Polymer Micelle Formulations of Proteasome Inhibitor Carfilzomib for Improved Metabolic Stability and Anticancer Efficacy in Human Multiple Myeloma and Lung Cancer Cell Lines

Lin Ao, Derek Reichel, Di Hu, Hyunyoung Jeong, Kyung Bo Kim, Younsoo Bae, and Wooin Lee

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky (L.A., D.R., K.B.K., Y.B.); Department of Pharmacy Practice, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois (D.H., H.J.); and College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea (W.L.)

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ABSTRACT

Carfilzomib (CFZ) is a second-generation proteasome inhibitor drug approved for the treatment of multiple myeloma. Contrary to its excellent antmyeloma activity, CFZ has shown only limited efficacy in patients with solid malignancies. This lack of efficacy has been attributed in part to rapid degradation of CFZ in the body, possibly hindering the ability of CFZ to access the proteasome target in solid tumors. We hypothesized that polymer micelles, a currently Food and Drug Administration–approved nanoparticle drug delivery formulation, may protect CFZ from metabolic degradation and thus expand the clinical utility of the drug as an anticancer agent. To test our hypothesis, we prepared CFZ-entrapped polymer micelle particles with various compositions and drug release profiles and examined the extent of the CFZ metabolism in vitro using mouse liver homogenates. We also assessed the cytotoxic activities of the CFZ-entrapped micelle formulations in human cancer cell lines derived from B lymphocytes (RPMI-8226) and the lung (H460). Our data indicated that polymer micelle-based formulations can improve metabolic stability and cytotoxic effects of CFZ compared with free CFZ in human cancer cell lines tested. Taken together, these results suggest that polymer micelles may have potential as a delivery system for CFZ with an extended therapeutic utility for nonhematologic malignancies in the future.

Introduction

The proteasome is a multimeric protease complex that is central to the highly-regulated ubiquitin-proteasome protein degradation system (Shen et al., 2013). The proteasome plays a key role in regulating numerous signaling pathways involved in cell proliferation, cell cycle control, and apoptosis, which are often found to be dysregulated in malignant cells (Adams, 2004; Mani and Gelmann, 2005). During the past decade, proteasome inhibition has proven to be an effective anticancer strategy with the Food and Drug Administration approval and revolutionary success of the first-in-class proteasome inhibitor agent bortezomib (BTZ; Velcade) in the treatment of multiple myeloma (Kumar et al., 2008). To improve upon the success of BTZ, a second-generation, more selective proteasome inhibitor carfilzomib (CFZ; Kyprolis) was approved recently (Arastu-Kapur et al., 2011; Chen et al., 2011). CFZ is a tetrapeptide equipped with an epoxyketone warhead that binds to the active site of the proteasome irreversibly (Kim and Crews, 2013). CFZ has demonstrated efficacy in both BTZ-naive and BTZ-resistant patients and possesses a more favorable toxicity profile compared with BTZ (Jagannath et al., 2012; Jakubowiak et al., 2012; Vij et al., 2012). With these improvements, CFZ, along with lenalidomide and dexamethasone, has been recently shown to provide unprecedented benefit in patients with multiple myeloma (Stewart et al., 2015).

Due to its promising anticancer activities and favorable toxicity profile, CFZ has also been explored as a potential therapeutic for malignancies other than multiple myeloma. However, despite the excellent efficacy observed in preclinical models of solid cancer (Yang et al., 2006; Demo et al., 2007; Ao et al., 2012), CFZ demonstrated disappointing results clinically when tested in patients with advanced solid tumors (Papadopoulos et al., 2013). Although the exact mechanisms underlying the discrepancies between the preclinical and clinical observations are currently unknown, one potential explanation is the rapid metabolic degradation of CFZ in vivo (Yang et al., 2011). CFZ degradation in humans is mainly due to peptide cleavage and epoxide ring opening, resulting in plasma half-life of less than 30 minutes (Wang et al., 2013). We postulated that the fast metabolic inactivation of CFZ in the body might hinder the ability of the active drug to accumulate in solid cancer tissues, leading to insufficient target inhibition.
and poor clinical efficacy. Thus, increasing the metabolic stability of CFZ may serve as a strategy to improve its overall anticancer efficacy.

