Synthesis of a copolymer carrier for anticancer drug luteolin for targeting human breast cancer cells

Mahin Maleki, Ali Aidy, Elahe Karimi, Shaahin Shahbazi, Nader Safarian, Naser Abbasi

Abstract

OBJECTIVE: To focus a new chemoprevention approach that uses nanotechnology to deliver luteolin to human breast cancer cells (MCF-7), and its underlying mechanism.

METHODS: Water-soluble copolymer-encapsulated nanoparticle-luteolin (CENL) was formulated using the hydrophobic drug luteolin. The ability to load and release the anticancer drug into/from the synthesized hyperbranched polyester was evaluated by high-performance liquid chromatography. The successful synthesis of CENL was supported by analytical techniques including Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, gel permeation chromatography, and dynamic light scattering. Cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide color method. Reactive oxygen species (ROS) were measured using a di-chlorofluorescein probe and intracellular calcium (Ca\(^{2+}\)) was evaluated with a fluo-3 AM probe.

RESULTS: The results showed that the drug delivery system is stable and that the loading capacity is high. Treatment with nanoparticles containing luteolin and free luteolin resulted in cell death in breast cancer cells at high concentrations ([IC\(_{50}\) (33 ± 4) and (48 ± 6) µM, respectively]. At high concentrations, CENL reduced cell viability and increased ROS and Ca\(^{2+}\) production.

CONCLUSION: Our results demonstrate that CENL has potential for human breast cancer treatment.

© 2019 JTCM. All rights reserved.

Keywords: Polymers; Materials testing; Drug delivery systems; Luteolin

INTRODUCTION

Cancer continues to cause a large number of deaths worldwide. Since 2008, approximately 7.6 million people have died as a result of cancer-related causes, and the number is estimated to reach 13.1 million by 2030. 1 Many anticancer therapies provide low efficacies and present large financial costs for companies and governments. One of the most significant problems is the lack of selectivity in administering anticancer drugs to the target tissue, which results in high incidences of side effects in patients. 2 This is often compounded by a large proportion of the molecules used as chemotherapy drugs having hydrophobic structures, which causes additional toxicity in non-target tissues. 3 To overcome
these problems, researchers have demonstrated the use of drug delivery carriers for drug transport. These delivery systems can be liposomes, polymers, viruses, and proteins. These vehicles can also improve drug effectiveness in target tissues while protecting the drug from degradation. The benefits of carrier encapsulation include increased solubility, increased duration of action, selective action on cancer cells, greater efficiency, fewer toxic effects, and less drug resistance. Citric acid, which is a biocompatible and inexpensive substance, is used on a large scale in the food and pharmaceutical industries, and glycerol is a key material in the synthesis of phospholipids. Therefore, polymers or copolymers based on citric acid and glycerol, which have biocompatible properties, could be considered for use as delivery vehicles. Step-by-step melting polycondensation of citric acid and glycerol was used to synthesize the hyperbranched polyester. Techniques including fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (NMR), gel permeation chromatography (GPC), and dynamic light scattering (DLS) were used to examine the physicochemical properties of the copolymer nanoparticles. The synthesized hyperbranched copolymers were shown to be able to load and deliver anticancer drugs and release them as cytotoxic agents. Nanoparticle toxicity has been shown to be related to oxidative stress and alteration of calcium homeostasis, gene expression, pro-inflammatory responses, and cellular signaling events. Luteolin belongs to a class of compounds known as flavonoids, which are generally characterized by polyphenol chemical structures derived from benzopyrone. It has been reported that luteolin is primarily found in fruits, vegetables, and medicinal plants. It has also been shown that plants rich in luteolin have an extensive variety of biological activities ranging from antioxidant, anti-inflammatory, and anti-allergy to anticancer effects. The antitumor effects of luteolin on different tumor cell lines have been shown to be associated with the activation of apoptosis, cell cycle disruption. There are several checkpoints in cell cycle when a cell may damage and thus may proceed to disintegrate itself which is termed apoptosis, metastasis, and angiogenesis inhibition. However, luteolin is not well dispersed in water, and it is challenging to make a luteolin formulation that can be injected intravenously or intraperitoneal administered. In this study, copolymer-encapsulated nanoparticle-luteolin (CENL) dispersed in water was studied and its anticancer effects and its mechanism of action on a breast cancer cell line were investigated.

