Molecular Cancer Therapeutics

# Dual Carfilzomib and Doxorubicin–Loaded Liposomal Nanoparticles for Synergistic Efficacy in Multiple Myeloma

Jonathan D. Ashley[1], Charissa J. Quinlan[2], Valerie A. Schroeder[3,4], Mark A. Suckow[3,4], Vincenzo J. Pizzuti[1], Tanyel Kiziltepe[1,5], and Basar Bilgicer[1,2,5]

## Abstract

Here, we report the synthesis and evaluation of dual drug–loaded nanoparticles as an effective means to deliver carfilzomib and doxorubicin to multiple myeloma tumor cells at their optimal synergistic ratio. First, various molar ratios of carfilzomib to doxorubicin were screened against multiple myeloma cell lines to determine the molar ratio that elicited the greatest synergy using the Chou–Talalay method. The therapeutic agents were then incorporated into liposomes at the optimal synergistic ratio of 1:1 to yield dual drug–loaded nanoparticles with a narrow size range of 115 nm and high reproducibility. Our results demonstrated that the dual drug–loaded liposomes exhibited synergy *in vitro* and were more efficacious in inhibiting tumor growth *in vivo* than a combination of free drugs, while at the same time reducing systemic toxicity. Taken together, this study presents the synthesis and preclinical evaluation of dual drug–loaded liposomes containing carfilzomib and doxorubicin for enhanced therapeutic efficacy to improve patient outcome in multiple myeloma. *Mol Cancer Ther; 15(7); 1452–9. ©2016 AACR.*



## Introduction

Multiple myeloma is the second most common hematologic malignancy in the United States, representing approximately 2% of all cancer-related deaths. The acquisition of drug resistance by multiple myeloma cells often requires that a combination of two or more drugs be prescribed to effectively promote cell death by synergistically disrupting different cellular mechanisms necessary for growth and survival (1). Although two drugs can demonstrate synergy, they are usually only synergistic, or exhibit the greatest synergy, at specific drug-to-drug molar ratios (2–4). Although the two drugs may be administered at the optimal drug ratio for synergy, this does not ensure that this ratio will be maintained at the tumor site due to differences in injection schedules, pharmacokinetic properties, metabolism, and nonuniform biodistribution (5–7). In order for combination therapies to achieve maximal antitumor effects and improved patient outcomes, it is imperative that the drugs reach the tumor at their optimal molar ratio. One strategy to enable this would be to use nanoparticles as drug delivery vehicles to control the release, biodistribution, and pharmacokinetic properties of the therapeutics, so that they reach the tumor at the desired molar ratio (6, 7).

In recent years, nanoparticle-based drug delivery systems have gained remarkable interest, as they have greatly improved the efficacy of traditional therapeutics through controlled release, improved circulations times, enhanced tumor accumulation, and reduced systemic toxicities (8–10). Importantly, by incorporating two or more drugs into the same nanoparticle, their rate of release, biodistribution, and metabolism can be controlled so that the optimal synergistic ratio can be attained at the tumor site for improved therapeutic efficacy (11). Thus, utilizing nanoparticles as drug delivery vehicles for combinatorial therapeutics can have a significant impact on patient outcomes.

PEGylated liposomal nanoparticles are attractive drug delivery vehicles for the significant advantages they possess over other nanoparticle types, such as high drug-loading capabilities, facile incorporation of different functionalities, improved biocompatibility, and precise control over particle size (9, 12). In addition, it is possible to incorporate drugs with various physiochemical properties in the different compartments of liposomes. Various chemotherapeutics have previously been incorporated into liposomes with remarkable clinical success, making liposomes an ideal candidate for combination drug delivery (10, 13, 14).

Two classes of drugs that are known to be synergistic with one another are anthracyclines and proteasome inhibitors (15–17). Specifically, doxorubicin and bortezomib have been shown to be synergistic and are FDA approved in combination for the treatment of multiple myeloma (15, 18, 19). Carfilzomib, a second-generation proteasome inhibitor, has shown to have reduced off-target activity compared with bortezomib, which can eliminate the dose-limiting side-effects seen with bortezomib, such as peripheral neuropathy (20–22). Although numerous studies have been performed with bortezomib and doxorubicin, combination studies involving carfilzomib and anthracyclines have not been pursued to date (23–25). In addition, the incorporation of these

[1]Department of Chemical and Biomolecular Engineering, University of Notre Dame, Notre Dame, Indiana. [2]Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana. [3]Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana. [4]Freimann Life Science Center, University of Notre Dame, Notre Dame, Indiana. [5]Advanced Diagnostics and Therapeutics, University of Notre Dame, Notre Dame, Indiana.

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**Corresponding Authors:** Basar Bilgicer, University of Notre Dame, 171 Fitzpatrick Hall, Notre Dame, IN 46556. Phone: 574-631-1429; Fax: 574-631-8366; E-mail: bbilgicer@nd.edu; and Tanyel Kiziltepe, tkiziltepe@nd.edu

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therapeutics into a single nanoparticle has the potential to further improve the therapeutic efficacy by selectively delivering the drugs to the tumor site at their optimal drug ratio.