To overcome its poor water solubility issues, CFZ is currently formulated with sulfobutylether β-cyclodextrin for clinical delivery. However, sulfobutylether β-cyclodextrin offers little protective effects against metabolic degradation of CFZ in vivo (Wang et al., 2013); thus, it would be useful to develop alternative CFZ formulations that can improve its metabolic stability in addition to solubility. Our current study explored the utility of polymer micelles in improving the metabolic stability of CFZ against enzyme-mediated degradation and delivering CFZ in a controlled manner. Polymer micelles are highly efficient in entrapping hydrophobic drug molecules inside the core to prevent the drugs from precipitating, binding to serum proteins, or being degraded by enzymes in the body (Sanvicencs and Marco, 2008). Additionally, polymer micelles are readily modified chemically or with attachment of surface ligands to control drug release patterns as well as cell-targeted drug delivery, making them versatile vehicles for delivery (Zhang et al., 2008; Ahmad et al., 2014). Previous studies have also shown that polymer micelles can significantly reduce toxicity and improve anti-cancer activity of chemotherapeutics by achieving sustained drug release and increasing drug exposure to cancer cells (Tan et al., 2013; Werner et al., 2013).

In our current study, we prepared micelle particles composed of biodegradable block copolymers poly- (ethylene glycol) (PEG) and poly-(caprolactone) (PCL), both of which are generally recognized as safe by the Food and Drug Administration. The hydrophilic PEG can improve the solubility as well as increase the circulation time of the particles inside the body (Knop et al., 2010), whereas the hydrophobic PCL portions readily interact with CFZ to allow efficient drug encapsulation (Adams et al., 2003). We prepared six different formulations with varying mol. wt. of the PEG-PCL block to improve drug loading and with calcium phosphate (CP) or deoxycholic acid (DCA) excipients to modulate drug release. To simplify the polymer micelle formulation, we used PEG end-capped with a methoxy group in our study.

In this study, we report our results demonstrating the potential of polymer micelle formulations of CFZ in improving metabolic stability and thereby extending therapeutic applicability of CFZ. These results may serve as the foundation for further optimization of CFZ formulations and investigations of their potential benefits for the treatment of various types of cancers.

All other reagents were obtained from Fisher Scientific (Waltham, MA), unless mentioned otherwise.

Preparation of CFZ-Loaded Polymer Micelles. We prepared a total of six CFZ-loaded polymer micelle formulations in this study, as summarized in Table 1: CFZ-loaded micelles, prepared from PEG-PCL 5-2 or PEG-PCL 5-5, with CP, DCA, or no excipient. In a 50 ml round-bottom flask, 1 mL CFZ (1 mg/ml ethanol) and 100 μl PEG-PCL (100 mg/ml ethanol) stock solutions were mixed at 60°C in the presence of additives: 20 μl ethanol was added for excipient-free micelles, 20 μl NaHPO4 (10 mg/ml in water) for CP-containing micelles, and 20 μl DCA (10 mg/ml in ethanol) for DCA-containing micelles. Ethanol was evaporated under reduced pressure by using a rotatory evaporator to create a thin film at the bottom of each flask. The thin film was rehydrated with deionized water and gently mixed to allow PEG-PCL to self-assemble into polymer micelles entrapping CFZ. For CP-containing micelles, 20 μl CaCl2 (10 mg/ml water) was added in this step to prepare CP-containing micelles. The flask was subsequently sonicated for 5 minutes, and the solution was transferred to a conical tube and centrifuged to remove insoluble free drug, insoluble excipients, and other impurities. The supernatant containing CFZ-loaded micelles was collected and divided into tubes for freeze drying. Freeze-dried micelles were weighed and stored at −20°C until use. To ensure no substantial degradation of CFZ occurs under these conditions, control experiments using free CFZ were carried out and CFZ integrity was assessed using high performance liquid chromatography (HPLC).

