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Expanding Therapeutic Utility of Carfilzomib for Breast Cancer Therapy by Novel Albumin-coated Nanocrystal Formulation

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Abstract

Carfilzomib (CFZ) is the second-in-class proteasome inhibitor with much improved efficacy and safety profiles over bortezomib in multiple myeloma patients. In expanding the utility of CFZ to solid cancer therapy, the poor aqueous solubility and *in vivo* instability of CFZ are considered major drawbacks. We investigated whether a nanocrystal (NC) formulation can address these issues and enhance anticancer efficacy of CFZ against breast cancer. The surface of NC was coated with albumin in order to enhance the formulation stability and drug delivery to tumors via interactions with albumin-binding proteins located in and near cancer cells. The novel albumin-coated NC formulation of CFZ (CFZ-alb NC) displayed improved metabolic stability and enhanced cellular interactions, uptake and cytotoxic effects in breast cancer cells *in vitro*. Consistently, CFZ-alb NC showed greater anticancer efficacy in a murine 4T1 orthotopic breast cancer model than the currently used cyclodextrin-based formulation. Overall, our results demonstrate the potential of CFZ-alb NC as a viable formulation for breast cancer therapy.

Keywords

proteasome inhibitor; carfilzomib; nanocrystal; albumin; breast cancer

1. Introduction

Breast cancer is ranked first in the incidence of disease and second in cancer-related deaths in women worldwide [1, 2]. Among different subtypes of breast cancer, triple-negative breast cancer (TNBC), which lacks the expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), is notorious for its aggressive behavior and resistance to endocrine and anti-HER2 therapies (e.g., tamoxifen, trastuzumab) [3, 4]. Thus, novel and effective therapies for TNBC are urgently needed. A growing body of preclinical evidence supports that the proteasome is an effective therapeutic target against breast cancers including TNBC [5, 6]. Bortezomib, the first-in-class proteasome inhibitor, reversibly inhibits the proteasome with its boronic acid pharmacophore. While bortezomib has shown remarkable successes in multiple myeloma therapy, it has brought only marginal therapeutic benefits to patients with solid cancers including breast cancer [7]. The lack of clinical efficacy of bortezomib is attributed in part to its poor penetration into the targeted solid tumor tissues and the narrow therapeutic window due to dose-limiting toxicities arising from proteasomal inhibition in normal tissues and off-target interactions with non-proteasomal targets [5, 8].

Carfilzomib (CFZ, Kyprolis®) is the second-in-class proteasome inhibitor and has received the FDA approval a decade after the approval of bortezomib. Unlike bortezomib, CFZ covalently and irreversibly inhibits the proteasome with its epoxyketone pharmacophore and displays minimal interactions with non-proteasomal targets, thereby improving safety profiles over bortezomib [9]. Due to the poor solubility of CFZ (log P = 4.13, ~ 10 μ M in PBS [10]), the current injectable formulation of CFZ contains 16-fold molar excess of sulfobutyl ether β -cyclodextrin (Captisol®) as a solubilizer. When administered as the current cyclodextrin (CD)-based formulation, CFZ has a very short circulation time (plasma half-lives of 0.5 – 1 h in humans and preclinical species) due to the extensive hepatic and extra-hepatic metabolism and wide tissue distribution [11]. As an alternative formulation, previous studies have surveyed the feasibility of CFZ nanoformulations including liposomes, polymer micelles, tethered polymer nanoassemblies, and neutrophil membrane-coated nanoparticles [12–15]. The shortcomings of these approaches however include the low drug loading efficiency, burst release of the drug in blood circulation, and no-to-marginal improvement in *in vivo* anticancer efficacy. Thus, there is a clear need for a new nanoformulation of CFZ with improved pharmacokinetic (PK) and biodistribution (BD) profiles as well as pharmacodynamics, such as anticancer efficacy and proteasome target modulation.

To develop a cost-effective nanoformulation with an improved circulation time and anticancer efficacy *in vivo*, we designed a CD-free, nanocrystal (NC)-based formulation of CFZ. NC formulations exploit poor water solubility of anticancer drugs to produce injectable nanoparticles with a high drug content [16–18]. NCs with sufficiently high lattice energy can circulate for a prolonged period and may gain selective access to solid tumors via leaky vasculature. To facilitate favorable interactions of NCs with cancer cells (thereby enhancing the delivery of NCs to cancer cells), the surface of NCs can be modified with various molecules. In particular, albumin has gained considerable interest as a potential

surface modifier, due to low toxicity, biocompatibility, and the ability to reduce interactions with phagocytes in the reticuloendothelial system (RES) [19–21]. Moreover, albumin can interact with cancer cells based on its increased use as an energy source in rapidly proliferating cancer cells [22]. It has been reported that nano-albumin-bound-drugs (nab-drugs) can aid drug permeation across tumor vessels [23, 24]. Albumin-bound paclitaxel (nab-paclitaxel) displayed promising efficacy and safety profiles in patients with breast cancers including TNBC [25]. Although the exact mechanisms by which nab-drugs reach cancer cells are not fully understood, it is suggested that albumin facilitates the movement across the endothelial cell membrane by binding to the gp60 receptor and sequentially interacting with other albumin-binding proteins such as SPARC (Secreted Protein Acidic and Rich in Cysteine) abundantly expressed in and near cancer cells [26]. Importantly, our previous studies support that albumin coating helps deliver paclitaxel and docetaxel NCs to solid tumors through the interaction with those albumin-binding proteins [27, 28].

In this study, we examined whether NCs with albumin coating could enhance the delivery of CFZ to breast cancers. Using albumin-coated CFZ NCs (CFZ-alb NC), we observed that CFZ-alb NC enhanced cytotoxic effects in breast cancer cell lines and tumor growth suppression in a murine 4T1 orthotopic breast cancer model, compared to the CD-based formulation of CFZ (CFZ-CD). We also observed the improved BD profiles of CFZ-alb NC in 4T1 orthotopic breast cancer model and investigated potential involvement of SPARC in the enhanced tumoral accumulation of CFZ-alb NC.

2. Material and methods

2.1 Materials

CFZ was purchased from Shenzhen Chemical Co. Ltd. (Shanghai, China). Pluronic F127 (F127) was a gift from BASF (New York, NY, USA). Human serum albumin (HSA; 96% purity assessed by agarose gel electrophoresis, Cat. No. A1653) and (2-hydroxypropyl)- β -cyclodextrin were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were from Thermo Fisher Scientific (Waltham, MA, USA).

2.2 Preparation of CFZ-alb NC

CFZ-alb NC was prepared by crystallizing CFZ in the presence of F127 and coating the resulting CFZ-NC with HSA, as reported previously [28]. Briefly, 6 mg of CFZ and 48 mg of F127 were dissolved in a mixture of 3 mL of chloroform and 1 mL of methanol and dried in a round-bottom flask by rotary evaporation at 40 °C for 10 min. The dry drug-polymer film was hydrated with 6 mL of water and sonicated in a water bath for 10 s. The NC suspension was further probe-sonicated for 5 min with an amplitude of 40% and a 4:1 duty cycle every 5 s in an ice bath. Subsequently, the suspension was incubated with 48 mg of HSA for 24 h at room temperature on a rotating shaker. Excess surfactant and albumin were removed by centrifugation at 135,700 g for 15 min at 4 °C.

Optionally, CFZ-alb NC was fluorescently labeled by adding rhodamine B as 0.1 mg/mL aqueous solution during the film hydration step. The rhodamine B-labeled CFZ-alb NC was called *CFZ-alb NC. To quantify the incorporated rhodamine B, the fluorescence intensity

($\lambda_{\text{ex}}/\lambda_{\text{em}} = 540 \text{ nm}/625 \text{ nm}$) of a known amount of CFZ-alb NC dissolved in acetonitrile (ACN) was measured with a SpectraMax M3 microplate reader (Molecular Devices, CA, USA). Most *in vitro* studies were performed with freshly prepared CFZ-alb NC. For *in vivo* studies, CFZ-alb NC was lyophilized with trehalose as a cryoprotectant (CFZ-alb NC:trehalose = 1:5 (w/w)) (Fig. S1). The lyophilized product of CFZ-alb NC was dissolved in PBS before administration. The concentration of trehalose in the dosing solution (containing 0.8 mg/ml of CFZ) was estimated to be around 4.3 mOsm/L, which is unlikely to compromise the isotonicity (approximately 300 mOsm/L).