MATERIALS AND METHODS

Materials

Glycerol (G) and monohydrate citric acid (CA) were purchased from Carlo Erba. Tetrahydrofuran (THF), Dimethyl sulfoxide (DMSO), and cyclohexane were purchased from Merk. Luteolin was purchased Sigma-Aldrich (St. Louis, MO, USA). The MCF-7 cell line (human breast carcinoma cell line) was purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Penicillin-streptomycin solution, trypsin-EDTA solution, Dulbecco’s modified Eagle’s medium (DMEM/F-12), and 1% antibiotic-antimycotic solution were obtained from Life Technologies GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) and the in vitro toxicology assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA), Dialysis membrane (MWCO 2000) was provided by Sigma-Aldrich (St Louis, Missouri).

Preparation of hyperbranched polyester

Hyperbranched polyester was synthesized using an esterification polymerization according to a reported method. In summary, the melting polycondensation method was used with G as monomer A, and CA monohydrate as monomer AB, at a mole ratio of 1:8, respectively. The amount of glycerol and CA monohydrate used was 0.5 mL (6.6 mM) and 11 g (33 mM), respectively. Glycerol and CA monohydrate were mixed in a polymerization ampoule with a gas and vacuum inlet and heated to 90-110 °C for 20 min with stirring. The mixture was then stirred under vacuum to remove the water generated during the reaction, at temperatures of 130, 140, 145, and 150 °C for 40, 40, 40, and 60 min, respectively, and then kept at room temperature until cool. The product was dissolved in THF and filtered to obtain a clear solution. The solution was then concentrated under reduced pressure and precipitated several times in n-hexane. The precipitated product was dissolved in 10 mL of THF and dialyzed against 150 mL of THF for 4 h. The whole system was then kept at room temperature before replacing with fresh THF. Dialysis was continued for 4 h and then the contents of the dialysis bag were transferred to the reaction flask. Finally, the THF was evaporated under reduced pressure and a pure viscous colorless compound was obtained.

Encapsulation of luteolin by copolymer

Copolymer was dissolved at a concentration of 1.67 × 10⁻⁷ mM in 5 mL of deionized water and mixed for one hour. Then, 0.1 mM luteolin in DMSO was prepared as a stock solution. Luteolin was then added dropwise to the above solution to achieve different luteolin concentrations (25, 50, 100, and 150 μM). The solution was stirred at room temperature for 24 h in the dark to obtain the final formulation.

FTIR spectroscopy

Infrared (IR) spectra of the copolymer were acquired using a Nicolet 320 spectrophotometer FT-IR. The specimens were prepared by mixing the fine powder with KBr and pressing. The spectra were obtained at a resolution of 4 cm⁻¹ in the range 4000-500 cm⁻¹.
NMR

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker DRX 400 (400 MHz) spectrometer in D.O. The samples were dissolved in D.O and the spectra were recorded at 400 MHz. The resulting data were processed and analyzed using ACDLABS/1D NMR software.\textsuperscript{25}

GPC

The molecular weight of the copolymer was determined using a Knauer GPC equipped with a Smartline Pump 1000 with a PL Aqua gel-OH mixed-H 8-μm column connected to a differential refractometer, with water as the mobile phase at 25 °C.\textsuperscript{26}

DLS

The particle size of the copolymer in water was determined using DLS (Malvern Instruments Ltd., Malvern, UK). The scattered light intensity was detected at 90° to the incident light. Measurements were carried out using samples of polymer dispersed in 1 mM NaCl at 25°C at a ratio of 0.01%, w/v. The mean size was determined from six measurements.\textsuperscript{27}