The extent of drug loading was determined by mass percentage of composition of CFZ in total CFZ-loaded micelles (w/w%). We quantified CFZ in micelles by [HPLC; Shimadzu LC20 system (Shimadzu, Kyoto, Japan); Agilent XDB-C18 column (Santa Clara, CA) equipped with a photo diode array detector (SPD-M20A using a mobile phase of H2O:CH3CN with 0.1% formic acid [45:55, v/v] at a flow rate of 1 ml/min, 40°C)]. Encapsulation efficiency was defined as the percentage of drug encapsulated to the weight of polymer added. According to the CFZ w/w%, we reconstituted freeze-dried micelles in water or buffer solutions, and serial dilutions were made to prepare CFZ concentrations for experiments described below.

In Vitro Metabolism of CFZ Polymer Micelles in Mouse Liver Homogenates. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Whole livers were harvested from five female C57BL/6J mice (8 weeks old, liver weight 1.203 ± 0.143 g) and washed three times with ice-cold phosphate-buffered saline (PBS; pH 7.4) in petri dishes. Livers were cut into small pieces and homogenized with a 15 ml glass dounce homogenizer (Kimble Chase Life Science, Rockwood, TN) in a 1:5 volume of ice-cold PBS. Liver homogenates (200 mg/ml) were preincubated at 37°C for 1 minute before the addition of free CFZ or micelle formulations of CFZ (final CFZ concentration of 1 μM). An aliquot of 40 μl reaction mixture was taken at 0, 5, 10, and 20 minutes at 37°C; quenched with 120 μl cold acetonitrile containing phenytoin (0.5 μM, an internal standard); and kept on ice for 30 minutes, followed by centrifugation at 16,100g for 15 minutes at 4°C. The concentrations of CFZ in the supernatants were measured using an Agilent 1200 HPLC interfaced with Agilent 6410 Triple Quadrupole tandem mass spectrometry equipped with an electrospray ion source. Briefly, chromatographic separation was carried out with a Waters X Terra MS C18 column (2.1 x 50 mm, 3.5 μm; Waters, Milford, MA). Mobile phase was delivered at 250 μl/min, and the gradient was initiated at 90% A–10% B [A, 0.1% (v/v) aqueous formic acid; B, acetonitrile]. The proportion of mobile phase B was increased to 90% over 1 minute, held constant for 2 minutes, and then restored to the initial composition. Following the injection of 10 μl samples, CFZ and phenytoin were detected by tandem mass spectrometry spectra obtained in the positive ion mode; CFZ by detecting the transitions 720.4 → 402.2 m/z; and phenytoin by detecting the transitions 253.2 → 182.2 m/z. All data were acquired employing Agilent 6410 Quantitative Analysis software.
In Vitro CFZ Release Profiles of Polymer Micelle Formulations. Each formulation was dissolved in PBS to an equivalent CFZ concentration of 100 μM. For each sample, 100 μl polymer micelle formulation was added to five dialysis cups and dialyzed against 1 l PBS at 37°C. A sample of 35 μl of each formulation was removed from the dialysis cups at 0, 1, 3, 6, 24, 48, and 72 hours. CFZ concentration was analyzed by HPLC equipped with a photo diode array detector described above. Percent changes in CFZ concentrations were obtained by normalization to the value obtained at 0 hour for each formulation. Drug release profiles for each formulation were analyzed by assuming second-order release kinetics, and by calculating the area under the curve (AUC) values.

Cell Viability Assay. H460 and RPMI-8226 cells were seeded in 96-well plates at 5000 and 10,000 cells/well, respectively. Following overnight incubation, H460 and RPMI-8226 cells were treated with free drug or the polymer micelle formulations at 50 and 5 nM concentrations, respectively. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) following manufacturer’s protocol. Relative cell viability was obtained from arbitrary luminescence units by normalization to drug-naive controls. Statistical analysis was carried out using GraphPad Prism (GraphPad Software, La Jolla, CA). One-way analysis of variance was used to compare multiple groups, and P < 0.05 was deemed to be statistically significant.