2.3. Characterization of CFZ-alb NC

2.3.1. Size, surface charge, and morphology—The size and surface charge of CFZ-alb NC diluted in phosphate buffer (1 mM, pH 7.4) were measured with a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). The morphology of CFZ-alb NC was examined with a FEI Tecnai G2 20 Transmission Electron Microscope (FEI Company, Hillsboro, OR, USA) at 200 keV. Samples were mounted on a 400-mesh Cu grid with Formvar/carbon supporting film, followed by negative staining with 1% uranyl acetate. Images were captured with a SIA L3-C2 megapixel CCD camera (Scientific Instruments and Application, Duluth, GA, USA). The length of CFZ-alb NC was analyzed from TEM images with ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.3.2. Powder X-ray diffraction (PXRD)—CFZ and CFZ-alb NC were analyzed with a Rigaku SmartLab diffractometer (Rigaku Americas, The Woodlands, TX, USA) equipped with a Cu-K α radiation source. The powder samples were placed on glass holder, and diffraction patterns were obtained from 5 to 40° 2 θ at a scan speed of 4° per min and a step size of 0.02°. The voltage and current used were 40 kV and 44 mA, respectively.

2.3.3. Contents of CFZ and albumin in CFZ-alb NC—The CFZ content in CFZ-alb NC was determined by HPLC. CFZ-alb NC with a pre-measured mass was dissolved in a 50:50 mixture of ACN and water and filtered with 0.45 μm syringe filter. HPLC was performed with an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA), equipped with Ascentis C18 column (25 cm $\times$ 4.6 mm, particle size: 5 μm). The column was initially equilibrated with 40% of ACN, followed by a linear gradient of ACN from 40% to 80% over 10 min and from 80% to 40% over the next 10 min at a flow rate of 0.7 mL/min. CFZ was detected with a UV detector at a wavelength of 210 nm.

The albumin content in CFZ-alb NC was quantified with SDS-PAGE. CFZ-alb NC with a known mass or albumin standard solutions were mixed with 4 $\times$ Laemmli sample buffer and heated at 95 °C for 5 min to separate albumin from CFZ-alb NC. The samples were resolved in a 12% polyacrylamide gel and stained with QC Colloidal Coomassie Stain (Bio-Rad, Hercules, CA, USA). The stained gel was imaged with Azure C300 (Azure Biosystems, Dublin, CA, USA), and albumin bands were subjected to densitometric analysis using the AzureSpot Analysis Software (Azure Biosystems, Dublin, CA, USA). The albumin content was determined by comparing the band intensity of CFZ-alb NC and albumin standards.

2.4. Stability of CFZ-alb NC

2.4.1. Physical stability—To predict the physical stability of CFZ-alb NC, we resorted to a linear relationship between the number of particles and light scattering intensity [29]. The derived count rate (i.e., absolute light scattering) of CFZ-alb NC suspension with a concentration equivalent to CFZ 7.2 – 72 µg/mL was measured in phosphate-buffered saline (PBS) or undiluted fetal bovine serum (FBS) at 37 °C over 24 h with a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). The derived count rate of FBS was subtracted from each measurement made in FBS. In a separate experiment, the dissolution of CFZ-alb NC in FBS was monitored by measuring the count rate continuously over 20 min at the final concentration equivalent to 7.2 or 30 µg/mL of CFZ. Measurements were made twice every two seconds at the measurement position of 4.65 nm and with an attenuator setting of 10.

2.4.2. *In vitro* metabolic stability—To compare the *in vitro* metabolic stability in CFZ formulations (CFZ solution, CFZ dissolved in dimethyl sulfoxide (DMSO); CFZ-CD, CFZ dissolved in 10 mM citrate buffer (pH 3.1) containing 20% (w/v) 2-hydroxypropyl-β-cyclodextrin); and CFZ-alb NC dissolved in PBS), the whole blood and the liver were collected from a male Sprague Dawley rat. The whole blood was treated with heparin (25 IU/mL) and kept on ice until the assay was performed. The isolated liver was washed and homogenized in ice-cold PBS (pH 7.4, a 1:5 ratio of liver weight (g) to PBS volume (mL)). Each of CFZ formulations (4 µL) was added to the whole blood (396 µL) or liver homogenates (396 µL) to result in the final CFZ concentration equivalent to 1 µM. A reaction mixture (40 µL) was taken at 0, 5, 10, 30, and 60 min at 37 °C, and snap-frozen in liquid nitrogen until the measurements of the remaining CFZ concentration.

2.5. Cell cultures and treatments

Human breast cancer cell lines (MDA-MB-231, MCF-7, HCC1943, and HCC1937) were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Mouse breast cancer cell line 4T1 was purchased from ATCC (Manassas, VA, USA). All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin (100 IU/mL-100 µg/mL) at 37 °C in a humidified incubator operating at 5% CO₂.

2.5.1. Cellular uptake study for rhodamine B-labeled CFZ-alb NC (*CFZ-alb NC)—Cells were seeded in a 35 mm glass bottom dish (MatTak, Ashland, MA, USA) at a density of 50,000 cells per dish. After 24 h, the medium was replaced with fresh medium, and the cells were incubated with *CFZ-alb NC equivalent to 30 µg/mL CFZ or free rhodamine B solution at a comparable level for 2 h in complete medium. After washing twice with PBS, the cells were fixed with 4% paraformaldehyde in PBS for 10 min. Following nuclear staining with Hoechst 33342 (2 µg/mL; Cat. No. H1399), cells were imaged with a Nikon A1R confocal microscope (Nikon America Inc., Melville, NY, USA). Hoechst 33342 and *CFZ-alb NC or free rhodamine B were detected with $\lambda_{ex}/\lambda_{em}$ of 407 nm/425 – 475 nm and 561 nm/570 – 620 nm, respectively. For quantitative analysis, cells were prepared in the same manner and analyzed with an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10,000 gated events were acquired, and FL-2

channel ($\lambda_{\text{ex}}/\lambda_{\text{em}}$ 488 nm/585 nm) was monitored to determine the cellular level of rhodamine B.

2.5.2. Cell viability assay—Cancer cells were seeded on 96-well plates (4T1, 2000 cells/well; MDA-MB-231 and MCF-7, 5000 cells/well; HCC1640, HCC1937, and U87MG, 8000 cells/well) 24 h prior to drug treatment. For the continuous drug treatment condition, cells were continuously exposed to CFZ solution or CFZ-alb NC for 72 h. For the pulse treatment condition, cells were treated with CFZ solution or CFZ-alb NC for 2 h, then washed twice with PBS, and grown in drug-free media for 72 h. Viable cells were measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) using Synergy HT plate reader (BioTek, Winooski, VT, USA).

2.6. Animal experiments

BALB/c mice (female, 5 – 6 weeks of age, 19 – 21 g), ICR mice (male, 8 weeks of age, 35 – 38 g), and Sprague Dawley rats (male, 8 weeks of age, 250 – 260 g) were obtained from Nara Biotech Co., Ltd. (Seoul, Korea) or Envigo (Indianapolis, IN, USA), and were acclimatized at the animal research facility in Seoul National University (SNU) or Purdue University (PU) for at least one week. All animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of SNU and PU (SNU-161205-2-1, SNU-170424-2-1, and PU-1503001212).

