Preparation of Caffeic Acid Phenethyl Ester-Incorporated Nanoparticles and Their Biological Activity

HYO-YOUNG LEE,[1] YOUNG-IL JEONG,[2] EUN JIN KIM,[3] KYUNG DONG LEE,[4] SEON-HEE CHOI,[1] YUN JIN KIM,[5] DA HYE KIM,[6] KI-CHOON CHOI[7]

[1]Department of Medical Science, Pusan National University School of Medicine, Yangsan, Gyeongnam 626-770, Korea
[2]Pusan National University Hospital, Pusan 602-739, Korea
[3]R&D Center, M.I.Tech Company, Ltd. Pyeongtaek, Gyeonggi-do 451-864, Korea
[4]Department of Oriental Medicine Materials, Dongsin University, Naju 520-714, Korea
[5]Department of Family Medicine, Pusan National University Hospital, Pusan 602-739, Korea
[6]Faculty of Life and Environmental Science, Shimane University, Matsue, Japan
[7]Grassland and Forages Division, National Institute of Animal Science, Rural Development Administration, Chungnam 331-808, Korea

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ABSTRACT: The aim of this study is to fabricate caffeic acid phenethyl ester (CAPE)-incorporated nanoparticles using methoxy poly(ethylene glycol)-*b*-poly(ε-caprolactone) (CE) copolymer and to study their antitumor activity against pulmonary metastasis model of CT26 colon carcinoma cells. CAPE-incorporated nanoparticles showed spherical shapes having small diameters less than 300 nm and CAPE was continuously released from CE nanoparticles over 4 days. CAPE-incorporated polymeric micelles properly inhibited proliferation and induced apoptosis of CT26 cells as well as CAPE itself. Furthermore, they showed similar anti-invasive and antimigrative effect against CT26 cells at *in vitro* compared with CAPE itself, indicating that CAPE-incorporated nanoparticles have at least equivalent anticarcinogenic activity against CT26 cells compared with CAPE itself. At pulmonary metastasis model of CT26 cells using nude mouse, CAPE-incorporated nanoparticles have superior antimetastatic efficacy against, that is, control treatment with pulmonary metastasis model showed significant increase of lung weight because of the metastasis of tumor cells, whereas CAPE or CAPE-incorporated nanoparticles properly inhibited metastasis of tumor cells. We suggest CAPE-incorporated nanoparticles as a promising candidate for antimetastatic chemotherapeutic agent. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:144–154, 2015
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INTRODUCTION

Caffeic acid phenethyl ester (CAPE) is a component of propolis of honeybee hives.[1] Various *in vitro* biological effect of CAPE has been reported, such as suppression of acute immune responses, antioxidant, antimitogenic, anticarcinogenic, anti-inflammatory, and antiallergic effect.[2–6] CAPE has activities in suppression of carcinogenic incidence and various anticancer effect by inducing apoptosis pathways in cancer cells.[4] Especially, CAPE is known to be a specific inhibitor of activation of nuclear transcription factor NF-κB.[7] CAPE was completely inhibited the activation of NF-κB by tumor necrosis factor in a dose- and time-dependent manner. Liao et al.[8] and Hwang et al.[9] described the inhibitory effect of CAPE against angiogenesis, tumor invasion, and metastasis against cancer cells. Furthermore, antibacterial and antiviral activity of CAPE has also been reported by several investigators.[10–13]