Results

Preparation of Polymer Micelle Particles. Our initial goal was to identify a polymer micelle formulation that will allow us to achieve improved metabolic stability of CFZ. We prepared six polymer micelle formulations of CFZ composed of PEG-PCL block copolymers with identical 5000 g/mol PEG portions and varying PCL portions to maximize drug loading (Table 1). Micelles containing short (2300 g/mol) or long (5500 g/mol) PCL portions are designated as PEG-PCL 5–2.3 (polymer micelles (PM1)) and PEG-PCL5–5 (PM2), respectively. We also incorporated excipients calcium phosphate (CP) or deoxycholic acid (DCA) into PM1 or PM2 formulations, with the goal of improving overall stability of the micelle particles. Formulations with added CP or DCA were found to have increased weights, consistent with successful incorporation of these excipients into the micelle particles. To ensure the final particles contained mainly fully incorporated particles, insoluble CP or DCA was removed by subsequent centrifugation of reconstituted micelle solution. The drug-loading efficiencies of micelle particles with differing sizes of the PCL portions and different excipients varied in the following order: PM1-DCA > PM1-CP > PM2-CP > PM2-DCA > PM2 (Table 1).

Polymer Micelle Formulations Improve CFZ Metabolic Stability to Varying Extents In Vitro. The metabolic stability profiles of the six CFZ-containing micelle formulations were compared against free CFZ solution by measuring the rate of CFZ disappearance in the presence of mouse liver homogenates (Fig. 1). Our results showed that free CFZ rapidly disappears in the presence of mouse liver homogenates, with less than 10% of the active drug remaining in 10 minutes of incubation. In contrast, all six micelle formulations of CFZ had improved stability profiles, demonstrated by five of the six formulations with at least 50% of the active CFZ remaining after 20 minutes of incubation (Fig. 1). Among the six different micelle formulations, PM1-CP particles demonstrated the least protective effect against CFZ degradation in the presence of mouse liver homogenates. All other micelle formulations displayed similar protective effects against the metabolic degradation of CFZ.

Polymer Micelle Formulations Exhibited Varying Rates of CFZ Release Over 72 Hours. Next, we characterized the CFZ release profiles of the micelle particles in vitro by measuring the rate of drug release from the particles over 72 hours. All six formulations demonstrated rapid CFZ release in the first 20 hours, followed by a slower, sustained release for up to 72 hours (Fig. 2). We fit the data to a two-phase decay model and obtained kinetic parameters for the fast- and slow-release phases (Table 2). The half-life values during the fast-release phase were similar among all six particles, averaging about 1 hour. In comparison, the slow-phase release profiles differed substantially among the six formulations, with PM1, PM2-CP, and PM2-DCA being the slowest-releasing formulations (Table 2). We also analyzed CFZ release from the polymer micelles by comparing AUC over 72 hours. AUC analysis is a model-independent analysis method that allows for direct comparison of release profiles. In the current study, the larger AUC values the formulations have, the more CFZ remained was measured at 0, 5 (blank bars), 10 (striped bars), and 20 minutes (checkered bars) following incubation with liver homogenates. Percentage of CFZ remaining values is normalized to 0-minute controls, and results are represented as means ± S.E.M. All polymer micelle groups significantly differ from CFZ control determined by one-way analysis of variance (P < 0.05).

![Fig. 1. In vitro metabolic stability of polymer micelle formulations containing CFZ in the presence of mouse liver homogenates. CFZ remaining was measured at 0, 5 (blank bars), 10 (striped bars), and 20 minutes (checkered bars) following incubation with liver homogenates. Percentage of CFZ remaining values is normalized to 0-minute controls, and results are represented as means ± S.E.M. All polymer micelle groups significantly differ from CFZ control determined by one-way analysis of variance (P < 0.05).](image-url)
would remain with polymer micelles over time. Our AUC analysis results showed that PM1, PM1-DCA, and PM2-DCA had the highest AUC values, corresponding to slower drug release, whereas PM1-CP, PM2, and PM2-CP had low AUC values, corresponding to faster drug release rates (Table 2).