High-performance liquid chromatography (HPLC) and loading capacity

HPLC analysis was carried out according to a previously reported procedure.\textsuperscript{29} To determine the amount of luteolin loaded in the nanoparticles, a reversed-phase HPLC with a Knauer liquid chromatograph (Smart line; Knauer, Berlin, Germany) was used, equipped with an ultraviolet detector (Well chrome, K-2600; Knauer) and a reverse-phase C18 column (Nucleosil H. P.; 25 cm × 0.46 cm internal diameter, pore size 100 Å; Knauer) using isocratic elution and UV absorbance detection. The mobile phase was made up of 40% methanol and 60% aqueous solution (0.1% phosphoric acid). The method was calibrated at a wavelength of 348 nm with a column temperature of 30°C, with a volume of injection of 1 μL, and a mobile phase flow rate of 1 mL/min.

After 24 h of stirring, encapsulation was considered complete for the aqueous solutions of copolymer and luteolin (25, 50, 100, and 150 μM). Some unencapsulated luteolin precipitated from the solution. Purification was carried out according to a reported procedure.\textsuperscript{28} To remove the unencapsulated luteolin residue, the solution was centrifuged at 6000 rpm and the supernatant was filtered using a Millipore Millex-HN syringe driven filter unit with 0.22 μm cut-off. An aliquot of the solution after filtration was injected into the HPLC to determine the amount of encapsulated luteolin.

Cell culture

MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% antibiotic-antimimetic solution. Cells were incubated in a humidified incubator containing 5% CO$_2$ at 37 °C. The cells were maintained in an exponential growth phase by periodic subcultivation. MCF-7 cells between the second and the fifth passages were used.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide color (MTT) assay

The cell viability and the cytotoxic effect of the drug delivery system at different concentrations were measured using the MTT method. This method is based on the ability of yellow tetrazolium salt to form purple formazan crystals as a result of enzymatic reduction by mitochondrial dehydrogenases.\textsuperscript{29} Briefly, the MCF-7 cells were plated in 96-well flat-bottom culture plates and treated with different concentrations of nanoparticles-luteolin (NP-L) (8, 17, 34, and 51 μm) and luteolin (8, 17, 34, and 51 μm). All cultured cells were incubated for 24, 48, and 72 h in a humidified incubator at 37°C. After 24, 46, and 72 h, 10 μL of the MTT labeling reagent was added to each well and then the plates were incubated at 37 °C in 5% carbon dioxide for 4 h. The resulting formazan was dissolved in 100 L of DMSO with gentle shaking at 37 °C, and the absorbance of the solutions was measured at 595 nm with an ELISA reader (Spectra MAX; Molecular Devices, USA).

Determination of ROS

The Intracellular H$_2$O$_2$ levels were determined by measuring the fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA), which is oxidized by nonspecific esterases to produce DCF, and quantitatively oxidized by ROS to produce fluorescent DCF. MCF-7 cells were incubated with 10 μM of DCFH-DA at 37 °C for 30 min and then analyzed using a bioassay multi-detection fluorescence plate reader (Biotek-FLx 800). DCF fluorescence was assessed at 485-nm excitation and 520-nm emission. ROS production was determined from an H$_2$O$_2$ standard curve (10-200 nM).