Here, we report the identification and incorporation of carfilzomib and doxorubicin into liposomes at their optimal synergistic ratio for improved therapeutic efficacy. In our approach, various molar ratios of carfilzomib-to-doxorubicin were first screened against multiple myeloma cell lines to determine the ratio that elicited the greatest synergy. Then, carfilzomib and doxorubicin were incorporated into liposomes at the optimal ratio and were further evaluated for improved synergy and therapeutic efficacy *in vitro* and *in vivo*. Our results demonstrated that the dual drug–loaded liposomes exhibited synergy *in vitro* and were more efficacious in inhibiting tumor growth *in vivo* than a combination of free drugs or single-agent liposomes. Taken together, this study presents the synthesis, characterization, and evaluation of carfilzomib and doxorubicin–loaded liposomes at their optimal synergistic drug ratio for enhanced therapeutic efficacy with the long-term goal of improving patient outcome in multiple myeloma.

## Materials and Methods

### Materials

Polycarbonate membranes (0.1 μm), mini-extruder, methoxy PEG2000-DSPE (mPEG2000) lipids, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl) (DPPE-GA) were from Avanti Polar Lipids, Inc. Carfilzomib was obtained from Chemie-Tek. All other chemicals were obtained from Sigma-Aldrich.

### Cell culture

MM.1S and NCI-H929 cell lines were obtained from and tested by ATCC in 2009. They were expanded and frozen immediately (within 2–3 weeks) upon receipt and then resuscitated only 2 to 3 months before experiments. Cell lines were cultured according to the vendor's instructions for a maximum of 3 to 4 months and testing was not necessary. No independent authentication was performed by the authors. Both cell lines were cultured in RPMI1640 media (CellGro) supplemented with 10% FBS, 2 mmol/L L-glutamine (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). NCI-H929 cells were supplemented with an additional 10% FBS and 55 μmol/L 2-mercaptoethanol.

### Cytotoxicity and synergy analysis

Cells ($2 \times 10^5$) per well were plated in a 96-well dish 16 hours prior to the experiment. Cells were treated with respective cytotoxic agents at varying concentrations. Cytotoxicity was assessed at 48 hours using Cell Counting Kit-8 Reagent (Dojindo Molecular Technologies). Combination index (CI) values were calculated using the Chou–Talalay method (26).

### Annexin V analysis

NCI-H929 ($2 \times 10^5$) cells were cultured in the presence of 12.5 and 25 nmol/L total drug equivalent concentrations of the different single-agent and combination formulations for 24 hours. Apoptotic cells were detected with Annexin V (FITC) antibody (BD Pharmingen) using a Guava easyCyte 8HT Flow Cytometer (EMD Millipore) as described previously (27).

### Synthesis of doxorubicin–lipid conjugate

The doxorubicin–lipid conjugate was synthesized as reported previously (28). Briefly, 1.14 mL of 25 mg/mL DPPE-GA (34.5 μmol) in chloroform was mixed with 21.4 μL of diisopropylcarbamide (137.9 μmol, 4 eq.) in a 5-mL glass vial and stirred for 5 minutes. Then, 21.4 μL of hydrazine (687 μmol, 20 eq.) was added to the solution, and then stirred for 5 hours at room temperature. Solvent was evaporated under vacuum. In a separate vial, 30 mg of doxorubicin (51.7 μmol, 1.5 eq.) was dissolved in 4 mL of methanol and was then added to the vial containing the dried lipid. The solution was stirred for 4.5 days in the dark at room temperature. Final product was isolated via extraction and characterized with MALDI-TOF-MS.

### Liposome preparation

Liposomes were prepared by dry film hydration as described previously (29). Briefly, the lipids, carfilzomib, and dox-lipid were mixed in chloroform, dried to form a thin film, and placed under vacuum overnight to remove residual solvent. The lipid films were hydrated at 65°C in PBS pH 7.4, gently agitated, and extruded at 65°C through a 0.1-μm polycarbonate filter. All liposomes adhered to the following molar formula: (95-x-y):5:*x*:*y* DSPC: mPEG-DSPE:carf:dox-lipid where $x$ and $y$ were either 0 or 1 depending on the desired drug loading of carfilzomib and doxorubicin, respectively.

### Particle sizing

Particle size was observed using dynamic light scattering (DLS) analysis via NanoBrook Omni Particle Size Analyzer (Brookhaven Instruments Corp.), using 658 nm light observed at a fixed angle of 90° at 25°C.

### Release analysis of carfilzomib and doxorubicin

Liposomes loaded with both carfilzomib and doxorubicin were diluted to 1 mmol/L total lipid concentration. To ensure no free drug was present, the liposome solution was purified via liposome extrusion purification method with a 30-nm polycarbonate membrane as described previously (30). Aliquots (100 μL) of the purified liposome solution were placed into each of the 3.5 kDa MWCO Slide-A-Lyzer dialysis units (Thermo Scientific). Dialysis units were dialyzed together in 1.5 L of PBS at 25°C. Samples (100 μL) were taken at $t = 0$, 1, 3, 6, 12, 24, 48, and 72 hours for DLS analysis and drug content characterization via reversed phase high performance liquid chromatography (RP-HPLC) on an Agilent 1200 series system with a semi-preparative Zorbax C3 column with isopropanol gradients.

