of 630nm.

Cell treatment

After determination of IC$_{50}$, an accounted cell was treated with β-Cyclodextrin-Helenalin complex. For control cells, the same volume of 10% DMSO without β-Cyclodextrin-Helenalin complex was added to flask that has control cells. Next, culture flasks were incubated for 24, 48 and 72h exposure duration.

RNA extraction and reverse transcription

For RNA extraction, cells were trypsinized, and then collected by centrifugation at 1500g for 5 min at 37°C. After that 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 primers (N6) and hexamer primers with purchased reverse transcriptase kit from fermentas, K1622.

Quantitative real-time PCR assay

Quantity of hTERT gene expression was determined by quantitative Real-time PCR technique using Syber Green-I by means of the Rotor-Gene™ 6000 machinery (Corbett research, Australia) according to the manufacturer's protocols. For real-time PCR, hTERT primers (Genbank accession: NM_198255, bp 2165-2362) 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 hTERT and β-actin used in real-time PCR were shown in Table 1. For hTERT, a 198bp 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 hTERT, 2X PCR Master Mix Syber Green I and 2μl of the cDNA was used. The Beta-Actin mRNA was calculated as the internal standard control gene by specific primers. The program for real-time PCR reaction consisted of an

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 curve analysis of 95-65°C. Changes that happen in telomerase expression amounts between the control and T47D cells that treated with β-Cyclodextrin Helenalin complex, normalized to β-Actin mRNA amounts, calculated with the $2^{-\Delta\Delta CT}$ method. Each DNA sample was divided so that hTERT and beta actin could be amplified, in parallel, the reaction were done in duplicate with equal amounts of starting material.

Statistical analysis

SPSS 14 has been used for statistical analysis and p value <0.05 was regarded statistically significant

Results

FTIR spectra analysis

The F-IR spectrum is consistent with the structure of the expected β-CD-Helenalin. FTIR spectroscopy was used to show the structure of β-CD and β-CD-Helenalin nanoparticles. The β-CD-Helenalin exhibited significant FTIR peak at wave number of 940, 1094, 1158, 1363,
1652, 2162, 2927 and 3372 cm^{-1}.
3372: ν (O-H) β-CD+Helenalin
2927: ν(C-H) β-CD+Helenalin
2162/ 1652: ν(C=O) Helenalin
1422: δ(C-H) from CH2 and CH3 β-CD+Helenalin
1363: coupled δ(C-C-H), δ(C-O-H), δ (H-CH) β-CD+Helenalin
1158 and 1094: coupled ν(C-O), ν(C-C), ν(C-O-H) β-CD+Helenalin
940: skeletal vibration involving α-1,4linkage.
*ν, stretching vibration; δ, bending vibration.

Determination of helenalin loading

Standard curve of helenalin concentration in DMSO was prepared via UV-Vis spectrophotometer at 450 nm. 1mg of β-Cyclodextrin-Helenalin complex contained 276.44μg Helenalin.

Results for MTT assay

T47D cells were exposed to variable concentrations of β-CD-Helenalin complex (1-1.8μM with six concentration) for 24, 48 and 72h and IC$_{50}$ of 2,1.64 and 1.4 was obtained for 24, 48 and 72h, respectively .Our Data analysis of cytotoxicity assay showed that IC$_{50}$ of β-Cyclodextrin-Helenalin complex on T47D breast cancer cell line was time and dose-dependent (Figure 4).

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

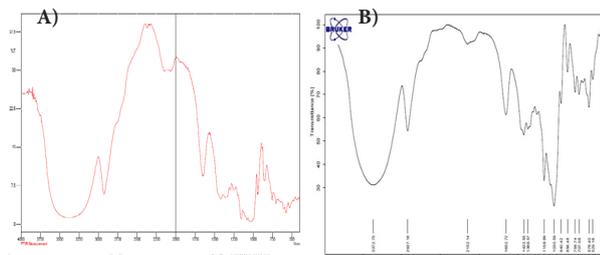| Oligonuleotide	Location	Sequence	PCR product size
hTERT			
Forward primer	2165	5'CCGCCTGAGCTGTACTTTGT3'	198(bp)
Reverse primer	2362	5'CAGGTGAGCCACGAACTGT3'	
Beta-actin			
Forward primer	787	5'TCCCTGGAGAAGAGCTACG3'	131(bp)
Reverse primer	917	5'GTAGTTTCGTGGATGCCACA3'	

100.0

6

75.0

Figure 3. FT-IR Spectra the Structure of Expected A) β-CD; and B) β-CD-Helenalin
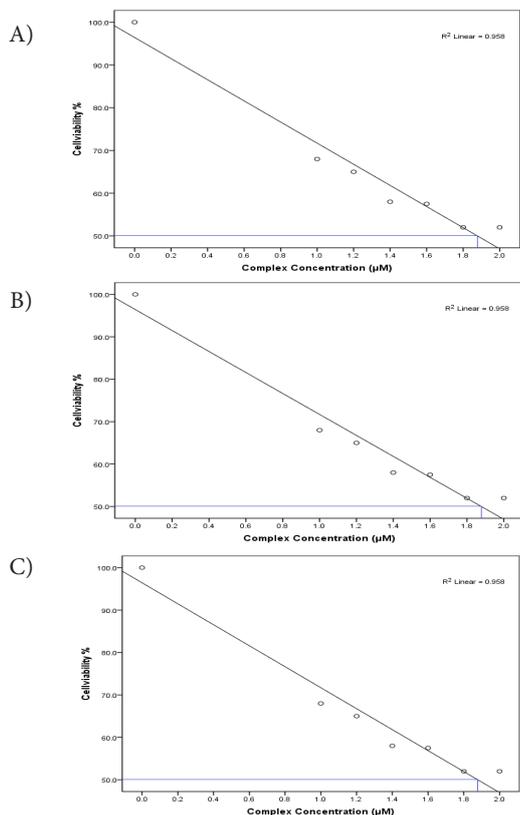