Intracellular calcium (Ca$^{2+}$) assay

Fluo-3-acetoxyethyl ester (Fluo-3/AM, 10 mM, Intercim) dissolved in DMSO was used to detect the intracellular Ca$^{2+}$ of MCF-7 cells. Fluo-3/AM binds free cytoplasmic calcium and emits green fluorescence (peak at 526 nm) detected using FL1.\textsuperscript{31} Briefly, 2 buffer solutions were prepared for washing: buffer 1 (Phenol red-free DMEM containing 10 mM HEPES [4-(2-hydroxyethyl)] piperazine-1-ethanesulfonic acid, pH 7.0) and buffer 2 (DMEM containing 10 mM HEPES and 5% fetal calf serum, pH 7.4). First, 1 mL aliquots of MCF-7 suspension (1 × 10$^6$ cells/mL) were washed with buffer 1 and resuspended in 200 μL of buffer 1. Then, 0.4 μL of Fluo 3-AM (1.0 M in DMSO) was added. The cells were incubated at room temperature for 30 min and washed with buffer 2 before the assay. Flow cytometry analysis of MCF-7 intracellular Ca$^{2+}$ was carried out using a FAC scan caliber™ flow cytometer (Becton Dickinson, California, USA).
Statistical analysis
Each experiment was carried out at least three times. The differences between treatments or groups were determined using $t$-tests and analysis of variance. Data are expressed as mean ± standard deviation ($\bar{x} \pm s$). For all tests, $P$ values of less than 0.05 were considered significant. The term half maximal inhibitory concentration (IC50) refers to the concentration of a drug or toxicant that induces a response halfway between the baseline and maximum after a specified exposure time.

RESULTS
Evaluation of hyperbranched polyester
The three expected absorption bands; O-H at 2977 cm$^{-1}$, COOH at 1632 cm$^{-1}$, and COOR at 1733 cm$^{-1}$ are present in the IR spectrum of the hyperbranched polyester (Figure 1). The $^1$H NMR spectrum shows 5 proton environments. Two multiplet signals for the glycerol hydrogens can be seen at 2.00-2.10 and 4.10-4.25 ppm for the methine and methylene protons, respectively, and three types for hydrogen for citric acid appeared at 2.6-2.9 ppm (methylene protons) and at 4.95 ppm (hydroxyl functional groups) (Figure 2A). The $^{13}$C NMR spectrum shows 6 types of carbon, with glycerol showing 2 signals and citric acid showing 4 signals. Signals C3 and C4 are carbonyl groups, signal C2 refers to the methylene groups, and signal C1 is the fourth carbon of citric acid. The C5 and C6 signals are assigned to the methylene and methine of glycerol, respectively (Figure 2B). The obtained molecular weight for the copolymer was ~5689 g/mol by GPC (Figure 3). The DLS experiments showed that the size of the hyperbranched polyester was 80-90 nm (Figure 4).

Nanoparticle-luteolin and luteolin effects on cell viability
There was no evidence of cell death in MCF-7 cells at free luteolin or nanoparticle-luteolin concentrations of 8 or 17 μM. However, incubation with free luteolin at high concentrations, 34 and 51 μM, led to a reduction in cell viability ($P < 0.05$ vs control). Incubation with docetaxel and nanoparticle-luteolin (34 and 51 μM) led to a reduction in cell viability ($P < 0.001$ vs control) (Figure 6).

Effect of nanoparticle-luteolin on ROS generation
Treatment with docetaxel and nanoparticle-luteolin (34 and 51 μM) significantly increased reactive oxygen species (ROS) levels in MCF-7 cells ($P < 0.001$ vs control). However, free luteolin and nanoparticle-luteolin at concentrations of 8 and 17 μM did not increase intracellular ROS (Figure 7).

Effect of nanoparticle-luteolin on Cai$^{2+}$
Cai$^{2+}$ significantly increased for docetaxel and nanoparticle-luteolin (34 and 51 μM) treated samples ($P < 0.001$ vs control). Free nanoparticles and free luteolin at concentrations of 8 and 17 μM did not increase Cai$^{2+}$ (Figure 8).