### Multiple myeloma xenograft mouse model

For all studies, C.B.-17 SCID mice (Charles River Laboratories) were irradiated with 150 rad and were inoculated subcutaneously with $5 \times 10^6$ NCI-H929 cells. When tumors reached a volume of 50 $mm^3$, mice were randomized into groups and treated intravenously via retro-orbital injections on days 1, 2, 8, and 9. For the combination dosing study, mice were distributed into 4 groups of 3 mice receiving PBS, 1 mg/kg carf + 0.8 mg/kg dox (1.8 mg/kg), 1.5 mg/kg carf + 1.2 mg/kg dox (2.7 mg/kg), or 2 mg/kg carf + 1.6 mg/kg dox (3.6 mg/kg). To compare the NP[carf+dox] and carf + dox formulations, 3 groups of 8 mice were treated with PBS, carf + dox, or NP[carf+dox] at a dose of 1.8 mg/kg total drug. For the comparison of the efficacy of the nanoparticle formulations NP[carf+dox] and NP[carf] +

NP[dox], mice were divided into 3 groups of 8 mice and were treated with PBS, NP[carf] + NP[dox], or NP[carf+dox] at a dose of 2.7 mg/kg carfilzomib and doxorubicin equivalents. Carfilzomib/NP[carf] and doxorubicin/NP[dox] were mixed together prior to each injection for carf + dox and NP[carf] + NP[dox], respectively. Animals were monitored for body weight and tumor volume. Tumor volume was measured via calipers (volume = 0.5 × length × width$^2$). Mice were treated humanely and in accordance with the protocol approved by the Institutional Animal Care and Use Committee at the Freimann Life Science Center (Notre Dame, IN).

## Results

### Determination of the optimal stoichiometric ratio of carfilzomib and doxorubicin for maximal synergy

It has been previously shown that anthracyclines and proteasome inhibitors demonstrate synergy in multiple myeloma (15–17). This provides the rationale for a combination treatment with carfilzomib and doxorubicin for synergistic outcomes (Fig. 1A). Although combination treatments can provide many advantages, the degree of synergism/antagonism between drugs in combination treatments can vary significantly with the drug ratio (2, 11). Hence, to determine the optimal drug loading of each therapeutic into the nanoparticle, it is essential to first determine the ratio of free drugs that will yield the maximum synergy *in vitro*. Therefore, we evaluated the cytotoxicity of different molar combinations ranging from 1:10 to 10:1 of free carfilzomib to free doxorubicin using NCI-H929 and MM.1S multiple myeloma cell lines. The Chou–Talalay method was employed to calculate the combination index to evaluate the synergism (CI < 1) or antagonism (CI > 1) for each drug combination (26). Our results demonstrated that in the carfilzomib-to-doxorubicin ratios of 1:1 (CI = 0.825 for NCI-H929 and 0.903 for MM.1S) and 2:1 (CI = 0.847 for NCI-H929 and 0.975 for MM.1S), there was sufficient synergy for the combination of these two drugs (Fig. 1B). At higher and lower carfilzomib-to-doxorubicin ratios, antagonism was observed, highlighting the significance of using optimal ratios for synergistic outcomes. The synergy of the 1:1 ratio is also demonstrated in Fig. 1C, where NCI-H929 and MM.1S cells were incubated with carfilzomib, doxorubicin, or the 1:1 combination at a concentration of 25 nmol/L for each drug. While each drug alone demonstrated only minimal cytotoxicity at 25 nmol/L, the 1:1 combination demonstrated significant synergistic cell death in multiple myeloma cells. Thus, given the synergy and cytotoxicity observed in multiple myeloma cell lines, the 1:1 molar drug ratio was selected for nanoparticle formulation.

### Preparation of carfilzomib and doxorubicin–loaded liposomal nanoparticles

On the basis of the optimal drug ratio analysis, both therapeutics need to be incorporated into liposomes at a 1:1 molar ratio with controlled drug release so they reach the tumor site at their optimal synergistic ratio for maximum therapeutic efficacy. To incorporate doxorubicin into the nanoparticles, first we conjugated doxorubicin to the polar head group of a DPPE lipid via a hydrolyzable hydrazone bond to create a doxorubicin–lipid prodrug conjugate (dox-lipid; Fig. 2A). The slow hydrolysis of this labile bond facilitates a controlled release of doxorubicin from the nanoparticle surface. The dox-lipid was purified via extraction, mixed with the other lipid constituents at a molar ratio of 94:5:1 DSPC:DSPE-PEG2000:dox-lipid to form the lipid film,