2.6.1. In vivo anticancer activity—To establish an orthotopic breast cancer mouse model, 4T1 cells (1.5×10^5 cells suspended in 50 μL of complete media) were inoculated into the inguinal mammary fat pad on both left and right sides of BALB/c mice (on day 0). When the palpable knob with a similar size appeared on day 4, the mice bearing 4T1 xenograft were randomly assigned to different groups ($n = 5 - 6$ per group) as follows: vehicle only group (10 mM citrate buffer (pH 3.1)), CFZ-CD at a dose of 3 mg CFZ/kg, and CFZ-alb NC at a dose of 3 mg CFZ/kg. The drug was administered two consecutive days a week for three weeks via tail vein injection. Tumor size was measured using a digital caliper; tumor volume = $\text{length} \times (\text{width})^2 / 2$. The body weight of mice was monitored every 2 – 3 days until day 22. Whole blood samples and harvested major organs were snap-frozen and stored at -80°C until analysis or fixed in 4% paraformaldehyde and embedded in paraffin for histological examination.

2.6.2. Plasma pharmacokinetic study—CFZ-alb NC and CFZ-CD were injected via tail vein into ICR mice at the dose of 3 mg/kg ($n = 3 - 4$ per group). At the pre-determined time points (2, 5, 20, 60, 120, 360, 600, and 1,440 min), blood samples (10 – 20 μL) were collected from the retro-orbital plexus of the mice using microhematocrit tubes. The detailed analytical conditions and assay validation parameters were previously reported [30]. PK parameters were calculated by non-compartmental method using WinNonlin software (Pharsight Corp., Version 5.0.1).

2.6.3. Biodistribution study—CFZ-alb NC and CFZ-CD were injected via tail vein into BALB/c mice harboring orthotopic xenografts (described in 2.6.1.) at an equivalent dose of 3 mg/kg when the tumor size became approximately 200 – 300 mm^3 . After 0.5, 2, and 6

h, the mice were anesthetized with isoflurane/oxygen mixture (3 – 5%:95 – 97%; v/v). At least 800 μ L of blood was collected with a heparinized syringe via cardiac puncture. Tumor and major organs were harvested, weighed, and snap-frozen until analysis. To measure the CFZ amount accumulated in tissues, the tissue homogenates were prepared on ice by adding the following proportion of PBS; 0.2 g tissue per mL PBS for all tissues except the lung (0.17 g tissue per mL PBS) and adrenal glands (0.1 g tissue per mL PBS). Tissue homogenates (100 μ L) were extracted with cold *tert*-butyl methyl ether (600 μ L) containing an internal standard (carfilzomib- d_8 250 ng/mL; Cayman Chemical, Ann Arbor, MI, USA) by vortex mixing. After centrifugation at 3,724 g for 10 min, 400 μ L of organic phase was transferred to a new tube, and evaporated. The samples were reconstituted in 100 μ L of ACN and transferred to a new vial for LC-MS/MS analysis. To measure the CFZ concentration remaining in plasma, 5 μ L of plasma was mixed with H₂O (15 μ L) and ACN (40 μ L) containing an internal standard (carfilzomib- d_8 250 ng/mL; Cat. No. 22558). After vortex mixing, samples were centrifuged at 13,000 g for 10 min. The supernatant (50 μ L) was transferred to a new vial for LC-MS/MS analysis.

2.6.4. Quantification of CFZ in tissue homogenates—Samples were analyzed by the HPLC system coupled to Agilent 6460 QQQ LC-MS/MS (Agilent, Palo Alto, CA, USA) equipped with an electrospray ionization interface operated in a positive ion mode. The chromatographic separation was performed on a C18 column (50 $\times$ 2.0 mm id, 3 mm) with a mobile phase that consisted of 0.1% formic acid in water (A)-0.1% formic acid in ACN (B) at a flow rate of 0.3 mL/min. The gradient was set up as follows: 0.5 min 85% A, 15% B; 3 min 100% B; 4 min 100% B; 5.5 min 85% A, 15% B; 6 min 85% A, 15% B. The autosampler temperature was set at 4 $^{\circ}$ C, and the injection volume was 10 μ L. The optimized source-dependent mass parameters were set as follows: nebulizing gas 45 psi; gas flow, 9 L/min; sheath gas flow, 7 L/min; capillary voltage, 4 kV; gas temperature, 330 $^{\circ}$ C. The compound-dependent parameters, fragment voltage and collision energy, were set at 180 V and 60 V for CFZ and carfilzomib- d_8 with the dwell time of 50 ms. Quantification was performed in the selected reaction monitoring (SRM) mode. The SRM transitions were as follows: for CFZ, m/z 720.3 > 100.1 (quantifier) and m/z 720.3 > 289.1 (qualifier); for the internal standard, m/z 728.3 > 108.1 (quantifier) and m/z 728.3 > 297.1 (qualifier).

2.6.5. Proteasome activity measurement in tissue homogenates from mice that received drug treatment—The homogenized tissue samples in PBS (prepared as described in the section 2.6.3.) were mixed with the same volume of ice-cold passive lysis buffer (Promega, Madison, WI, USA; Cat. No. E1941). After centrifugation of tissue mixtures at 3,000 g for 10 min at 4 $^{\circ}$ C, the supernatant was collected and used for proteasome activity assay (50 – 150 μ g total protein/ μ L verified to be within the linear range of the assay using serial diluted samples). For blood samples, the whole blood (0.2 μ L) was mixed with ice-cold passive lysis buffer (1.8 μ L), and used for proteasome activity assay. To assess the proteasome activity, the tissue supernatant or blood mixture (2 μ L) was incubated with 100 μ M Suc-LLVY-AMC (Bachem, Bubendorf, Switzerland) in the assay buffer (48 μ L; 20 mM Tris-Cl buffer (pH 8.0) and 500 μ M EDTA). The proteasome activity was determined by monitoring the initial cleavage rate of fluorescent 7-amino-4-methylcoumarine from Suc-LLVY-AMC. Fluorescence signals were detected every min for

60 min using a SpectraMax M3 microplate reader (Molecular Devices, CA, USA) at the wavelengths of $\lambda_{\text{ex}}/\lambda_{\text{em}} = 360/460$ nm with the cut-off filter set at 420 nm.

2.7. Involvement of SPARC in the cellular uptake of CFZ-alb NC

2.7.1. Quantification of mRNA expression of hSPARC—Total RNA of breast cancer cells was isolated using PureLink RNA Mini Kit and transcribed to cDNA using SuperScript III First-Strand Synthesis kit. The mRNA level of hSPARC was assessed by quantitative real-time PCR using TOPreal™ qPCR 2X PreMIX (Enzynomics, Seoul, Korea), the primer pair (forward, 5′-tcgacagtcagccgcactct-3′; reverse, 5′-ccgttgactccgacctca-3′), and StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster city, CA, USA). The hGAPDH level was similarly measured as a house-keeping control (forward primer, 5′-gggacttcgagaagaac-3′; reverse primer, 5′-agaggtaccgtaatg-3′), and the relative hSPARC levels were then calculated using the Ct method.

2.7.2. Immunoblotting analysis for hSPARC—Cell lysates were prepared in RIPA buffer, mixed with 4× Laemmli sample buffer, and heated at 95 °C for 5 min. Cell lysates containing the equivalent protein amount of 30 µg were resolved using 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking using 5% milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), the membranes were probed with the antibodies for hSPARC (1:200; R&D Systems, Minneapolis, MN, USA; Cat. No. AF941) and β-actin (Cell Signaling Technology, Danvers, MA, USA; Cat. No. 4970). After washing with TBST, the membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase. Bound antibodies were visualized with an enhanced chemiluminescence substrate using ImageQuant™ LAS4000 (GE Healthcare, Little Chalfont, United Kingdom).

2.7.3. Immunohistochemical analysis for mSparc—Tissue antigen was retrieved by boiling deparaffinized slides in 10 mM Tris, 1 mM EDTA, and 0.03% Tween 20 (pH 9.0) using a pressure cooker. After washing with water and blocking with 4% bovine serum albumin in PBS Tween-20 (PBST), slides were incubated with the mSparc antibody (1:20; R&D Systems, Minneapolis, MN, USA; Cat. No. AF942) for 60 min at room temperature. After washing slides with PBST, a streptavidin-biotin system was used according to the manufacturer's instructions (BioGenex, San Ramon, CA, USA). The slides were counterstained with Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). All sections were examined under a Vectra imaging system (PerkinElmer, Waltham, MA, USA).