Figure 4. Cytotoxicity Effect of β-Cyclodextrin-Helenalin Complex on T47D for A) 24h ; B) 48h ; C) 72h Exposure
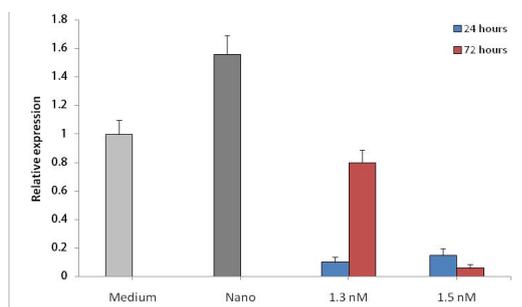

Figure 5. Amplification Column for hTERT Expression

Results for quantitative real-time RT-PCR

The expression of hTERT mRNA levels was calculated via q-RT PCR. The level of hTERT mRNA was normalized to mRNA level of the uniformly expressed housekeeping gene, beta actin, within each sample. The difference of $2^{-\Delta\Delta Ct}$ value was calculated. Data analysis of q-RT PCR showed that with increasing concentration of β-Cyclodextrin-Helenalin complex, a decreasing trend was appeared in mRNA level of hTERT. Each sample

was repeated two times. Q-RT PCR results showed a considerable decrease in hTERT gene expression in the treated cells in comparison with the control cells. When we treated T47D cells with 1.3 and 1.5 nm concentrations of β-Cyclodextrin-Helenalin complex for 24 and 72 hours, expression of hTERT was significantly decreased (Figure 5).

Discussion

Although chemotherapy is used in treatment of approximately 50% of human cancers (Gottesman et al., 2002), most of chemotherapeutic agents have toxic side effects in healthy tissues and sometimes, chemotherapy is limited by selection of drug-resistant cells expressing the multidrug resistance (MDR) phenotype (Szakacs et al., 2006). Nanotechnology provides an alternative strategy to overcome these problems by encapsulating or attaching drugs to nonmaterial such as lipids, polymers and solid-core nanoparticles which are resistant to drug efflux (Davis et al., 2008; Sun et al., 2008; Veiseh et al., 2010). Lately, Cyclodextrinnanoparticles are used broadly as biodegradable transporters for drug delivery and enhance the solubility of non-polar substances. Cyclodextrins are a group of compounds made up of sugar molecules combined in a circle. There is a balance between electrostatic and hydrophobic agents. Studies have shown that encapsulating drugs to Cyclodextrinpolymers reduces adverse side effects of the drugs and causes to use low dosage of drug. Helenalin is a nontoxic agent, suitable and proficient drug for remedial uses and has significant anticancer activity over different epithelial cancers, including breast cancer cells (Tyagi et al., 2003; Kim et al., 2009; Wang et al., 2010). Besides, Helenalin decreases telomerase activity by inhibition of hTERT gene expression in breast cancer cells (Choudhuri et al., 2002; Ramachandran et al., 2002). In this study, we used β-Cyclodextrin-Helenalincomplex nanoparticles to inhibit T47D breast cancer cell lines. To show the efficiency of our nanoparticle, we compared our results (T47D cell lines treated with β-Cyclodextrin-Helenalincomplex) with Nasiri et al. (2013) experiments that they studied the effect of pure Helenalin on T47D cell lines. Our experiments demonstrated that when we treat cell lines with the same values of β-Cyclodextrin-Helenalin complex and Helenalin-free, under the same conditions, β-Cyclodextrin-Helenalin complex is more effective and kill some more breast cancer cells. Our study shows that β-Cyclodextrin-Helenalin complex nanoparticles significantly inhibit hTERT mRNA gene expression.

In conclusion, our data show that β-Cyclodextrin-Helenalin complex had inhibitory effect on breast cancer T47D cell line. This inhibition was time and dose-dependent too. Cytotoxic effect of β-Cyclodextrin-Helenalin complex in the cells was increased with increasing concentration of β-Cyclodextrin-Helenalin complex. Data analysis showed that with increasing concentration of β-Cyclodextrin-Helenalin complex, decreasing trend of telomerase expression was observed. In summary, our results showed that low dosage of β-Cyclodextrin-Helenalin complex has more inhibitory

effect on expression of hTERT mRNA than Helenalin free. Besides, β-Cyclodextrin-Helenalin complex has fewer side effects on T47D cell lines than Helenalin free and we can use this complex (β-Cyclodextrin-Helenalin) as a new anti cancer compound in breast cancer treatment.

Acknowledgements

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