**Polymer Micelle Formulations Display Comparable or Improved Anticancer Activities Compared with Free CFZ.** When tested in H460 human lung cancer cells, we found that four of the polymer micelle formulations had more potent cell-killing effects compared with free CFZ solution (Fig. 3A). To exclude the possibility that components of the micelle particles themselves contributed to the overall cytotoxicity, we also measured the cell-killing effects of empty micelle particles of both mol. wt. compositions in H460 cells. Our results indicated that micelle particles without CFZ entrapment did not possess any cytotoxic effects (Fig. 4A: PM1 empty, PM2 empty). As another control, we also measured the effect of the physical mixture of empty particles and free CFZ on H460 cell viability. No statistically significant difference was observed between the viabilities of cells treated with the physical mixture of particles and CFZ compared with those treated with free CFZ alone (Fig. 4A: PM1 empty + free CFZ, PM2 empty + free CFZ). Because CFZ is currently indicated for the treatment of multiple myeloma in the clinic, we also verified that the polymer micelle formulations are also effective in RPMI-8226 multiple myeloma cells using select formulations. Lower concentrations were used in this case because RPMI-8226 cells are inherently more sensitive to the effects of CFZ compared with H460 cells. Similar cell-killing trends of PM1-DCA and PM2 were observed in RPMI-8226 cells (Figs. 3B and 4B).

**Discussion**

In the current study, we assessed the potential of polymer micelle formulations to improve metabolic stability and overall anticancer efficacy of the proteasome inhibitor CFZ. Our results demonstrated that CFZ-loaded PEG-PCL polymer micelles were more metabolically stable than free CFZ solution, supporting an extended circulation of the active drug in vivo. In addition, several of our micelle formulations of CFZ demonstrated potential to enhance anticancer activity compared with free CFZ solution. The current study is the first to report on the feasibility of polymer micelles in improving the delivery of proteasome inhibitor agents. Further investigations are warranted to examine the utility of these new formulations in improving the antitumor activity of CFZ in vivo for the treatment of solid cancers by examining metabolic stability, target modulation, and tumor accumulation in preclinical animal models.

Several other studies have previously explored similar strategies of utilizing nanoparticle systems to deliver proteasome inhibitor agents. For example, Ashley et al. demonstrated improved efficacy and reduced toxicity of VLA-4 targeted liposomal CFZ particles in multiple myeloma cell lines and xenograft models (Ashley et al., 2014). Similarly, Swami et al. used a targeted liposomal approach to deliver BTZ to the bone microenvironment (Swami et al., 2014). Although the latter study did not achieve direct enhancement in BTZ efficacy, their results supported the utility of nanoparticle systems by demonstrating successful delivery to the targeted bone tissues. Findings from both of these studies support the promising utility of nanoparticle-mediated delivery of proteasome inhibitors by demonstrating the potential therapeutic advantages over the currently existing system. Unlike the previous studies, which focused on PEGylated liposome delivery systems, we explored the capabilities of a much simpler micelle-based system to serve our goals. There are several advantages to micelle-based delivery over liposome-based platforms, including better efficiency in the
loading, carrying, and releasing of hydrophobic drug molecules (Nishiyama and Kataoka, 2006). Furthermore, micelles have been shown to have better tumor-infiltrating abilities, most likely due to their smaller sizes compared with liposomes (Tsukioka et al., 2002; Bae et al., 2005). However, as with any nanoparticle delivery system, polymer micelles have drawbacks associated with their use in drug delivery. One of the major concerns with utilizing micelle-based delivery systems is their lack of stability in vivo due to disintegration of the particles in the body. In this regard, previous studies have demonstrated that PEG-PCL-based polymer micelle particles carrying the anticancer agent paclitaxel exerted more potent anticancer activity in a mouse xenograft model than taxol alone (Tan et al., 2013). These results suggest that PEG-PCL-based micelles are structurally stable enough in vivo to reach the tumor sites.