2.7.4. Association between hSPARC expression and overall survival in the TCGA dataset—To explore a potential association between the hSPARC expression and the outcomes in breast cancer patients, the datasets available from the ONCOMINE (www.oncomine.org) were analyzed; the datasets were sorted based on the gene name (hSPARC), the analysis type (cancer vs normal analysis), and the cancer type (breast cancer). The case dataset containing the hSPARC expression levels and overall survival of breast cancer patients was downloaded from a breast cancer meta-data set (reported in TCGA, The Cancer Genome Atlas) [31, 32].

2.7.5. Impact of hSPARC knockdown on cellular uptake of CFZ-alb NC in U87MG cells

—To achieve the stable knockdown of hSPARC, U87MG cells were transduced using a lentiviral vector-based shRNA system targeting hSPARC (obtained from Santa Cruz Biotechnology, Dallas, TX, USA) (Detailed experimental methods were reported in the thesis of JH Jo and will soon be published; the thesis available at [<http://hdl.handle.net/10371/138009>]). To assess the cellular uptake of CFZ-alb NC, U87MG cells with hSPARC knockdown or wildtype control were seeded on a poly-L-lysine-coated 12-well plate (at a density of 1×10^5 cells/well) in complete media containing FBS. After 24 h, cells were rinsed with Opti-MEM media, exposed to unformulated CFZ or CFZ-alb NC in Opti-MEM media for 2 h, and then washed 3 times with PBS. After harvesting cells with 50% methanol (200 μ L), aliquots of cell lysates were transferred to separate tubes for the measurement of CFZ via LC-MS/MS analysis (100 μ L) or the protein quantitation assay (30 μ L). For LC-MS/MS analysis, cell lysates (100 μ L) were subject to probe sonication and extracted with ice-cold ACN (100 μ L) containing an internal standard by vortex mixing. After centrifugation at 3,000 g for 10 min, 150 μ L of supernatant was transferred to a new vial for LC-MS/MS analysis.

2.8. Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD) of the replicates from independent experiments unless stated otherwise. Student's *t*-test or ANOVA with a *post-hoc* test was performed to determine statistical significance using GraphPad Prism (GraphPad Software, version 7.0.3.). A *p*-value < 0.05 was considered significant.

3. Results and discussion

3.1. Preparation and characterization of CFZ-alb NC

CFZ-alb NC was prepared following the two-step method as reported previously [28]. In the first step, CFZ and F127 (an amphiphilic triblock co-polymer of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)) were dissolved in a mixture of chloroform and methanol and subsequently evaporated to form a dry film. Incipient CFZ nanocrystal (CFZ-NC) was formed by hydration of the film. F127 played dual roles: it helped hydrate and retrieve the film from the container (without F127, a dry film of CFZ resisted hydration and only 0.02 wt% of CFZ was retrieved from the film) and prevented excessive crystal growth of CFZ-NC by binding to the NC surface [17, 33]. In the second step, the incipient CFZ-NC was further functionalized with HSA, and the excess HSA and F127 were removed by multiple washing.

The TEM images of CFZ-alb NC showed a rod-like structure with an average length and width of 352 ± 195 nm and 58 ± 15 nm, respectively ($n=135$, analyzed using the Image J software) (Fig. 1A). When measured with a dynamic light scattering (DLS), the size and polydispersity index of CFZ-alb NC were 270.8 ± 21.5 nm and 0.27 ± 0.06 , respectively ($n = 10$ independently prepared batches) (Fig. 1B and Table 1). Although the average size of CFZ-alb NC is relatively large compared to other nanoformulations, the current size may still be sufficient for extravasation through peritumoral vasculature with the reported cut-off size in the range of 200 – 1,200 nm [34–36]. Even if CFZ-alb NC does not freely traverse

the tumor interstitium, CFZ-alb NC may undergo gradual disintegration and release CFZ near cancer cells. The shape of NC appears to depend on the drug itself rather than process parameters. In the previous reports, docetaxel and paclitaxel form crystals with sheet- and rod-like structures, respectively, even with similar procedural methods [27, 28]. The elongated shape of CFZ-alb NC is also considered advantageous for transvascular flux as compared to the spherical shape at an equivalent volume [37, 38] and for resisting uptake by macrophages [39]. CFZ-alb NC was lyophilized with trehalose as a cryoprotectant for a long-term storage of the formulation and verified to redisperse to the original size (Fig. S1).

The zeta potential of CFZ-alb NC was -13.7 ± 3.2 mV, likely due to the HSA present on the surface of NCs (Fig. 1B and Table 1). The surface-bound HSA protected F127-coated CFZ-NC from aggregation (Fig. 1C). In the absence of HSA, CFZ-NC aggregated upon repeated centrifugation and washing procedures, as F127 was gradually removed from the NC surface [40]. Under the same conditions of multiple centrifugation and washing, the size of CFZ-alb NC did not increase. When PXRD patterns were assessed, CFZ powder displayed multiple sharp peaks, indicative of crystalline solids, while CFZ-alb NC showed much attenuated peak intensities, indicative of surface-bound HSA (Fig. 1D) [28]. The contents of CFZ and albumin in CFZ-alb NC were 74 ± 6 % (w/w) and 20 ± 4 % (w/w), respectively ($n = 6$ independently prepared batches) (Fig. 1E and Table 1). When the weight ratio of CFZ to albumin is considered, it is estimated that the albumin content corresponds to approximately 1.5 continuous layers of albumin covering the NC surface (Table S1).

3.2. Enhanced physical and metabolic stability of CFZ-alb NC

We next examined whether CFZ-alb NC can display the physical and metabolic stability. In assessing the physical stability of NCs in aqueous media, light scattering intensity can serve as a convenient and reliable indicator [28, 29]. Stable NCs maintain the light scattering capability, thereby a constant derived count rate, whereas disintegrating or dissolving NCs show a decreasing derived count rate. When tested in PBS, CFZ-alb NC maintained a linear relationship between the derived count rates the concentration within the tested range (7.2 to 72 $\mu\text{g/mL}$ CFZ) up to 24 h (Fig. S2A). In undiluted FBS, similar trends were observed with comparable slopes up to 4 h and slightly decreasing slopes at 7 or 24 h (Fig. 2A). When the derived count rates were continuously monitored over 20 min, CFZ-alb NC (7.2 $\mu\text{g/mL}$ CFZ, Fig. 2B; 30 $\mu\text{g/mL}$ CFZ, Fig. S2B) yielded the signals consistently greater than those of FBS alone. These results support the physical stability of CFZ-alb NC at its concentrations as low as 7.2 $\mu\text{g/mL}$ CFZ, even in the presence of serum proteins. When CFZ-alb NC (3 mg/kg, based on the CFZ content) is administered to a mouse with a typical blood volume of 1.5 – 2.5 mL [41], the initial blood concentration of CFZ-alb NC can be roughly estimated to be 24 – 40 $\mu\text{g/mL}$ CFZ. If the current *in vitro* results measured in undiluted serum were to reflect the physical stability of CFZ-alb NC *in vivo*, one may expect CFZ-alb NC to circulate as intact particulate forms in blood, especially during the early hours after drug administration.

CFZ displays a very short circulation time due to its rapid metabolic inactivation by epoxide ring opening and peptide hydrolysis [42]. To assess whether CFZ-alb NC can improve metabolic stability, CFZ-alb NC was incubated with rat liver homogenates. As a control,

separate reactions were performed with CFZ solution (dissolved in DMSO) or clinically used CD-based CFZ formulation (CFZ-CD, CFZ dissolved in 10 mM citrate buffer (pH 3.1) containing 20% (w/v) 2-hydroxypropyl- β -cyclodextrin). At a concentration equivalent to CFZ 1 μ M, both CFZ solution and CFZ-CD displayed very rapid disappearance of CFZ (<10% remaining after 10 min) in the presence of rat liver homogenates (Fig. 2C). These results are consistent with the previous reports showing the half-lives of CFZ in various rat tissue homogenates in the ranges of 4 – 39 min [41]. On the other hand, CFZ-alb NC displayed much improved stability with approximately 40% of the active drug remaining even at 60 min in the liver homogenates (Fig. 2C). The disappearance of CFZ after addition of CFZ-alb NC and CFZ-CD was quite rapid at early time points and became slower at later time points. The rapid disappearance of CFZ at early time points may arise from the readily available portion of CFZ near the outer layer of NC formulation. At later time points, the drug release from the disintegration of NC formulation may take longer time. Given the results were obtained using liver homogenates (containing a variety of metabolizing enzymes and proteins including the proteasome), it is unknown what contributes to this biphasic pattern of CFZ disappearance and further investigations are warranted. The metabolic stability profiles in whole blood displayed similar trends, finding CFZ-alb NC most stable (Fig. 2D). These results indicate that CFZ-alb NC can confer both physical and metabolic stability, rendering a promising potential to extend the circulation time of CFZ *in vivo*.

