1521-0103/355/2/168–173$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 2015 by The American Society for Pharmacology and Experimental Therapeutics
http://dx.doi.org/10.1124/jpet.115.226993
J Pharmacol Exp Ther 355:168–173, November 2015

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.)

Received June 25, 2015; accepted August 25, 2015

ABSTRACT

Carfilzomib (CFZ) is a second-generation proteasome inhibitor drug approved for the treatment of multiple myeloma. Contrary to its excellent antimyeloma 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

This work was supported by the National Institutes of Health [Grants R01-CA128903 and R15-CA156601]; American Foundation for Pharmaceutical Education Pre-Doctoral Fellowship; and Basic Science Research Program, National Research Foundation of Korea, Ministry of Science, ICT and Future Planning [NRF-2014R1A1A3050645].
dx.doi.org/10.1124/jpet.115.226993.

ABBREVIATIONS: AUC, area under the curve; BTZ, bortezomib; CFZ, carfilzomib; CP, calcium phosphate; DCA, deoxycholic acid; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PCL, poly-(caprolactone); PEG, poly-(ethylene glycol); PM, polymer micelle.

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 (Sanvicens 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 anticancer 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.

Materials and Methods

Cell Lines and Reagents. PEG *N*-hydroxysuccinimide ester was obtained from NanoCS (Boston, MA). Branched poly(ethylene imine) (25,000 mol. wt.), palmitoyl chloride, HEPES buffer, dimethyl sulfoxide, tetrahydrofuran, diethyl ether, dimethyl formamide, and ethanol were purchased from Sigma-Aldrich (St. Louis, MO). PEG-PCL block copolymers with mol. wt. 5000–2300 (PEG-PCL 5-2) or 5000–5500 (PEG-PCL 5-5) were purchased from Polymer Source (Montreal, QC, Canada). CFZ was purchased from LC Laboratories (Woburn, MA). All other reagents used in the metabolism studies were purchased from Sigma-Aldrich. Established human cancer cell lines derived from lung (H460) and B lymphocytes (RPMI-8226) were purchased from American Type Culture Collection (Manassas, VA) and maintained according to American Type Culture Collection–recommended conditions. 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 Na_2HPO_4 (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 $CaCl_2$ (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 H_2O:CH_3CN 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 drug added. Drug-loading efficiency was defined as the weight 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 1.203–1.431 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 16,100*g* 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 XTerra MS C18 column (2.1 × 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.

TABLE 1
Description of the six polymer micelle formulations containing CFZ and their respective drug-loading and encapsulation efficiencies

Formulation	Description	Drug-Loading Efficiency	Encapsulation Efficiency
		%	
PM1	PEG-PCL 5–2.3 kDa	4.0	40.0
PM1-CP	PEG-PCL 5–2.3 kDa, calcium phosphate added	3.9	38.6
PM1-DCA	PEG-PCL 5–2.3 kDa, deoxycholic acid added	4.4	44.3
PM2	PEG-PCL 5-5.5 kDa	1.0	9.9
PM2-CP	PEG-PCL 5–5.5 kDa, calcium phosphate added	3.1	31.0
PM2-DCA	PEG-PCL 5–5.5 kDa, deoxycholic acid added	2.3	23.4

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 equivalent CFZ 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 ≈ 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

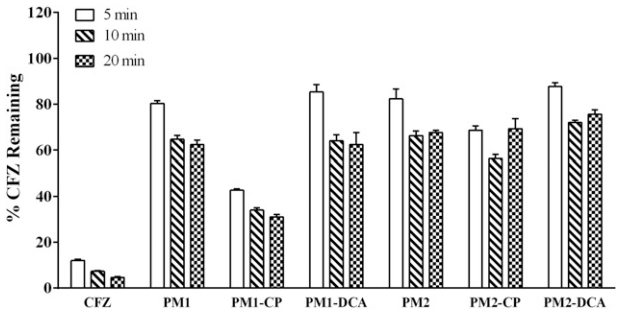

Fig. 1. In vitro metabolic stability of polymer micelle formulations containing CFZ in the presence of mouse liver homogenates. CFZ remaining was measured at 0, 5 (blank bars), 10 (striped bars), and 20 minutes (checkered bars) following incubation with liver homogenates. Percentage of CFZ remaining 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 ($P < 0.05$).