We have made efforts to address concerns with micelle stability in our current study by including two PEG-PCL micelle particles with the added excipients CP and DCA. Our goals by incorporating such additives into our particles were to further stabilize micelle core structures and to control drug release. CP is a main biologic component of bone and teeth, which has been investigated as a surface modification due to its biocompatibility and rigidity to improve the stability of the hydrophobic drug-loaded nanoparticle cores (Li et al., 2015). In solution, calcium ions and phosphate ions react to form solid CP. Micelles or other nanoparticles can act as nucleation sites for this reaction, which results in the formation of a mineralized surface on the nanoparticle core (Perkin et al., 2005). CP formed away from the micelle particles can be removed easily, due to the insolubility of CP. DCA is a sterol-based bile acid that acts as an endogenous emulsifying agent by forming micelles to aid in interactions with insoluble compounds inside the body. The addition of sterol compounds to micelles has been previously shown to improve micelle stability and in vivo delivery (Oe et al., 2014), as well as reduce drug release rates (Vakil and Kwon, 2008).

As discussed previously, rapid CFZ metabolism in the body may lead to an insufficient accumulation of active drug in distal tumor sites. To better understand the relationship between the interactions of CFZ with the polymer micelle particles and the overall metabolic stability profile, we characterized the release of CFZ from each particle over time. Assuming drug release is dependent on the concentration of the drug inside the particles relative to its surroundings, measuring drug release can help us approximate the strengths of interactions between CFZ and the varying micelle core environments. All six of the polymer micelle formulations demonstrated sustained CFZ release over 72 hours in rapid- and slow-release phases. Differences in the rate of release were observed mainly in the slow-release phases, which most likely derived from stronger and more specific interactions between CFZ and the polymer micelle particles. Based on our results, both PM1 and PM2 were able to release CFZ in a sustained manner, suggesting that the difference in PCL content may not be the dominant factor in controlling CFZ release. Furthermore, PM1-DCA and PM2-DCA appeared to release CFZ more slowly compared with polymer micelles that did not contain DCA, suggesting that interactions between DCA and CFZ may play a role in slowing CFZ release from micelle particles.

Our findings indicated that both the release rate and the metabolic profile of CFZ may be important in determining the overall efficacy of CFZ, but not with a straightforward relationship between rate of CFZ release and metabolic degradation. We found that PM1-CP and PM2-CP, which released CFZ quickly, had the least anticancer activities compared with other formulations. In contrast, PM1-DCA and PM2-DCA, which had slower CFZ release, achieved better anticancer activity. These observations suggest that the rate of drug release plays a role in determining the overall efficacy of the drug, and that micelle particles with slower CFZ release may be better in inducing cancer cell death. In addition to having the quickest release profile, PM1-CP also demonstrated least...
in vitro metabolic stability among the six formulations. This may be an effect of the fast release of CFZ or instability in the structure of the particles that resulted in the breakdown of CFZ and thus poor efficacy in cells. PM2 particles were most effective in inducing cell death among all formulations, despite also possessing a fast CFZ release profile similar to that of PM1-CP. Interestingly, PM2 particles had good metabolic stability despite having quick CFZ release rates, suggesting that metabolic stability may depend on factors other than the rate of drug release. This is also consistent with the fact that differences observed in CFZ release in our study did not necessarily correspond to those observed in metabolic stability profiles. To better understand the necessary balance between drug stability and release rate, further optimizations in particle design and more in-depth assessments of metabolic stability and drug release in vivo will be necessary.

In our current study, we compared six polymer micelle formulations that displayed substantial protection of CFZ molecules from degradation in the presence of liver homogenates. The excipients CP or DCA added with an intent to stabilize the micelle core appeared to have an impact on drug release profiles. For example, PM1-DCA and PM2-DCA appeared to release the drug more slowly than polymer micelles without DCA added (Fig. 2; Table 2). It is plausible that additional favorable hydrophobic interactions between DCA and CFZ may slow down the drug release from polymer micelles. Interestingly, there was no apparent correlation between the extent of protective effects against metabolism and the rate of drug release in vitro. Further investigations with a more extensive library of micelle structures may be necessary to shed light on the relationship between drug-micelle interactions and metabolic stability. Taken together, the results from the current study support the feasibility and potential utility of polymer micelle formulations in treating solid malignancies, and further investigations are warranted for in vivo efficacy studies using animal models of solid malignancies.

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Address correspondence to: Dr. Wooin Lee, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-Gu, Seoul 151-742. E-mail: wooin.lee@snu.ac.kr

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