3.3. Enhanced cellular uptake and cytotoxic effects of CFZ-alb NC in breast cancer cell lines

In order to assess its cellular uptake, CFZ-alb NC was fluorescently labeled using a small quantity of rhodamine B. The rhodamine B-labeled CFZ-alb NC (*CFZ-alb NC) was comparable to the unlabeled CFZ-alb NC in terms of the average size, zeta potential, and morphology (Table 1 and Figs. S3A and S3B), similar to the previous reports with other drugs and nanoformulations [43, 44]. The content of incorporated rhodamine B in *CFZ-alb NC was 0.34 ± 0.11 wt% ($n = 3$). Established breast cancer cell lines (murine, 4T1; human, MCF7, HCC1937) were incubated with *CFZ-alb NC (30 μ g/mL) for 2 h and observed by confocal microscope imaging (Fig. 3A and Fig. S3C). Little signal was obtained from the incubation of *CFZ-alb NC in the dish without cells (data not shown), ruling out the possibility that the signal obtained with 4T1 cells may come from protein adsorption and subsequent fixation of protein. The incubation with *CFZ-alb NC yielded red fluorescence signals localized in the cytoplasm (Fig. 3A, d-f), indicating cellular uptake of *CFZ-alb NC (merged images shown in Fig. 3A, f). As a control, free rhodamine B solution was tested at an equivalent dye concentration (0.1 μ g/mL). As shown in Fig. 3A (g-i), the treatment of free rhodamine B showed little signal in all three cell lines (Fig. 3A and S3C). No signal from the incubation of *CFZ-alb NC in the dish without cells (data not shown) ruled out the plausible signal from protein adsorption and subsequent fixation of protein. In quantitative analysis by flow cytometry, the fluorescent signal intensities were higher in cells treated with *CFZ-alb NC than those in the control groups (no treatment or free rhodamine B) for all three cell lines (Fig. 3B). Of the *CFZ-alb NC-treated cell lines, HCC1937 cells showed the highest fluorescence signal intensity, followed by 4T1 and MCF-7 cells.

Next, we tested whether the enhanced cellular uptake of CFZ-alb NC leads to greater cytotoxic effects in breast cancer cells. When treated with unformulated CFZ, five breast cancer cell line models (human: MCF7, MDA-MB-231, HCC1143, and HCC1937; murine: 4T1) displayed IC₅₀ values of low nano-molar ranges for CFZ (Fig. S4A). With regard to the expression levels of the proteasome catalytic subunits (β 1, β 2, and β 5), the four human breast cancer cell lines tested were comparable to human multiple myeloma RPMI8226 cells (well-known for its abundant expression of the proteasome and sensitivity to proteasome inhibitor drugs) (Fig. S4B). In addition, the four human breast cancer cells displayed comparable proteasome activities (chymotrypsin-like, trypsin-like, and caspase-like activity, typically attributed to β 5, β 2, and β 1 subunits, respectively) to the RPMI8226 cells (Fig. S4C). Although the expression and activity of proteasomes were similar across the tested cell lines, MCF7 cells were least sensitive to CFZ based on 2D colony formation assay (Fig. S4E). MCF7 cells were also shown to be resistant to bortezomib [5]. The mechanism of MCF7 resistance to proteasome inhibitors remains unclear. Since CFZ is a known substrate of P-glycoprotein (P-gp) [44], the P-gp mediated drug efflux was initially suspected as a possible resistance mechanism in MCF7 cells, but this was excluded given the lack of P-gp expression (Fig. S4D). Cells under 3D spheroid culture conditions are known to enrich stem-like cancer cells [45, 46], and the 3D spheroid formation was highest with CFZ-treated MCF7 cells (Fig. S4F). These results can be cautiously interpreted that a sub-population of stem-like cancer cells existing in MCF7 cells may play a role in the resistance to proteasome inhibitors.

The cytotoxic effects of CFZ-alb NC was tested with either continuous or pulse treatment. Upon continuous exposure at low CFZ concentrations (equivalent to 20 and 50 nM for 72 h), CFZ-alb NC and unformulated CFZ solution (dissolved in DMSO) displayed similar cytotoxic effects (Fig. 4A). These results were expected in that CFZ-alb NC would dissolve over time at sufficiently low CFZ concentrations. Upon intravenous injection, however, CFZ-alb NC will be present initially at high concentrations and rapidly decline, featuring more dynamic changes over time [47, 48]. To mimic such conditions, cells were subject to a pulse treatment at high CFZ concentrations (equivalent to 200 or 400 nM) and grown without CFZ: cells were initially exposed to CFZ-alb NC or CFZ solution for 2 h, rinsed, and grown in drug-free medium for 72 h prior to the measurement of cell viability. Upon a pulse-treatment, CFZ-alb NC displayed a greater cytotoxic effect than unformulated CFZ solution (Fig. 4B). When the intracellular CFZ content was determined by LC-MS/MS after a pulse treatment in HCC1937 cells, CFZ-alb NC led to a greater amount of CFZ than unformulated CFZ solution (Fig. S7E; CFZ-alb NC vs unformulated CFZ solution; 12.7 vs 7.0 pmol/mg protein). These results collectively suggest that CFZ-alb NC gains cellular entry during the initial short exposure to a greater extent than unformulated CFZ solution and possibly serves as an intracellular drug reservoir during the incubation in drug-free medium.

3.4. Enhanced *in vivo* anticancer efficacy of CFZ-alb NC in BALB/c bearing 4T1 breast cancer cells

The *in vivo* anticancer efficacy of CFZ-alb NC was assessed using the murine 4T1 orthotopic breast cancer model established by the transplantation of 4T1 cells in the

mammary fat pad of BALB/c mice, which mimic human cancer pathology with tumors growing at the location of human disease in the presence of the proper stromal environment compared to the subcutaneous model [49]. Similar to the clinical dosing schedule of CFZ [50], intravenous injections of CFZ-alb NC and CFZ-CD (at the equivalent CFZ dose of 3 mg/kg) were carried out on two consecutive days per week for three weeks (Fig. 5A). CFZ-alb NC or CFZ-CD suppressed tumor growth compared to the vehicle only (Fig. 5B). On day 19, the tumor volumes of the mice receiving CFZ-alb NC were smaller than those receiving CFZ-CD ($p = 0.0024$) or the vehicle only ($p < 0.0001$), while the vehicle- and CFZ-CD-treated groups did not show statistical difference. On day 22, all three groups showed difference from each other: CFZ-CD ($p < 0.0001$ vs the vehicle only; $p < 0.0001$ vs CFZ-alb NC) and CFZ-alb NC ($p < 0.0001$ vs the vehicle only; $p < 0.0001$ vs CFZ-CD). Similar results were obtained for the weight of dissected tumor tissues (Figs. 5C and S5A) and specific tumor growth rates (Fig. S5B). During the span of experiment, the body weight of mice showed no major difference among the three groups (the overall changes from the initial weight was $\pm 10\%$, Fig. 5D), suggesting no signs of gross toxicity. The weight loss observed near the end of experiment (in all three groups) are likely due to distant metastasis commonly occurring in 4T1 tumor models (Fig. S5C). Histological examination of major organs (liver and lung) also showed no signs of major toxicities in three groups (Fig. S5D).