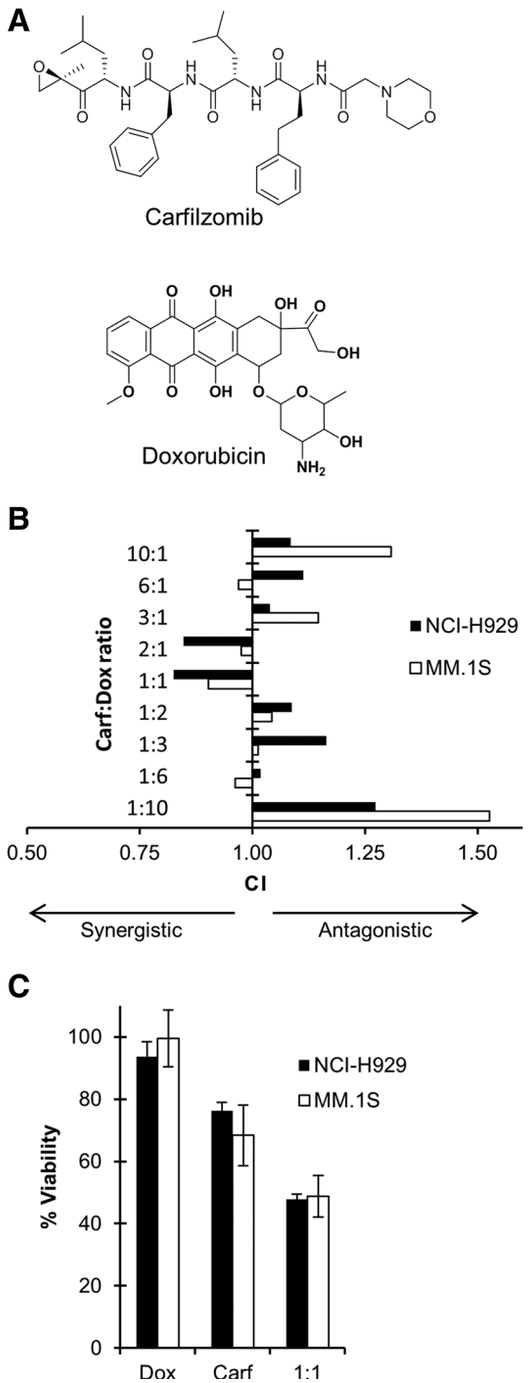


**Figure 1.**
Characterization of the synergistic activity of free carfilzomib (Carf) and free doxorubicin (Dox) combination treatment at different molar ratios. A, chemical structures of carfilzomib and doxorubicin. B, CI values of the different combinations of free carfilzomib and free doxorubicin were calculated based upon their respective $IC_{50}$ values to measure the level of synergism (CI < 1) or antagonism (CI > 1) using the Chou–Talalay method. C, cytotoxicity of free carfilzomib, free doxorubicin, and a combination of 1:1 molar ratio at a concentration of 25 nmol/L of each drug against NCI-H929 and MM.1S cell lines was determined at 48 hours. The combination treatment with 1:1 molar ratio demonstrated synergy in both cell lines. Data, means of triplicate cultures (±SD).

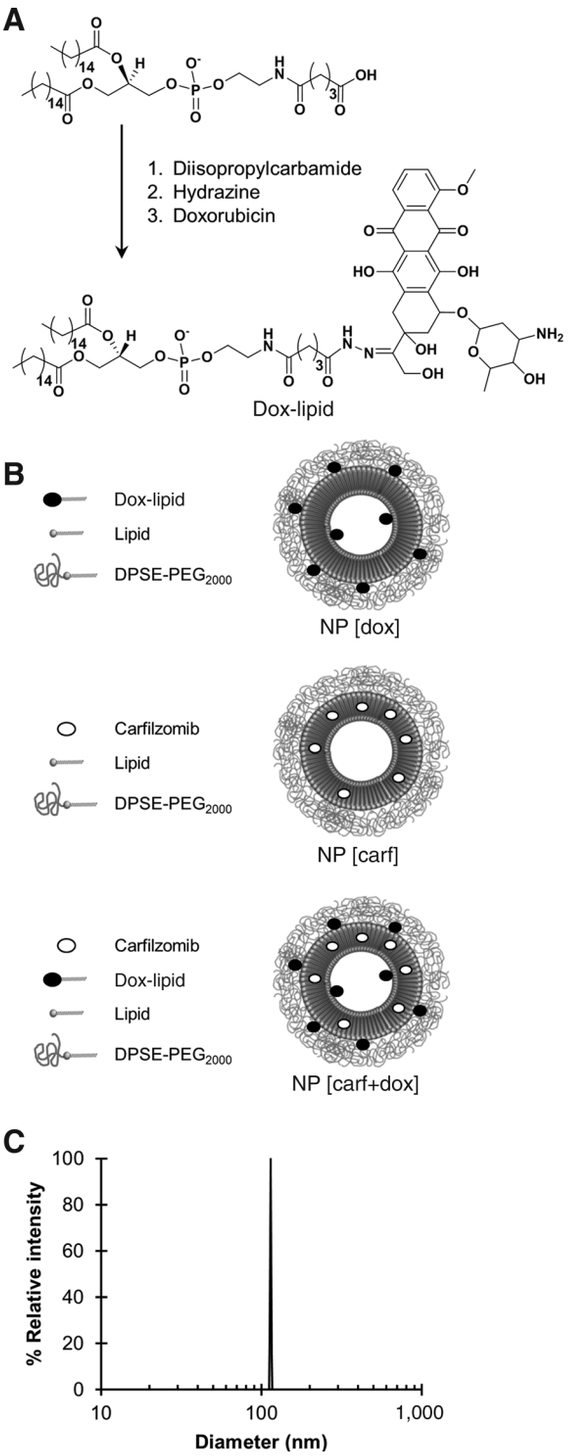


**Figure 2.**
Synthesis and characterization of carfilzomib (Carf) and doxorubicin (Dox) dual drug–loaded liposomes. A, schematic of the conjugation of doxorubicin to DPPE-GA via a labile hydrazone bond. B, illustrations of the single agent–loaded liposomes, NP[dox] (top) and NP[carf] (middle), and the dual drug–loaded liposome, NP[carf+dox] (bottom). C, representative DLS analyses of the different liposomal nanoparticles. NP[carf], NP[dox], and NP[carf+dox] yielded the same average diameter of approximately 115 nm. Data shown are from a representative experiment.

and then hydrated to form doxorubicin-loaded liposomes (NP[dox]; Fig. 2B). This allowed precise control over the molar ratio of doxorubicin presented on the nanoparticle and ensured high nanoparticle purity and reproducibility.