Nanoparticles or colloidal carriers have been extensively investigated in biomedical field.[14–16] Because of its small particle sizes, nanoparticles or colloidal carriers are regarded to an ideal vehicle for site-specific drug delivery and tumor targeting.[17] Sakhrani and Padh[17] described the efficacy of nanoparticulate-based drug delivery, that is, nanoparticles is a promising vehicle for the site-specific delivery of anticancer drugs to the disease site, whereas systemic injection of drug induces systemic toxicity and side effects because of its lack of target-specific affinity. Especially, nanoparticles are studied to amplify biological activity of antineoplastic drug and natural products. For example, tea polyphenol-loaded poly(lactide-co-glycolide) (PLGA) nanoparticles revealed 30-fold higher prevention advantages on the DNA damage than native tea polyphenols such as theaflavin and epigallocatechin-3-gallate.[18] Nanoparticles also enhanced anti-invasive efficacy of all-trans retinoic acid against CT26 colorectal cancer cells.[19] In other words, nanoparticles are appropriate vehicle for solubilization of lipophilic bioactive agents and to use as an intravenous injection of such compounds. As CAPE is a hydrophobic compound, nanoparticles can be used to solubilize CAPE into an aqueous solution and to amplify biological activity in an *in vitro–in vivo* system.[20,21] Liposomal formulation of CAPE was studied to solve solubility problem of it and tested *in vivo* tumor-bearing mice.[22]

The aim of this study is to prepare CAPE-incorporated nanoparticles using poly(ε-caprolactone)/poly(ethylene glycol) (CE) block copolymer and to investigate their biological activity. Properties of CAPE-incorporated nanoparticles were studied and their biological activity was studied in the terms of antioxidant activity, anti-invasive activity against CT26 colorectal carcinoma cells, and antibacterial activity.

Correspondence to: Ki-Choon Choi (Telephone: +82-41-580-6752; Fax: +82-41-580-6779; E-mail: choiwh@rda.go.kr); Kyung Dong Lee (Telephone: +82-61-330-3261; Fax: +82-61-330-3261; E-mail: leekd@dsu.ac.kr); Young-Il Jeong (Telephone: +82-55-360-3873; Fax: +82-55-360-3879; E-mail: nanomed@naver.com)

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EXPERIMENTAL

Materials

Caffeic acid phenethyl ester, methoxy poly(ethylene glycol) (MPEG) ($M_n = 5000$ g/mol), ε-caprolactone, thiazolyl blue tetrazolium bromide (MTT), and dialysis membranes (molecular weight cut off : 2000 g/mol) were purchased from Sigma Chemical Company (St. Louis, Missouri). Polyoxyethene (60) hydrogenated caster oil (HCO-60) was a product of Nikko Chemical Company Ltd., (Tokyo, Japan). Acetone, methanol (MeOH), methylene chloride (MC), and dimethyl sulfoxide (DMSO) were used as an extrapure grade.

Synthesis of CE Block Copolymer

The CE block copolymers were synthesized as reported previously.[23] Mixtures of MPEG and ε-caprolactone in round-bottomed flask were cooled and degassed with a pump. This was then sealed off and placed in an oil bath at 180°C. Polymerization was performed for 2 days, and then the product was dissolved in MC. This solution was poured into an excess amount of cold MeOH to precipitate block copolymer and this solution was filtered off to remove unreacted MPEG or ε-caprolactone. The filtered products were washed with MeOH three times and the final products were obtained by drying at reduced pressure for 2 days.

The molecular weight (MW) of CE block copolymer was determined using 400 MHz ^{1}H NMR spectroscopy (Varian 400 MHz NMR; Agilent Technology Inc., Santa Clara, California) in chloroform-d form.

As diblock copolymers have amphiphilicity in aqueous solution, critical association concentration (CAC) of CE was estimated by fluorescence spectroscopy (Shimadzu F-7000 Spectrofluorometer; Shimadzu Company Ltd., Tokyo, Japan) using pyrene as a probe. Various concentrations of CE diblock copolymer in pure water was prepared and then pyrene was added to this solution (final concentration: 6.0×10^{-7} M). This solution was equilibrated for 3 h at 65 C. Then, excitation fluorescence spectra were measured (emission wavelength: 390 nm). Excitation and emission bandwidths were 1.5 and 1.5 nm, respectively.