At the end of the *in vivo* anticancer efficacy experiments described above, we collected the tumor tissues and blood samples on day 22 (3 days following the last drug dosing on day 19) and attempted to quantify the CFZ levels by LC-MS/MS. The levels of CFZ in tumor tissues were below the lower limit of quantitation (< 1 ng/mL), but those of CFZ in plasma samples were higher in mice receiving CFZ-alb NC than CFZ-CD (Fig. 5E). Because CFZ is a covalent modifier, which forms an irreversible CFZ-proteasome complex undetectable by LC-MS/MS [51, 52], the drug amount measured by LC-MS/MS may not accurately represent the total amount of CFZ reaching a particular tissue (as depicted in Fig. 6B). The recovery of the proteasome activity following inhibition is known to take at least several days due to the slow *de novo* biogenesis rates of proteasomes [53]. Thus, we examined the inhibitory extent of the proteasomal activity in tissues as another indicator of the CFZ distribution. In whole blood samples, the inhibitory extent of the proteasome activity was greater in the CFZ-alb NC group than in the CFZ-CD group ($p < 0.0001$) (Fig. S5B), consistent with the blood levels of CFZ (Fig. 5E). These results suggest that CFZ-alb NC increased the formation of the irreversible, long-lasting complex between CFZ and the proteasome in whole blood (mainly located in red blood cells), likely from the enhanced stability and cellular uptake of CFZ-alb NC over CFZ-CD.

3.5. Comparison of PK and BD profiles of CFZ-alb NC with CFZ-CD

In the previous study, albumin-coated paclitaxel NC was superior in anticancer efficacy and showed more favorable plasma PK and BD profiles compared to Abraxane® at the equivalent paclitaxel dose level [21]. To examine whether CFZ-alb NC confers similar advantages, we first examined the plasma PK profiles of CFZ-alb NC relative to those of CFZ-CD (an equivalent CFZ dose of 3 mg/kg) in ICR mice. Following a single intravenous administration, the plasma CFZ concentrations declined very rapidly in both groups (Fig. 6A). The PK parameters obtained via non-compartmental analysis did not show major

differences between the two groups (Table 2). The volume of distribution at steady-state (V_{ss} , 19.85 ± 0.04 L/kg and 17.01 ± 9.40 L/kg for CFZ-alb NC and CFZ-CD, respectively) and the plasma half-lives ($t_{1/2, initial}$, 21 ± 4 min and 19 ± 5 min for CFZ-alb NC and CFZ-CD, respectively). The V_{ss} and $t_{1/2, initial}$ values of CFZ-CD were comparable to the previously reported values [11, 41, 48]. The systemic exposure of CFZ (assessed by AUC_{inf}) was slightly greater with CFZ-alb NC (57.5 ± 14.0 min·nmol/mL) than with CFZ-CD (45.4 ± 6.9 min·nmol/mL), although the difference did not reach statistical significance. Likewise, the systemic clearance was slightly slower with CFZ-alb NC than with CFZ-CD but did not show statistical significance (76 ± 21 mL/min/kg and 94 ± 15 mL/min/kg for CFZ-alb NC and CFZ-CD, respectively). Similar to the previous reports [11, 41], the clearance of both CFZ-alb NC and CFZ-CD was considerable fast, almost approximating to the hepatic blood flow in mice [54]. These results suggest that the extrahepatic metabolism likely plays an important role in the elimination of CFZ in both groups. Overall, CFZ-alb NC appeared to have a modest improvement over CFZ-CD with regard to the plasma PK profiles.

To examine whether CFZ-alb NC can enhance tumor distribution of CFZ, BALB/c mice bearing 4T1 tumors were injected with a single intravenous dose of CFZ-alb NC or CFZ-CD (an equivalent CFZ dose of 3 mg/kg). Tumor tissues and major organs were harvested at 0.5, 2, or 6 h post-injection, processed to make tissue homogenates, and subsequently analyzed with respect to the CFZ levels by LC-MS/MS (the remaining CFZ quantity as either NC or released CFZ) and the proteasome activity by the enzyme kinetics assay (likely representing the cumulative CFZ access to a given tissue and the formation of the CFZ-proteasome complex in a given tissue). In tumor tissues, the CFZ-alb NC group displayed significantly higher CFZ levels than the CFZ-CD group at 0.5 h post-dosing (9.2 ± 1.7 vs 6.8 ± 1.1 nM CFZ/g tissue, $p = 0.028$) (Fig. 6C). However, this trend was reversed at 2 and 6 h post-dosing, where CFZ-alb NC group displayed lower CFZ levels than the CFZ-CD group (at 2 h, 4.3 ± 0.4 vs 8.2 ± 2.2 nM CFZ/g tissue, $p = 0.0003$; at 6 h, 1.4 ± 0.5 vs 7.0 ± 1.2 nM CFZ/g tissue, $p < 0.0001$). When we compared the extent of proteasomal activity inhibition (by forming the irreversible CFZ-proteasome complex) in tumor tissues, the CFZ-alb NC group displayed a greater or comparable extent of the proteasomal inhibition than the CFZ-CD group (2 h post-dosing, $27\% \pm 5\%$ for CFZ-CD vs $45\% \pm 9\%$ for CFZ-alb NC, $p = 0.0112$) (Fig. 6D). The CFZ levels and proteasome activity in tumors, taken together, suggest that CFZ-alb NC was rapidly entrapped into tumor tissues after administration, subsequently releasing CFZ, and forming the CFZ-proteasome complex.

In both CFZ-alb NC and CFZ-CD groups, CFZ was predominantly distributed to the liver, the lung, and the spleen at 0.5 h post-dosing with a minor distribution into the kidney, the heart, adrenal glands, and the brain (Figs. 6E and S6A). CFZ-alb NC recognized and sequestered by mononuclear phagocytic system (MPS) as exogenous materials likely to be accumulated in MPS-rich organs, such as the liver, the lung, and the spleen [18]. Similar to the plasma PK profiles, CFZ levels in various tissues rapidly declined within 6 h post-injection. At 2 and 6 h post-injection, the CFZ-CD group resulted in CFZ levels in the liver, lung, and spleen comparable to or even higher than CFZ-alb NC group (Figs. 6E and S6A). In the liver (at 2 and 6 h post-dosing), the CFZ-alb NC group showed more pronounced inhibition of the proteasome activity than the CFZ-CD group (Fig. S6B). CFZ-alb NC group had lower CFZ levels than CFZ-CD group in the liver, reminiscent of the results obtained in

tumor tissues (Figs. 6C and 6D). No apparent organ toxicity of CFZ-alb NC was observed based on histological examinations (Fig. S5D). For CFZ-alb NC, the initial accumulation in the RES organs and very limited distribution to the brain were observed as expected from typical nanoparticle interactions and uptake by liver sinusoidal endothelial cells and Kupffer cells, mechanical filtration [55, 56], and poor penetration through the blood brain barrier [57].

The results in CFZ-CD group were rather surprising. Currently, the quantitative data of CFZ BD profiles by LC-MS/MS is limited. An early study used whole-body autoradiogram in rats after an intravenous injection of [^3H]-CFZ (a total CFZ dose of 2 mg/kg, prepared as an injectable CFZ-CD solution) and found predominant distribution of the radioactivity in the liver and lung [58]. It is unknown whether these observed profiles are attributable to the intrinsic properties of CFZ or CD itself. CD derivatives are generally assumed to have a minimal impact on the PK profiles of drugs [59]. However, there are cases where the complexation with CD alters the systemic exposure of certain compounds, especially those with high affinity for CD derivatives [60]. It remains to be determined whether the complexation with CD enhanced the distribution of CFZ to the liver and lung.

