Synthesis, Characterization and Anti-Cervical Cancer Cell Properties of Bovine Serum Albumen Curcumin Conjugate

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Abstract — Curcumin, which has been used in ancient Indian Ayurvedic system of medicine, has recently been shown to have anti-cancer activity. However, its poor solubility in water has restricted its development as an effective anti-cancer agent for clinical use. We report the synthesis of a well-defined water soluble Bovine Serum Albumin (BSA)-curcumin conjugate. The conjugate was characterized via Gel electrophoresis and Fast Protein Liquid Chromatography (FPLC). The drug loading was determined by MALDI-TOF. The potent anti-cervical cancer activity was validated by cytotoxicity properties against the HeLa cancer cell line via the MTT assay.

Keywords — Curcumin, Bovine Serum Albumin, Conjugate

INTRODUCTION

Curcumin, the primary active ingredient in the spice turmeric, is a hydrophobic polyphenol derived from the rhizome of the herb Curcuma longa and has been shown to have a wide spectrum of biological and pharmacological activities. Traditionally, turmeric has been used as a spice in South Asian cooking, as a cosmetic and in the ancient Ayurvedic system of medicine particularly as an anti-inflammatory agent. Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antimicrobial, anti-carcinogenic and anti-Alzheimer’s disease activity. Various animal models and human studies have shown that curcumin is extremely safe even at very high doses. The pharmacological safety and potential efficacy of curcumin makes it a potential compound for treatment and prevention of a wide variety of human diseases. Despite these promising studies, curcumin has not yet been approved as a therapeutic agent; this is mainly due to its low plasma solubility and bioavailability. Three broad approaches toward increasing the bioefficacy of curcumin have been reported. (A) In the first approach curcumin is formulated via encapsulation in liposomes, phospholipid complexes or nanoparticles; all of these methods have been designed to increase its circulation time, permeability and the resistance to metabolic degradation. The leaching of curcumin from the formulations, poor release of encapsulated curcumin and the ill defined loading of the drug in the formulation are some of the problems associated with this approach. (B) The second approach involves curcumin analog synthesis to improve its water/plasma solubility and bioefficacy. The synthesis of analogs has an inherent limitation: the safety and bioactivity of curcumin has already been established and it is FDA GRAS (generally regarded as safe); with analogs one is around 15 years behind in the clinical approval process, in vitro and in vivo efficacy has to be first established. (C) The third technique is the synthesis of curcumin based prodrug conjugates. This is the major focus of our research endeavors. The advantages of prodrug conjugates arise from the facts that (1) they are well defined constructs where the amount of drug loaded can be estimated correctly, (2) macromolecular conjugates will be targeted to tumors which have leaky vasculature (Enhanced Permeability and Retention (EPR) effect), and (3) they confer enhanced water/plasma solubility and are designed to release the drug at the site of tumor. We have recently reported a general route to reactive monofunctional curcumin derivatives. The reactive groups can be used to attach curcumin to various water/plasma solubilizing agents to produce new generations of curcumin based prodrugs. We have recently reported the synthesis of a curcumin labeled water soluble dendrimer and an antibody curcumin conjugate which eliminates brain tumors in vivo in the mouse model.

Albumin has been used for decades as a carrier for various therapeutic drugs for the treatment of diseases like cancer, diabetes. There are a number of approved drugs in the market which include the taxol albumin nanoparticle Abraxane® for treating metastatic breast cancer, Leveimir® the albumin-binding derivative of human insulin, for treating...
types 1 and 2 diabetes mellitus. Also, there are albumin-conjugated drugs which are in clinical trials such as INNO-206, an albumin-binding prodrug of doxorubicin entering phase II studies against sarcoma and gastric cancer, and CJC-1134-PC, an albumin-modified exendin-4 conjugate for treating type 2 diabetes mellitus in phase-I clinical trials. The maleimide derivative of these drugs binds selectively to the cysteine-34 position endogenous albumin. In the current study we present the synthesis of water soluble Bovine Serum Albumin (BSA)-curcumin conjugate which serves as model system for a curcumin based anti-cancer drug. We have chosen BSA because several conjugates based on albumin are already FDA approved. We have developed a synthetic methodology to produce water-soluble BSA-curcumin conjugate via the synthesis of a monofunctional curcumin derivative in which one of the phenolic groups of curcumin has been chemically modified with a reactive carboxylic acid. This was further reacted to afford an N-hydroxysuccinimide derivative of curcumin, which was then coupled with the lysine residues of BSA to form the BSA-curcumin conjugate (Fig. 1). The bioactivity of conjugate was found to be significantly better than curcumin and its derivatives (Fig. 2) when evaluated against the HeLa cervical cancer cell line.