Preparation of CAPE-Incorporated Nanoparticles

The CAPE-incorporated nanoparticles using CE block copolymers were fabricated as follows: 5–10 mg of CAPE was dissolved in 0.5 mL of DMSO. Fifty milligram of CE block copolymer was separately dissolved in 5 mL of acetone. These solutions were mixed and poured into 20 mL of deionized water. After that, nanoparticles were immediately formed and solvent was removed at reduced pressure using rotary evaporator. To remove residual solvent, resulting solution was introduced into dialysis tube (MWCO = 2000 g/mol) and dialyzed against deionized water for 1 day. Resulting nanoparticle solution was used for analysis or lyophilization.

Empty nanoparticles were prepared by similar procedure in the absence of CAPE.

Drug contents and loading efficiency of CAPE in the nanoparticles were evaluated as follows: 5 mg of lyophilized nanoparticles were dissolved in 10 mL of DMSO and then diluted with DMSO. Contents of CAPE in the nanoparticles were estimated using an UV-spectrophotometer (UV Spectrophotometer 1800, Shimadzu Company Ltd.) at 336 nm. To avoid interference of polymer, empty nanoparticles were dissolved in DMSO and then used for blank test. Drug contents and loading efficiency were calculated as following equation:

$$\text{Drug contents} = \frac{\text{Amount of CAPE in the nanoparticles}}{\text{Weight of nanoparticles}} \times 100$$

$$\text{Loading efficiency} = \frac{\text{Residual amount of CAPE in the nanoparticles}}{\text{Feeding amount of CAPE}} \times 100$$

Characterization of Nanoparticles

The morphological observation of the nanoparticles was performed with transmission electron microscope (TEM; JEOL JEM-2000 FX II; JEOL, Tokyo, Japan). One drop of nanoparticle solution was placed onto a carbon film coated on a copper grid for TEM. Three hours later, phosphotungstic acid [0.05% (w/w) in deionized water] solution was dropped onto them. Observation of nanoparticles was performed at 80 kV.

Particle size of empty and CAPE-incorporated nanoparticles was measured with a dynamic laser scattering (DLS-7000; Otsuka Electronics Company, Tokyo, Japan).

Aqueous Solubility of CAPE

Aqueous solubility of CAPE was determined as follows: 10 mg of CAPE was added to phosphate-buffered saline (PBS; 0.01 M, pH 7.4) as a solid and this solution was magnetically stirred for 12 h at 36°C. Then, this solution was filtered using filter paper (Whatman International Ltd., Maidstone, England) to remove large precipitates. This solution was filtered again using syringe filter (0.8 μm, Minisart®; Sartorius Stedim Biotech, UK). After that, this solution was used to measure solubility test. Concentration of CAPE was measured using an UV-spectrophotometer (UV spectrophotometer 1800; Shimadzu Company Ltd.) at 336 nm. For comparison, CAPE dissolved in DMSO was diluted with PBS and then used to draw standard curves. Final DMSO concentration was maintained at 10% (v/v) at standard samples and test samples. Standard curves were drawn at 0.005–0.1 mg/mL.

For comparison, more than 50 mg of lyophilized nanoparticle solid was reconstituted into 4 mL PBS and then filtered with syringe filter (0.8 μm, Minisart®; Sartorius Stedim Biotech). Then, aliquots of this solution were dissolved in DMSO and then diluted with PBS 10 times. Then, this solution was used to CAPE concentration.

Drug Release Study *In Vitro*

The release rate of CAPE from nanoparticles was performed *in vitro*. Five milligram of nanoparticles reconstituted in 5 mL of PBS (0.01 M, pH 7.4) was put into dialysis tube. Then, this was introduced into 50 mL tube with 45 mL of PBS. After that, release experiment was performed in a shaking incubator (stirring speed: 100 rpm; maintenance temperature: 37°C). The whole media were taken at specific time intervals to measure concentration of CAPE in the media. To avoid saturation of CAPE in the release media, whole media was exchanged with fresh PBS. The concentration of released CAPE was measured