3.6. Potential involvement of SPARC in the uptake of CFZ-alb NC to breast cancer and glioma cells

In line with the proposed role of SPARC present in the tumor microenvironment facilitating tumor accumulation of nab-drugs [23, 61], a positive association was reported between SPARC expression and response to nab-paclitaxel in multiple types of cancers (e.g., human head-and-neck cancer, non-small cell lung cancer, HER2-positive breast cancer) [62–64]. However, SPARC-independent pathways may also play a role in delivering nab-drugs to cancer cells [65, 66]. The relative importance of SPARC and non-SPARC pathways may well vary depending on cellular context and cancer types. In the case of breast cancer, the results from publicly available databases indicated that the hSPARC expression was higher in breast cancer tissues than in non-malignant control tissues (Fig. 7A). From the dataset collected from 498 breast cancer patients, the sub-groups with high and low hSPARC levels (top 10% and bottom 10% in the SPARC expression levels, respectively; $n = 50$ each) were identified and compared for their median survival. The higher hSPARC expression level was associated with poorer outcomes (median survival times of 23 and 31 months in the high and low hSPARC expression groups, respectively) (Fig. 7B; Fig. S7A showed similar trend in another dataset ($n = 418$)).

To investigate the potential involvement of SPARC in the delivery of CFZ-alb NC to cancer cells, we first compared the SPARC level among four human breast cancer cell lines used in our study. HCC1937 cells were highest in the mRNA and protein level of hSPARC, followed by HCC1143, MDA-MB-231, and MCF7 cells (Figs. 7C and 7D). The protein level of mSparc in 4T1 cells cannot be directly compared to human breast cancer cells due to species differences. However, the previous analysis confirmed the expression of mSparc in 4T1 cells cultured as monolayer *in vitro* [27].

The hSPARC level showed an apparent (not necessarily implying causality) positive correlation with cellular uptake of CFZ-alb NC (HCC1937 taking up a greater amount of

CFZ-alb NC than MCF7) (Fig. 3B) and also with cytotoxicity of CFZ-alb NC (HCC1937 displaying the greatest enhancement of cytotoxicity by CFZ-alb NC over the unformulated CFZ solution, followed by HCC1143, MDA-MB-231, and MCF7) (Figs. 4B and S7B). hSPARC expression was detected mainly on the plasma membrane of HCC1937 cells (Fig. S7C), and mSparc was abundantly expressed in the 4T1 tumor tissue sections (Fig. 7E). Compared to the 4T1 tumor tissue sections, the immunopositive signal intensity for mSparc was low in normal lung tissue sections. Although there may well exist inter-tissue differences in mSparc levels, the 4T1 tumor tissue sections did show abundant mSparc expression. It was thus postulated that the SPARC expressed in or near cancer cells may facilitate cellular interactions and internalization of CFZ-alb NC, potentially accounting for the enhanced inhibition of the proteasome activity shown *in vivo* (Figs. 5B and 5C).

To obtain experimental evidence on the involvement of SPARC in the cellular uptake of CFZ-alb NC, we examined changes in cellular uptake and cytotoxicity of CFZ-alb NC in breast cancer cells by modulating their SPARC levels. When SPARC was partially knocked down by transient siRNA transfection in HCC1937 cells (Fig. S7D), no appreciable change was observed in cellular uptake (Fig. S7E) or cytotoxicity (Fig. S7F) of CFZ-alb NC. Boosting SPARC levels using decitabine (Fig. S7G) [63] also did not change the cellular uptake (Fig. S7H) and cytotoxic effects (Fig. S7I) of CFZ-alb NC in HCC1143 cells.

In order to assess the involvement of SPARC in the cellular uptake of CFZ-alb NC, we employed a model system that allows for a complete knockdown of SPARC from its high basal expression level. High levels of endogenous SPARC have been reported in glioma [67, 68], especially in U87MG cell lines derived from human glioblastoma. Upon lentiviral transduction of SPARC-targeting shRNA, U87MG cells showed an almost complete knockdown of SPARC at the protein level (Fig. 7F), making it suitable for the proof-of-concept study. When the cellular uptake and cytotoxicity of CFZ-alb NC were assessed in U87MG cells with complete SPARC knockdown, CFZ-alb NC displayed dramatic increases in both the cellular uptake (Fig. 7G) and cytotoxicity (Fig. 7H) compared to the control U87MG cells. These results support the important role of SPARC in delivering albumin-containing nanoparticles to U87MG cells and may warrant further exploration of the SPARC involvement in additional models and the potential utility of albumin coating for the therapy of SPARC-overexpressing tumors including gliomas.

Taken together, our results support that CFZ-alb NC enhances pharmacodynamics of CFZ compared to CFZ-CD formulations by improving its physical and metabolic stability and enhancing cellular uptake by cancer cells. The extent by which CFZ-alb NC improved the anticancer efficacy compared to CFZ-CD was not as pronounced as albumin-coated paclitaxel NC (reported as Cim-F-alb [21, 28]). Possible reasons include the followings: (a) The size of CFZ-alb NC was larger than Cim-F-alb (270 vs 198 nm). It was previously shown that the cellular uptake of paclitaxel NCs significantly decreased as the particle size increased [28]. (b) The conformational status of surface-bound albumin was shown to be an important factor to determine the efficacy of NCs [21]. When the extent of proteolysis by thermolysin was compared using the pulse proteolysis method [28], the surface-bound albumin in CFZ-alb NC was comparable to native albumin ($23.9 \pm 3.9\%$ vs $15.8 \pm 5.1\%$, $n=3$), whereas denatured albumin (boiled at 95 °C for 10 min as a positive control) showed

significantly higher extent of proteolysis than native albumin ($42.0 \pm 9.5\%$ vs $15.8 \pm 5.1\%$, $n=3$). Given the slightly elevated proteolysis, the possible impact of albumin conformation changes on the performance of CFZ-alb NC may not be completely ruled out, but the majority of albumin in CFZ-alb NC appears to maintain its native conformation. (c) Different levels of albumin-interacting proteins including SPARC may impact the extent by which the surface-bound albumin facilitates cellular uptake. Cim-F-alb was tested in C57BL/6 mice harboring xenografts of B16F10 melanoma cells [21], which showed much greater SPARC expression level than 4T1 cells used in our current study [69]. (d) The improvement in the circulation time might have been less with CFZ-alb NC than albumin-coated paclitaxel nanocrystal. Given that paclitaxel has aqueous solubility even lower than CFZ, albumin-coated paclitaxel nanocrystal may have had a longer circulation time as NCs than CFZ-alb NC. Moreover, albumin-coated paclitaxel nanocrystal was administered at 30 mg/kg, much higher than 3 mg/kg for CFZ-alb-NC. Consideration of these possibilities may assist in optimizing CFZ formulations containing albumin for the treatment of breast cancer and other types of solid cancer. Nonetheless, our results indicate a promising potential for CFZ-alb NC as an alternative, CD-free formulation which can improve *in vivo* anticancer efficacy.

4. Conclusion

In summary, CFZ-alb NC was successfully developed as a novel CD-free formulation of CFZ for systemic delivery with a promising potential in breast cancer therapy. Compared to the CD-based formulation, CFZ-alb NC showed enhanced cellular interaction, uptake, and cytotoxicity in breast cancer models *in vitro*. With improved physical and metabolic stability, CFZ-alb NC improved tumor accumulation of CFZ at early time points in a murine 4T1 orthotopic breast cancer model, accompanied by a pronounced inhibition of the proteasomal activity in tumor tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

CFZ	carfilzomib
NC	nanocrystal
CFZ-alb NC	albumin-coated nanocrystal
TNBC	triple-negative breast cancer

HER2	human epidermal growth factor receptor 2
CD	cyclodextrin
PK	pharmacokinetic
BD	biodistribution
RES	reticuloendothelial system
nab-drug	nano-albumin-bound-drug
SPARC	Secreted Protein Acidic and Rich in Cysteine
CFZ-CD	CD-based formulation of CFZ
F127	Pluronic F127
HAS	human serum albumin
ACN	acetonitrile
TEM	transmission electron microscopy
PXRD	powder X-ray diffraction
PBS	phosphate-buffered saline
FBS	fetal bovine serum
DMSO	dimethyl sulfoxide
*CFZ-alb NC	rhodamine B-labeled CFZ-alb NC
SRM	selected reaction monitoring
SD	standard deviation
CFZ-NC	CFZ nanocrystal
DLS	dynamic light scattering
P-gp	P-glycoprotein

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Figure 1.