**Methods**

**Reagents and Chemicals**

Curcumin, α-cyano-4-hydroxycinnamic acid (4-HCCA), trifluoroacetic acid (TFA), acetonitrile (ACN), formic acid, bovine serum albumin (BSA), isopropanol and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyterrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Curcumin mono-alkyne was synthesized in our lab in accordance with literature procedure. (Fig. 2)

**Synthesis of Curcumin mono-carboxylic acid (5-(4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenoxy)-5-oxopentanoic acid)**

A solution of 2.01 g (5.46 mmol) of curcumin, 112 mg (0.92 mmol) of DMAP and 1.33 mL (9.55 mmol) of Et$_3$N was prepared in 100 mL THF. A solution of 0.685 g (6 mmol) of glutaric anhydride (95%) in 5 mL THF was added dropwise to the above mentioned curcumin solution. The mixture was stirred and refluxed under argon atmosphere overnight. THF was removed under vacuum, 55 mL EtOAc was added, followed by the addition of 15 mL of 1M HCl; the mixture was stirred for 10 minutes. The organic phase was separated and extracted with EtOAc three times; the solvent was then removed and dried with anhydrous sodium sulfate. The product was purified via column chromatography, eluting with CH$_2$Cl$_2$:MeOH (95:5). Yield: 69 %. $^1$H NMR (CDCl$_3$), δ (ppm): 2.10-2.12 (t, 2H); 2.56-2.58 (t, 2H); 2.69-2.72 (t, 2H); 3.87 (s, 3H); 3.94 (s, 3H); 5.83 (s, 2H); 6.48-6.57 (t, 2H); 6.48-6.57 (m, 1H); 6.94-7.16 (m, 5H); 7.59-7.62 (d, 2H). $^{13}$C NMR (CDCl$_3$), δ (ppm): 19.87; 32.65; 55.70; 101.48; 109.73; 111.23; 114.91; 120.83; 121.42; 123.02; 124.03; 127.22; 133.76; 133.90; 139.25; 141.06; 146.89; 148.09; 151.09; 170.85; 177.24; 181.59; 184.51.

**Synthesis of Curcumin mono-NHS (2,5-dioxocyclopentyl 4-((1E,6E)-7-(4-hydroxy-3-methoxyphenyl)-3,5-dioxohepta-1,6-dienyl)-2-methoxyphenoxy glutarate)**