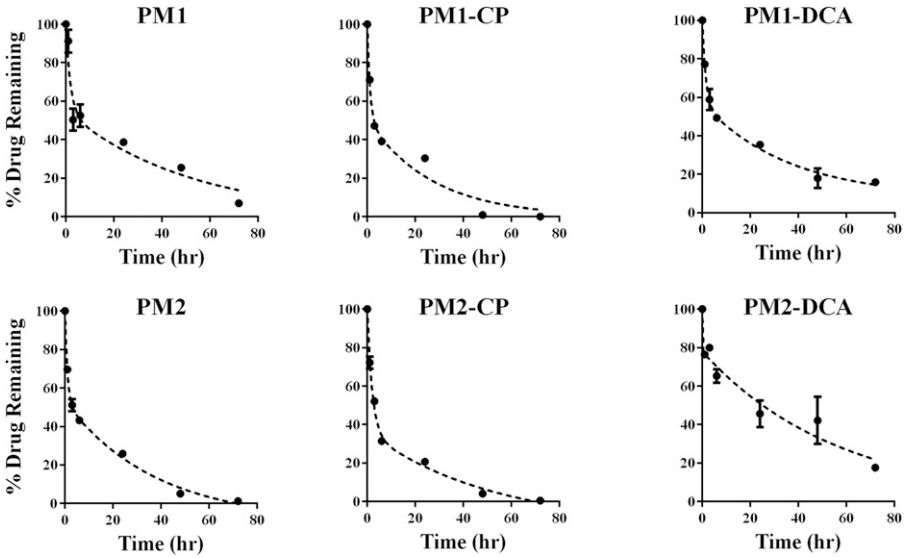

Fig. 2. In vitro drug release profiles of polymer micelle formulations. CFZ release was measured over 72 hours and represented as percent drug remaining of control. Results are represented as means ± S.E.M., and each data set is fitted to a two-phase decay model (dotted curve).

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).

TABLE 2
Kinetic parameters of in vitro CFZ release from the six polymer micelle particles based on two-phase decay modeling and AUC analyses

Formulation	Half-Life Fast	Half-Life Slow	AUC
	h		$\mu mol/l \times h$
PM1	1.3 ± 0.7	36.0 ± 13.7	2372
PM1-CP	0.8 ± 0.3	18.8 ± 5.1	1346
PM1-DCA	1.0 ± 0.4	25.0 ± 12.5	2195
PM2	0.7 ± 0.2	26.1 ± 8.0	1419
PM2-CP	1.4 ± 0.4	36.4 ± 22.4	1160
PM2-DCA	0.1 ± 0.1	39.2 ± 10.0	3229

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

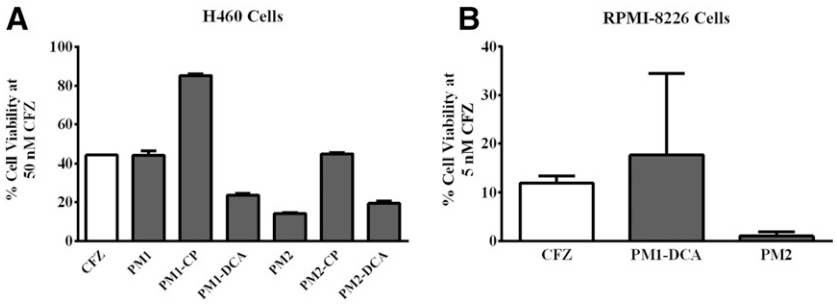

Fig. 3. Cytotoxic activities of polymer micelles containing CFZ in H460 (A) and select micelle formulations in RPMI-8226 (B) cell lines. Results are represented as percentage of cell viability of vehicle-only control. Micelle formulations containing CFZ were compared at 50 and 5 nM of equivalent CFZ concentration in H460 and RPMI-8226 cells, respectively. Data are represented as means ± S.E.M.

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

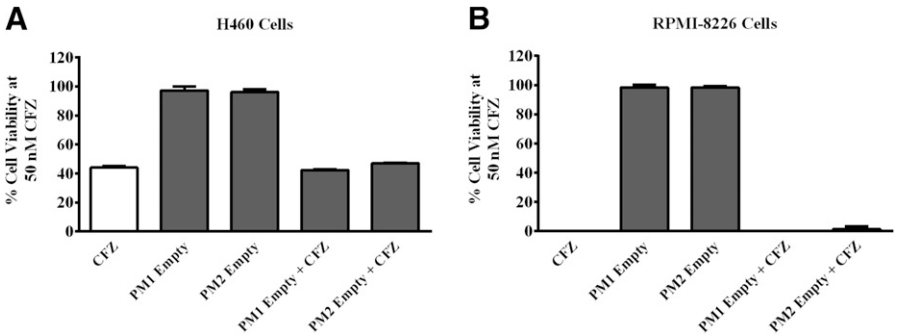

Fig. 4. Cytotoxic effects of PM1 and PM2 empty particle controls and coincubation of CFZ with empty particles tested in H460 (A) and RPMI-8226 (B) cells. Results are represented as percentage of cell viability of vehicle-only control. Empty particle controls and coincubation controls were compared at 50 nM equivalent CFZ concentration in both cell lines. Data are represented as means ± S.E.M.