Carfilzomib can be embedded into the lipid bilayer of liposomes with high efficiency due to its hydrophobicity as we have previously shown (29). Hence, carfilzomib was loaded into liposomes by mixing it with the other lipids prior to film formation at the following molar ratio of 94:5:1 DSPC:DSPE-PEG2000: carfilzomib. This method yielded nanoparticles with high stability, purity, and reproducibility. The drug loading for the carfilzomib-loaded liposomes (NP[carf]) was 1 mol%, equal to NP[dox] (Fig. 2B).

To make dual drug–loaded liposomes (NP[carf+dox]), carfilzomib and the dox-lipid were passively loaded into liposomes with high purity and exact stoichiometry. Specifically, carfilzomib and the dox-lipid were mixed with the other lipids at the molar ratio of 93:5:1:1 DSPC:DSPE-PEG2000:carfilzomib:dox-lipid prior to film formation to facilitate their insertion into the bilayer (Fig. 2B). This method ensured that the drugs and lipids were incorporated into the liposomes at precise stoichiometric ratios.

The liposomes were extruded through a 100-nm polycarbonate membrane to yield unilamellar liposomes. NP[carf+dox], NP[carf], and NP[dox] yielded the same DLS results with an average diameter of 115 $\pm$ 1.36 nm with high reproducibility and stability [Fig. 2C; zeta ($\zeta$) potential in Supplementary Table S1]. This was also consistent with non-drug–loaded liposomes, showing that the presence of the therapeutics does not affect the size of the liposomes. Importantly, the diameter of these liposomes falls within the particle size range required for the passive targeting of tumors via the enhanced permeability and retention (EPR) effect (12, 31).

The loading efficiency for both drugs is critical to maintaining the precision of the molar drug ratio and minimizing the variability and impurities during nanoparticle formation. To maintain high loading efficiencies, 1 mol% drug loading into nanoparticles was selected for both carfilzomib and doxorubicin, which yielded loading efficiencies >95% for both drugs (Supplementary Fig. S1). This precluded the requirement for any purification after particle formation to remove any free drug in solution. The synthetic approach used to prepare NP[carf+dox] enabled high drug loading efficiencies, narrow size range precision, and homogenous particle populations with minimal batch-to-batch variability.

### Release of carfilzomib and doxorubicin from the dual drug–loaded liposomes

After loading carfilzomib and doxorubicin into liposomes at the optimal synergistic ratio, we evaluated their release from NP[carf+dox] using dialysis in conjunction with HPLC analysis. The results showed that both drugs were retained and released slowly from the nanoparticle over a 72-hour period (Fig. 3). Doxorubicin was released more rapidly than carfilzomib from NP[carf+dox], which could be attributed to the differences in the mechanism of release for each drug. Doxorubicin, being surface conjugated to the nanoparticle, requires the hydrolysis of the hydrazone bond before being released, whereas carfilzomib is released via diffusion out of the lipid bilayer, which enables the slow release of the drug. Although carfilzomib and doxorubicin were released at different rates, the nanoparticles were able to maintain a synergistic drug ratio between 1:1 and 2:1, starting at

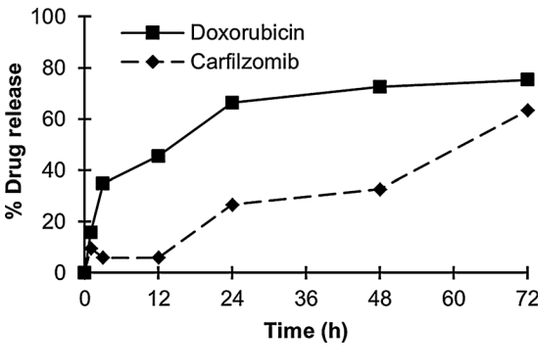


**Figure 3.**
Release of carfilzomib and doxorubicin from dual drug–loaded liposomes. Drug release from NP[carf+dox] was performed in PBS pH = 7.4 over a 72-hour period. Data shown are from a representative experiment.

24 hours when the nanoparticles maximally accumulate in the tumor (8, 32). These results demonstrate that NP[carf+dox] released both drugs in a controlled manner to facilitate the delivery of therapeutics to the tumor site at their optimal synergistic ratio.

### *In vitro* evaluation of the dual drug–loaded liposomes

After having engineered NP[carf+dox], we evaluated its efficacy on multiple myeloma cells *in vitro*. First, the cytotoxicity of the free drugs was compared with their respective single-drug nanoparticle formulation. While NP[carf] ($IC_{50}$ = 37.4 nmol/L) exhibited an approximate 2-fold decrease in $IC_{50}$ value relative to free carfilzomib ($IC_{50}$ = 63.5 nmol/L; Fig. 4A), NP[dox] and free doxorubicin had very similar $IC_{50}$ values of 220 and 235 nmol/L, respectively (Fig. 4B). Next, the cytotoxicity of NP[carf+dox] was evaluated and compared with carf + dox as well as NP[carf] + NP[dox]. The results demonstrated that NP[carf+dox] ($IC_{50}$ = 18.7 nmol/L) was more cytotoxic than carf + dox ($IC_{50}$ = 45 nmol/L) and NP[carf] + NP[dox] ($IC_{50}$ = 23.1 nmol/L; Fig. 4C).