Curcumin mono-carboxylic acid (235 mg, 0.486 mmol), N-hydroxysuccinimide (83.91 mg, 0.729 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (112.97 mg, 0.583 mmol) were dissolved in 10 mL anhydrous THF in a 50 mL round bottom flask. The reaction was allowed to run at room temperature for 12 h. The solvent was evaporated and the reaction mixture was dissolved in dichloromethane. The resulting reaction mixture was washed with water and sat. aq. NaHCO$_3$, followed by 20 mL of brine; dried under vacuum to yield an off yellow solid. The crude product was finally purified via column chromatography using CH$_2$Cl$_2$:MeOH (95:5). Yield: 212 mg (76.4%). $^1$H NMR (600 MHz; CDCl$_3$), δ (ppm): 1.11-1.15 (t), 1.23-1.33 (t), 1.57-1.59 (d), 1.67-1.69 (m), 1.90-1.91 (t), 2.15-2.19 (m), 2.65-2.94 (m), 2.41 (s), 3.79-3.90 (m), 5.82-5.85 (d), 6.48-6.58 (m), 6.89-6.91 (d), 6.03-7.15 (m), 7.54-7.61 (m), 8.0 (s). $^{13}$C NMR (150 MHz; CDCl$_3$), δ (ppm): 19.92, 20.28, 24.83, 25.33, 25.50, 25.57, 29.78, 32.40, 32.78, 33.66, 49.33, 55.07, 55.80, 55.83, 101.55, 101.84, 111.38, 111.42, 114.54, 120.95, 121.07, 121.62, 123.06, 123.15, 124.22, 127.42, 133.06, 134.10, 133.94.
141.02, 141.15, 141.23, 146.97, 148.17, 151.22, 151.24, 151.29, 151.32, 157.63, 166.11, 169.12, 169.21, 170.42, 170.85, 172.20, 181.74, 183.11, 184.80. 

Synthesis of BSA-Curcumin Conjugate and Purification
5 mg BSA was dissolved in 1 mL sodium bicarbonate buffer (pH 8.5) at room temperature. 50 mg curcumin mono-NHS was dissolved in 100 µL anhydrous DMSO. Then two solutions were mixed and stirred at 0°C for 2 h. The reaction mixture was centrifuged at 5,000 rpm for 5 min. The clarified supernatant was collected and dialyzed with a cellulose membrane (MWCO 3.5 kDa) against phosphate buffer solution (PBS) (pH 7.4) for 24 h. The dialyzed product was characterized via Fast Protein Liquid Chromatographic system (Akta Purifier, Amersham Biosciences) using HiPrep Sephacryl S-200 (GE Healthcare) size-exclusion column in PBS (pH 7.4) as the eluent. The peak with maximum absorbance at 415nm and 280nm was collected using a peak fractionation system. The product (BSA-curcumin conjugate) was lyophilized and further characterized by MALDI-TOF mass spectrometry.

Matrix-assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)
The thin layer solution was prepared as a saturated solution of 4-HCCA in TFA/Acetonitrile/water (0.1%:2:1). One part of this saturated solution was combined with three parts of isopropanol. Using a micropipette 30µL of this solution was spread as thin layer on the target plate. One milligram of the BSA-curcumin conjugate was dissolved in 100µL PBS. BSA was used as control. The sample was diluted [1 part into 20 parts of matrix solution (saturated sinnapic acid in FWI (3:1:2) (formic acid: water: isopropanol)]. 1 µL sample/matrix mixture was spotted on the target plate. After the matrix crystallized the excess liquid was removed by vacuum aspiration. Mass Spectroscopy (MS) experiments were performed using a MALDI-time-of-flight mass spectrometer (Bruker Daltonik, Bremen, Germany).

SDS-PAGE
SDS-PAGE was performed with BSA-curcumin conjugate and BSA as controls using a 4-20% PAGE gel SDS Cassette Gel. The gel cassette was visualized using a Fischer Scientific trans illuminator with a 312nm UV lamp, prior to staining the gel with Coomassie brilliant blue.

Cytotoxicity assay
HeLa cells were maintained in DMEM (Dulbecco’s Modified Eagle Medium): Nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 1% ITS (human insulin, human transferrin, sodium seleite), 0.1% penicillin.

Cytotoxicity of the BSA-curcumin was analyzed by the MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. HeLa cells were plated in a 96-well plate at a density of 5,000 cells/well. Cells were then incubated for 24 h at 37 °C in presence of 5% CO₂ until they were well flattened and distributed evenly in the plates. Cells were washed with media and then treated with various concentrations of curcumin/curcumin derivatives dispersed in media. Next, treated cells were further incubated for 24 h at 37 °C and in presence of 5% CO₂. MTT solution (5 mg/mL dissolved in PBS) was then added to each well in an amount equal to 10% of the culture media volume. The MTT treated cells were then incubated for 2 h. After the incubation, formazan crystals in each well were dissolved by adding an amount of MTT solubilization solution (isopropanol-HCl) equal to the initial culture media volume. Each well then was carefully mixed to form a homogeneous solution by pipetting up and down several times. The plate was covered with aluminum foil and allowed to shake for 15 minutes at room temperature. The absorbance of each well was measured at 690nm and 570nm.