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.

Acknowledgments

The authors thank all members of the Lee, Kim, and Bae laboratories at University of Kentucky and the Jeong laboratory at University of Illinois at Chicago for helpful feedback and suggestions.

Authorship Contributions

Participated in research design: Ao, Reichel, Hu, Jeong, Kim, Bae, Lee.

Conducted experiments: Ao, Reichel, Hu.

Performed data analysis: Ao, Reichel, Hu.

Wrote or contributed to the writing of the manuscript: Ao, Reichel, Jeong, Kim, Bae, Lee.

References

Adams J (2004) The development of proteasome inhibitors as anticancer drugs. *Cancer Cell* **5**:417–421.

Adams ML, Lavasanifar A, and Kwon GS (2003) Amphiphilic block copolymers for drug delivery. *J Pharm Sci* **92**:1343–1355.

Ahmad Z, Shah A, Siddiq M, and Kraatz HB (2014) Polymeric micelles as drug delivery vehicles. *RSC Advances* **4**:17028–17038.

Ao L, Wu Y, Kim D, Jang ER, Kim K, Lee DM, Kim KB, and Lee W (2012) Development of peptide-based reversing agents for p-glycoprotein-mediated resistance to carfilzomib. *Mol Pharm* **9**:2197–2205.

Arastu-Kapur S, Anderl JL, Kraus M, Parlati F, Shenk KD, Lee SJ, Muchamuel T, Bennett MK, Driessen C, and Ball AJ, et al. (2011) Nonproteasomal targets of the proteasome inhibitors bortezomib and carfilzomib: a link to clinical adverse events. *Clin Cancer Res* **17**:2734–2743.

Ashley JD, Stefanick JF, Schroeder VA, Suckow MA, Alves NJ, Suzuki R, Kikuchi S, Hideshima T, Anderson KC, Kiziltepe T, and Bilgicer B (2014) Liposomal carfilzomib nanoparticles effectively target multiple myeloma cells and demonstrate enhanced efficacy in vivo. *J Control Release* **196**:113–121.

Bae Y, Nishiyama N, Fukushima S, Koyama H, Yasuhiro M, and Kataoka K (2005) Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. *Bioconjug Chem* **16**:122–130.

Chen D, Frezza M, Schmitt S, Kanwar J, and Dou QP (2011) Bortezomib as the first proteasome inhibitor anticancer drug: current status and future perspectives. *Curr Cancer Drug Targets* **11**:239–253.

Demo SD, Kirk CJ, Aujay MA, Buchholz TJ, Dajee M, Ho MN, Jiang J, Laidig GJ, Lewis ER, and Parlati F, et al. (2007) Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. *Cancer Res* **67**:6383–6391.

Jagannath S, Vij R, Stewart AK, Trudel S, Jakubowiak AJ, Reiman T, Somlo G, Bahlis N, Lonial S, and Kunkel LA, et al. (2012) An open-label single-arm pilot phase II study (PX-171-003-A0) of low-dose, single-agent carfilzomib in patients with relapsed and refractory multiple myeloma. *Clin Lymphoma Myeloma Leuk* **12**:310–318.

Jakubowiak AJ, Dytfeld D, Griffith KA, Lebovic D, Vesole DH, Jagannath S, Al-Zoubi A, Anderson T, Nordgren B, and Detweiler-Short K, et al. (2012) A phase 1/2 study of carfilzomib in combination with lenalidomide and low-dose dexamethasone as a frontline treatment for multiple myeloma. *Blood* **120**:1801–1809.

Kim KB and Crews CM (2013) From epoxomicin to carfilzomib: chemistry, biology, and medical outcomes. *Nat Prod Rep* **30**:600–604.

Knop K, Hoogenboom R, Fischer D, and Schubert US (2010) Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew Chem Int Ed Engl* **49**:6288–6308.

Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, and Lust JA, et al. (2008) Improved survival in multiple myeloma and the impact of novel therapies. *Blood* **111**:2516–2520.

Li L, Zhang L, Wang T, Wu X, Ren H, Wang C, and Su Z (2015) Cancer therapy: facile and scalable synthesis of novel spherical au nanocluster assemblies@polyacrylic acid/calcium phosphate nanoparticles for dual-modal imaging-guided cancer chemotherapy (Small 26/2015). *Small* **11**:3082.