The reduced efficacy of NP[carf] + NP[dox] relative to NP[carf+dox] can be attributed to the differing rate at which NP[dox] is taken up by the cells relative to NP[carf]. The hydrophobic patches were created by doxorubicin on the surface of NP[dox], which could increase its nonspecific cellular interactions and facilitate endocytosis. This would change the therapeutic molar ratio within the cells, as doxorubicin would be preferentially taken up mitigating the observed synergy. However, the optimal synergistic ratio is delivered to the cells with NP[carf+dox], as both drugs would be taken up at the same rate. Synergistic analysis of the cytotoxicity data for the both NP[carf+dox] and NP[carf]+NP[dox], based upon the activity of NP[carf] and NP[dox], showed that NP[carf+dox] (CI = 0.584) exhibited more synergy than NP[carf] + NP[dox] (CI = 0.721). Furthermore, NP[carf+dox] had a lower CI value than carf + dox (CI = 0.898), suggesting that NP[carf+dox] has improved synergy over the other formulations.

To validate these results, flow cytometric analysis of the early apoptosis marker, Annexin V, was performed. NCI-H929 cells were incubated with different free and liposomal formulations of carfilzomib and doxorubicin at a concentration of 12.5 nmol/L for each drug (Fig. 4D). The results demonstrated that the liposomal formulations elicited higher expression levels of Annexin V than the free drug formulations. NP[carf] + NP[dox] had a minimal increase in Annexin V expression compared with NP[carf]. However, NP[carf+dox] had significantly higher expression levels than NP[carf] and NP[carf] + NP[dox], demonstrating the advantages that can be gained by using this formulation. Taken together, NP[carf+dox] demonstrated improved efficacy relative to the other combinatorial formulations.

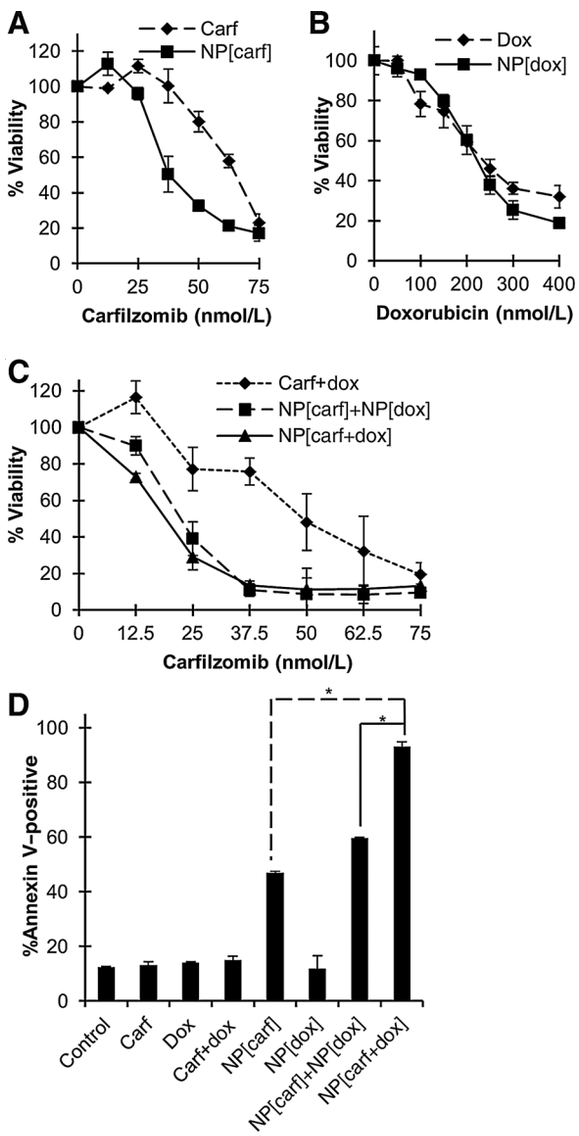


**Figure 4.**
Cytotoxicity of the dual drug–loaded liposomes. A, cytotoxicity of free carfilzomib (Carf) and NP[carf]. B, cytotoxicity of free doxorubicin (Dox) and NP[dox]. C, cytotoxicity of carf+dox, NP[carf]+NP[dox], and NP[carf+dox]. All of the cytotoxicity assays were determined at 48 hours with NCI-H929 cells. D, apoptosis in NCI-H929 cells was assessed by flow cytometry following Annexin V staining. Cells were incubated with the different free and liposomal formulations of carfilzomib and doxorubicin at a concentration of 12.5 nmol/L for each drug. The molar drug ratio of carfilzomib to doxorubicin of 1:1 was used for all combinations. Data, means of triplicate cultures (±SD). *, $P < 0.05$.