Physicochemical properties of CFZ-alb NC. (A) Representative TEM image of CFZ-alb NC and size distribution (analyzed from TEM images, $n=135$). (B) The average size and zeta potential of CFZ-alb NC, measured by DLS. Mean values $\pm$ SD ($n=10$ identically prepared batches). (C) The particle size of CFZ NC with or without human serum albumin added during the three repeated procedures of centrifugation and washing, measured by DLS. (D) Powder X-ray diffraction of CFZ powder (black) and CFZ-alb NC (red). Arbitrary unit (a.u.). (E) Content (%) of CFZ and albumin in CFZ-alb NC. Mean values $\pm$ SD ($n = 6 - 10$ independently prepared batches).

Figure 2.

Physical and metabolic stability profiles of CFZ-alb NC. (A) In the presence of undiluted FBS, the derived count rates of CFZ-alb NC maintained linear relationships over the concentrations ranges tested (7.2 to 72 $\mu\text{g/mL}$ CFZ) for 24 h. (B) The derived count rates of CFZ-alb NC (equivalent to 7.2 $\mu\text{g/mL}$ CFZ, in the presence of undiluted FBS) were obtained by continuous monitoring for the first 20 min. (C and D) Remaining amount of CFZ following the incubation with CFZ-alb NC, cyclodextrin-based CFZ formulation (CFZ-CD), and unformulated CFZ solution in rat liver homogenates (C) and whole blood (D). Mean values $\pm$ SD (n=3 independently prepared batches of CFZ-alb NC).

Figure 3.

Cellular uptake of rhodamine B-labeled CFZ-alb NC (*CFZ-alb NC) in breast cancer cell lines. (A) Representative confocal microscopy images of HCC1937 cells following 2 h incubation with *CFZ-alb NC (30 μ g/mL CFZ) (d, e, and f). Free rhodamine B (Rho B) was used as a negative control (g, h, and i). Scale bar = 10 μ m. (B) Intensities of fluorescent signals associated with breast cancer cells (4T1, MCF7, HCC1937) after 2 h incubation with *CFZ-alb NC, measured by flow cytometry. Arbitrary unit (a.u.). Two-way ANOVA followed by Tukey's *post hoc* test. ** $p < 0.0021$, *** $p < 0.0002$. Mean values $\pm$ SD (n= 3 independently and identically performed experiments).

Figure 4.

Cytotoxic effects of CFZ-alb NC in breast cancer cell lines. Cell viability was measured following the treatment of human breast cancer cell lines with CFZ-alb NC or unformulated CFZ solution under continuous treatment (Tx) conditions for 72 h (A) or pulse Tx conditions for 2 h followed by washout (B). Two-way ANOVA followed by Sidak's *post hoc* test. * $p < 0.0332$, ** $p < 0.0021$, **** $p < 0.0001$. Mean values SD (n= 4~5 independently and identically performed experiments).

Figure 5.

In vivo anticancer efficacy of CFZ-alb NC in BALB/c mice bearing 4T1 orthotopic xenograft tumors. (A) Experiment schedule including drug treatments. Mice received intravenous injections of CFZ-alb NC ($n = 5$; 3 mg/kg), CFZ-CD ($n = 5$; 3 mg/kg), or vehicle control ($n = 6$) on two consecutive days per week for three weeks. (B) Tumor growth curves. Two-way ANOVA followed by Tukey's post hoc test. On day 19: CFZ-alb NC vs vehicle, * $p < 0.0001$; CFZ-alb NC vs CFZ-CD, † $p = 0.0024$. On day 22: CFZ-alb NC vs vehicle, * $p < 0.0001$; CFZ-CD vs vehicle, § $p < 0.0001$; CFZ-alb NC vs CFZ-CD, †† $p < 0.0001$. (C) The weight of tumor tissues harvested on day 22. One-way ANOVA followed by Tukey's post hoc test. ** $p < 0.0021$, **** $p < 0.0001$. (D) The body weight of mice during the span of experiment. (E) The concentration of CFZ from plasma collected on day 22 (n.d.; not detected). Mean values $\pm$ SD.

Figure 6.

Comparison of pharmacokinetic and biodistribution profiles between CFZ-alb NC and CFZ-CD following the intravenous administration (a single equivalent CFZ dose of 3 mg/kg). (A) The plasma concentration-time profiles of CFZ after the administration of CFZ-alb NC ($n = 3$) or CFZ-CD ($n = 4$) in ICR mice. Inset, plasma concentration-time profiles of CFZ for the first 2 h. (B) Diagram depicting the irreversible binding of CFZ with the proteasome in tissues. The resulting CFZ-proteasome complex is not measurable by LC-MS/MS, but can be indirectly assessed by measuring the inhibitory extent of the proteasomal activity. (C, D, and E) The results showing the measured amount of CFZ and the inhibitory extent of the proteasome activity in tumor tissues and major organs in BALB/c mice harboring 4T1 xenograft at 0.5, 2, and 6 h following the administration of CFZ-alb NC or CFZ-CD ($n = 4 - 5$ per group). The measured amount of CFZ (C) and the inhibitory extent of the proteasomal activity (D) in tumor tissues. (E) The measured amount of CFZ in major organs. Two-way

ANOVA followed by Sidak's post hoc test. * $p < 0.0332$, *** $p < 0.0002$, **** $p < 0.0001$.
Mean values $\pm$ SD.

Figure 7.

Role of SPARC in the uptake of CFZ-alb NC into cancer cells. (A) The relative expression of hSPARC gene in normal vs cancerous breast tissues (from a dataset in the TCGA database, $n = 64$; the detailed description provided in the methods). (B) The Kaplan-Meier curves depicting overall survival for breast cancer patients with a high and low levels of hSPARC ($n = 498$, the dataset from the previous report [31]). (C and D) The mRNA (C) and protein (D) expression level of hSPARC in breast cancer cell lines. (E) Immunohistochemical staining of mSPARC (positive staining visualized as brown colors using 3,3'-diaminobenzidine) in 4T1 tumor tissue sections. Normal lung tissue section showed a weak staining signal for mSPARC under the same experimental condition (representative images, scale bar = 100 μ m). (F) The protein levels of hSPARC in HCC1937 and U87MG cells following the lentiviral transduction of SPARC-targeting shRNA. (G and H) Comparison of the cellular uptake of CFZ (G) and cell viability (H) in U87MG cells

where SPARC was completely knocked down following exposure to CFZ-alb NC or unformulated CFZ solution. Two-way ANOVA followed by Tukey's post hoc test. *** $p < 0.0002$, **** $p < 0.0001$. Mean values $\pm$ SD.

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Table 1.

Physicochemical properties of CFZ-alb NC and *CFZ-alb NC.

Formulation	Size (nm)	Polydispersity Index	Zeta potential (mV)	Formulation content (wt%)		
				CFZ	albumin	rhodamine B
CFZ-alb-NC	270.8 ± 21.5	0.27 ± 0.06	-13.7 ± 3.2	74 ± 6	20 ± 4	-
*CFZ-alb NC	328.0 ± 48.6	0.21 ± 0.09	-8.5 ± 1.1	82 ± 4	16 ± 3	0.34 ± 0.11

Mean values ± SD from different batches for CFZ-alb NC (n = 10) and *CFZ-alb NC (n = 3).

Table 2.

Pharmacokinetic (PK) parameters following the intravenous administration of CFZ-alb NC or CFZ-CD at an equivalent CFZ dose of 3 mg/kg to ICR mice.

PK parameter (unit)	CFZ-alb NC (n = 3)	CFZ-CD (n = 4)
AUC _{inf} (min•nmol/mL)	57.5 ± 14.0	45.4 ± 6.9
CL (mL/min/kg)	76 ± 21	94 ± 15
V _{ss} (L/kg)	19.85 ± 0.04	17.01 ± 9.40
t _{1/2, initial} (min)	21 ± 4	19 ± 5
MRT (min)	192 ± 125	274 ± 67

AUC_{inf}, area under the concentration-time curve from time 0 to infinity; V_{ss}, volume of distribution at steady state; t_{1/2, initial}, half-life from the initial decline phase (0 – 120 min); MRT, mean residence time; CL, clearance. Student's *t*-test indicated no significant difference. Mean values ± SD.