Mani A and Gelmann EP (2005) The ubiquitin-proteasome pathway and its role in cancer. *J Clin Oncol* **23**:4776–4789.

Nishiyama N and Kataoka K (2006) Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacol Ther* **112**:630–648.

Oe Y, Christie RJ, Naito M, Low SA, Fukushima S, Toh K, Miura Y, Matsumoto Y, Nishiyama N, and Miyata K, et al. (2014) Actively-targeted polyion complex micelles stabilized by cholesterol and disulfide cross-linking for systemic delivery of siRNA to solid tumors. *Biomaterials* **35**:7887–7895.

Papadopoulos KP, Burris HA, 3rd, Gordon M, Lee P, Sausville EA, Rosen PJ, Patnaik A, Cutler RE, Jr, Wang Z, and Lee S, et al. (2013) A phase I/II study of carfilzomib 2-10-min infusion in patients with advanced solid tumors. *Cancer Chemother Pharmacol* **72**:861–868.

Perkin KK, Turner JL, Wooley KL, and Mann S (2005) Fabrication of hybrid nanocapsules by calcium phosphate mineralization of shell cross-linked polymer micelles and nanocages. *Nano Lett* **5**:1457–1461.

Sanvicens N and Marco MP (2008) Multifunctional nanoparticles–properties and prospects for their use in human medicine. *Trends Biotechnol* **26**:425–433.

Shen M, Schmitt S, Buac D, and Dou QP (2013) Targeting the ubiquitin-proteasome system for cancer therapy. *Expert Opin Ther Targets* **17**:1091–1108.

Stewart AK, Rajkumar SV, Dimopoulos MA, Masszi T, Špička I, Oriol A, Hájek R, Rosiñol L, Siegel DS, and Mihaylov GG, et al.; ASPIRE Investigators (2015) Carfilzomib, lenalidomide, and dexamethasone for relapsed multiple myeloma. *N Engl J Med* **372**:142–152.

Swami A, Reagan MR, Basto P, Mishima Y, Kamaly N, Glavey S, Zhang S, Moschetta M, Seevaratnam D, and Zhang Y, et al. (2014) Engineered nanomedicine for myeloma and bone microenvironment targeting. *PNAS* **111**:10287–92.

Tan C, Wang Y, and Fan W (2013) Exploring polymeric micelles for improved delivery of anticancer agents: recent developments in preclinical studies. *Pharmaceutics* **5**:201–219.

Tsukioka Y, Matsumura Y, Hamaguchi T, Koike H, Moriyasu F, and Kakizoe T (2002) Pharmaceutical and biomedical differences between micellar doxorubicin (NK911) and liposomal doxorubicin (Doxil). *Jpn J Cancer Res* **93**:1145–1153.

Vakil R and Kwon GS (2008) Effect of cholesterol on the release of amphotericin B from PEG-phospholipid micelles. *Mol Pharm* **5**:98–104.

Vij R, Wang M, Kaufman JL, Lonial S, Jakubowiak AJ, Stewart AK, Kukreti V, Jagannath S, McDonagh KT, and Alsina M, et al. (2012) An open-label, single-arm, phase 2 (PX-171-004) study of single-agent carfilzomib in bortezomib-naive patients with relapsed and/or refractory multiple myeloma. *Blood* **119**:5661–5670.

Wang Z, Yang J, Kirk C, Fang Y, Alsina M, Badros A, Papadopoulos K, Wong A, Woo T, and Bomba D, et al. (2013) Clinical pharmacokinetics, metabolism, and drug-drug interaction of carfilzomib. *Drug Metab Dispos* **41**:230–237.

Werner ME, Cummings ND, Sethi M, Wang EC, Sukumar R, Moore DT, and Wang AZ (2013) Preclinical evaluation of Genexol-PM, a nanoparticle formulation of paclitaxel, as a novel radiosensitizer for the treatment of non-small cell lung cancer. *Int J Radiat Oncol Biol Phys* **86**:463–468.

Yang J, Wang Z, Fang Y, Jiang J, Zhao F, Wong H, Bennett MK, Molineaux CJ, and Kirk CJ (2011) Pharmacokinetics, pharmacodynamics, metabolism, distribution, and excretion of carfilzomib in rats. *Drug Metab Dispos* **39**:1873–1882.

Yang W, Monroe J, Zhang Y, George D, Bremer E, and Li H (2006) Proteasome inhibition induces both pro- and anti-cell death pathways in prostate cancer cells. *Cancer Lett* **243**:217–227.

Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, and Farokhzad OC (2008) Nanoparticles in medicine: therapeutic applications and developments. *Clin Pharmacol Ther* **83**:761–769.

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