**Determination of the MTD for the free carfilzomib and free doxorubicin combination treatment *in vivo***

To test whether NP[carf+dox] is more efficacious than the other formulations, it is imperative to ultimately evaluate them *in vivo*. To do this, we first determined the MTD (<15% body mass loss) of free carfilzomib and doxorubicin combination treatment at the identified optimal synergistic molar ratio of 1:1. Although this will not accurately represent MTD for NP[carf+dox], it can still be used to set the dosing parameters for comparison studies, as we expect the nanoparticle formulations to be less toxic relative to the free drug formulations (5, 9, 12). SCID mice were subcutaneously injected with NCI-H929 cells. When the tumors reached a volume of 50 $mm^3$, the mice were randomized into treatment groups and received one of the regimens: (i) control (PBS), (ii) 1 mg/kg carf + 0.8 mg/kg dox combination (1.8 mg/kg carf + dox), (iii) 1.5 mg/kg carf + 1.2 mg/kg dox (2.7 mg/kg carf + dox), or (iv) 2 mg/kg carf + 1.6 mg/kg dox (3.6 mg/kg carf + dox). Treatments were given intravenously on days 1, 2, 8, and 9, modeling the clinical dosing schedule for carfilzomib. Tumor growth and body mass were monitored throughout the study as a measure of therapeutic efficacy and systemic toxicity, respectively. Results demonstrated that mice in the 2.7 and 3.6 mg/kg carf + dox groups demonstrated significant tumor growth inhibition relative to those that received 1.8 mg/kg (Fig. 5A). While the 2.7 and 3.6 mg/kg doses demonstrated similar responses in tumor growth, they differed significantly in systemic toxicity based on average body mass assessment (Fig. 5B). The mice that received 2.7 mg/kg lost, at most, approximately 10% body mass and were able to recover most of it by the end of the study. In contrast, mice that received 3.6 mg/kg lost substantially more mass (~20%) throughout the study and were not able to recover it. Thus, the MTD for carf + dox was determined to be 2.7 mg/kg.

***In vivo* efficacy of the dual drug–loaded liposomes**

To evaluate the therapeutic potential of NP[carf+dox], subcutaneous NCI-H929 tumor–bearing SCID mice were randomized into treatment groups when tumors reached a volume of 50 $mm^3$ and were intravenously injected with PBS (control), carf + dox, or NP[carf+dox] at a dose of 1 mg/kg carfilzomib + 0.8 mg/kg doxorubicin equivalents (1.8 mg/kg total drug) on days 1, 2, 8, and 9. Our results demonstrated that NP[carf+dox] significantly inhibited tumor growth inhibition (Fig. 6A) and reduced systemic toxicity relative to carf + dox (Fig. 6B). We anticipate that the increased efficacy of NP[carf+dox] over carf + dox was due to the delivery of the drugs at their optimal ratio as well as the other advantages gained from nanoparticle incorporation (10, 12).

To determine that the efficacy of NP[carf+dox] is attributed to the delivery of the therapeutics at their optimal synergistic ratio via single nanoparticle incorporation and not simply the result of the enhanced drug delivery properties gained by nanoparticles, the therapeutic efficacy of NP[carf+dox] was compared with NP[carf] + NP[dox]. Mice were injected with PBS (control), NP[carf+dox], or NP[carf] + NP[dox] to evaluate tumor growth inhibition and systemic toxicity. Although both formulations inhibited tumor growth, NP[carf+dox] demonstrated greater tumor growth inhibition than NP[carf] + NP[dox] (Fig. 6C) while maintaining a similar systemic toxicity profile (Fig. 6D). As expected, both nanoparticle treatment regimens showed minimal weight loss, demonstrating their ability to reduce the overall systemic toxicities associated with the free drugs. However, NP[carf+dox] demonstrated significantly greater tumor growth inhibition relative to NP[carf] + NP[dox], which suggests that NP[carf+dox] was able to deliver both therapeutics to the tumor at their synergistic ratio for an improved effect. The reduced efficacy of NP[carf] + NP[dox] could be attributed to suboptimal drug ratios at the tumor as a result of the differing circulation clearance rates between NP[carf] and NP[dox]. Specifically, the surface-conjugated doxorubicin in NP[dox] may facilitate opsonization and increase its clearance rate relative to NP[carf]. Although surface-conjugated doxorubicin is also present in NP[carf+dox], and still may increase the nanoparticle clearance rate, this affects both therapeutics equally, which does not impact the drug ratio delivered to the tumor. Taken together, these results further validate the potential impact that the NP[carf+dox] may have in the clinic.