DISCUSSION
In this study, we synthesized hyperbranched polyester with high water solubility and biocompatibility that is a promising material for biomedical applications. It has been shown that nanoparticles can penetrate the cell through various mechanisms, including direct diffusion across the plasma membrane or via receptors.35-37 In a previous investigation, citric glycerol hyperbranched polyester was loaded with different concentrations of the anticancer drug cisplatin.13 In this study, to determine the copolymer structure we used different analytical techniques including Fourier transform infrared spectroscopy (FTIR) and $^1$H and $^{13}$C nuclear magnetic resonance spectroscopy ($^1$H NMR and $^{13}$C NMR). The FTIR spectrum showed hydroxyl and carbonyl groups at 2977, 1632, and 1733 cm$^{-1}$, The $^1$H NMR spectrum showed that there are 5 types of hydrogen. Two multiplet signals for the hydrogens of glycerol were detected at 2.00-2.10 and 4.10-4.25 ppm for the methine and methylene protons, respectively, and three types of hydrogen for citric acid were identified at 2.6-2.9 ppm for the methylene protons and at 4.95 ppm for the hydroxyl functional groups. The $^{13}$C NMR spectrum showed 6 types of carbon. Glycerol gives rise to 2 signals and citric acid leads to 4 signals. Signals C3 and C4 are attributed to carbonyl groups, signal C2 is assigned to the methylene groups and the C1 signal is the fourth carbon of citric acid. The C5 and C6 signals are assigned to the methylene and methine of glycerol, respectively. The obtained molecular
weight (GPC) for the copolymer was ~5689 g/mol. The DLS findings showed that the hydrodynamic diameter of the hyperbranched polyester is around 80-90 nm. A previous report found that the luteolin encapsulation efficiency of the polymer, measured by UV-Vis spectrophotometry, was approximately 9%. In this study, a HPLC method was used to determine the loading capacity of the copolymer. The standard curve indicated that the luteolin loading capacity of the hyperbranched polyester obtained was 34%. In a previous study, the effects of various concentrations of luteolin on normal human umbilical vein endothelial cells demonstrated that high concentrations of luteolin increase ROS generation and intracellular calcium, and reduce cell viability, however at low luteolin concentrations these findings were reversed. The alignment of cell death and intracellular calcium indicates that calcium plays an important role in cell death. The nifedipine effect suggests that the pores of calcium entering the cell are elevated. Plasma membrane pores that can be done by the ROS, may also contribute to the entry of calcium into the cell. On the other hand, it is known that increasing the production of ROS can suppress the balance of the cell as a result of oxidative stress. In addition, nanoparticles can induce cell death through ROS production. Similarly, the change in calcium ions leads to reduced cellular function, and energy and metabolic imbalance, leading to cell death. A number of environmental toxicants cause cell death by increasing the entry of calcium into the cell and releasing calcium from intracellular sources. In a previous study, zinc nanoparticles were shown to cause cell death through the release of calcium from intracellular sources in Chinese hamster ovary cells, as a result of the effect of muscarinic receptors. In this work, our results demonstrated that nanoparticle-luteolin at high concentrations (34 and 51 μM) was cytoxic to MCF-7 cells and reduced cell viability and increased ROS generation and intracellular calcium at 48 h. However, at lower luteolin concentrations these find-
ings were reversed. In addition, the anticancer drug delivery system described led to water-soluble nanoparticle-luteolin and is a powerful vector for killing cancer cells in vitro by increasing intracellular ROS and Ca^{2+} levels.

In conclusion, we observed that nanoparticle-luteolin at high concentrations (34 and 51 μM) reduced the growth of MCF-7 breast cancer cells. It appeared to exert cytotoxic activity through activation of ROS generation and increases in Ca^{2+}. The experimental results demonstrated that nanoparticle-luteolin is a potential therapeutic agent for human breast cancer treatment.

ACKNOWLEDGMENT

The authors are grateful for the financial and technical support of Ilam University of Medical Science.

REFERENCES

37 Abbasi N, Akhavan MM, Rahbar-Roshandel N, Shafiei M. The effects of low and high concentrations of luteolin...


Bootman MD, Collins TJ, MacKenzie L, et al. 2-aminothoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca\(^{2+}\) entry but an inconsistent inhibitor of InsP\(_3\)-induced Ca\(^{2+}\) release. FASEB J 2002; 16(10): 1145-1150.