Results
BSA was modified by reacting the surface lysine groups with curcumin mono-NHS (1b) to produce the BSA-curcumin conjugate. The formation of the conjugate was confirmed via size exclusion Fast Protein Liquid Chromatography (FPLC) where the conjugate BSA-curcumin eluted earlier (due to its higher molecular weight) in comparison to the control BSA sample. The BSA-curcumin conjugate peak had significant absorbance at 280nm arising from the protein component and at 430nm arising from curcumin at the 137.6mL elution volume in the FPLC chromatogram; this confirms the attachment of curcumin to BSA. The control BSA peak at the elution volume 142.6mL does not have any absorbance at 430nm (Fig.3).

Fig.3: FPLC chromatogram of BSA (control) and BSA Curcumin conjugate (1c).

MALDI-TOF was used to estimate the average loading of curcumin per BSA in the conjugate. Mass spectra were collected and a profile over the mass range 10,000 – 90,000 m/z was created from the average spectra of each sample.
Effect of BSA-curcumin conjugate on HeLa cells

To investigate the cytotoxicity of the BSA-curcumin conjugate, we performed the MTT assay on HeLa cells with BSA-curcumin conjugate, curcumin and its derivatives. We dissolved/dispersed BSA-curcumin, curcumin, curcumin mono-carboxylic acid, and curcumin mono-alkyne directly in media at various concentrations (10mg/mL, 5mg/mL, 2.5mg/mL and 1.25mg/mL). The result showed only the BSA-curcumin conjugate had significant inhibitory effect against HeLa cells (Fig. 6). The reason is curcumin and its derivatives were insoluble in water and could not inhibit the growth of HeLa cells. In contrast BSA-curcumin was readily soluble in water; this increased solubility amplified the bioactivity against HeLa cells and inhibited their growth. At the concentration of 2.5 mg/mL (~36.6µM) of BSA-curcumin, only 25% of the HeLa cells survived, while curcumin has much a lower inhibitory effect.

Discussion:
The therapeutic activity of curcumin as a potent anti-cancer agent is limited by its poor water solubility resulting in low...
bioavailability and absorption. Curcumin can be attached to the protein via its various functional groups but previous reports indicate that curcumin’s functional groups are important for its activity. (37-41) Here we have developed a novel methodology to produce a well defined completely water soluble curcumin-albumin conjugate. Curcumin is attached to the BSA via a cleavable ester linkage which would be hydrolyzed by cytosolic esterases, releasing free curcumin inside the target cells; the conjugate serves as a prodrug. The conjugates would be targeted to tumors via the EPR effect. The cleavage of the payload in vivo from the curcumin-albumin conjugates is not likely to result in adverse side effects because it results in the release of a harmless FDA approved nutraceutical curcumin. This is important, as current chemotherapy drugs used to treat cancer patients are limited by their indiscriminate toxicity to normal tissues. Moreover, our evaluation of the cytotoxicity of the water soluble BSA-curcumin conjugate indicates that it has excellent anti-cancer activity against HeLa cells. In conclusion, we have successfully synthesized a well-defined water soluble curcumin-albumin conjugate. The conjugate was characterized by FPLC, SDS PAGE and MALDI-TOF. The BSA curcumin conjugate can specifically target tumor cells via the EPR effect and will ultimately serve as a convenient and highly efficient platform towards a potent, non-toxic treatment of cancer.

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REFERENCES


[38] Wright JS. Predicting the antioxidant activity of curcumin and curcuminoids. THEOCHEM 2002; 91:207–17.