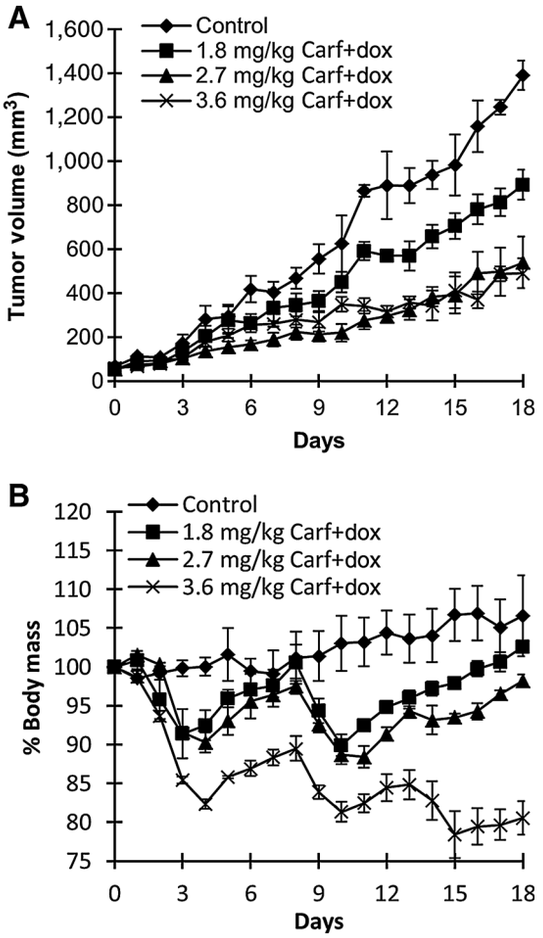


**Figure 5.**
Determination of MTD of free carfilzomib (Carf) and free doxorubicin (Dox) combination treatment *in vivo*. Tumor-bearing SCID mice were injected intravenously on days 1, 2, 8, and 9 with PBS (control), 1 mg/kg carf + 0.8 mg/kg dox (1.8 mg/kg carf + dox), 1.5 mg/kg carf + 1.2 mg/kg dox (2.7 mg/kg carf + dox), or 2 mg/kg carf + 1.6 mg/kg dox (3.6 mg/kg carf + dox). A, tumor growth inhibition was measured via calipers. B, percent body weight of the animals was used as a measure of systemic toxicity to determine the MTD.

## Discussion

Combinatorial therapies continue to play a critical role in the treatment of cancers and in multiple myeloma. Formulations that

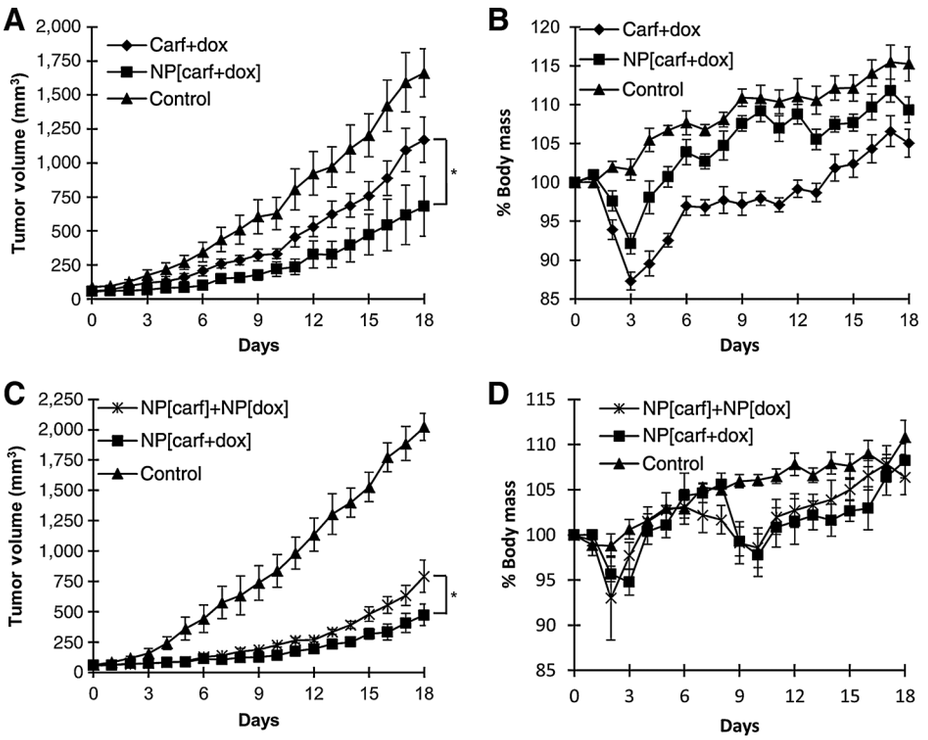


**Figure 6.**
*In vivo* efficacy of combination formulations. A and B, tumor-bearing SCID mice were injected intravenously on days 1, 2, 8, and 9 with PBS, carf + dox, or NP[carf+dox] with 1 mg/kg carfilzomib (Carf) + 0.8 mg/kg doxorubicin (Dox; total drug dose of 1.8 mg/kg). A, tumor growth inhibition was measured via calipers. B, percent body weight of the animals was used as a measure of systemic toxicity. C and D, tumor-bearing SCID mice were injected intravenously on days 1, 2, 8, and 9 with PBS, NP[carf] + NP[dox], or NP[carf+dox]. C, tumor growth inhibition was measured via calipers. D, percent body weight of the animals was used as a measure of systemic toxicity. *, $P < 0.05$.

deliver the drugs at their optimal synergistic ratios at the tumor site are critical for harnessing maximum efficacy of combination treatments. Although current combination therapies are effective, controlling the drug ratio at the tumor is extremely difficult due to differences in the pharmacokinetics, biodistribution, and metabolism of each drug (5–7). Nanotechnology can overcome these problems by loading the therapeutics into nanoparticles at the optimal ratio to facilitate their controlled release and increase their accumulation in the tumor due to the EPR effect enabled by angiogenic blood vessels (12, 31). Recent studies have established that angiogenesis plays a critical role in various hematologic malignancies including multiple myeloma, providing a strong rationale to exploit nanotechnology in managing this disease (33, 34). Hence, the unique advantages provided by nanotechnology can be used to formulate more effective combination therapies in multiple myeloma with enhanced synergy at the tumor site with the long term goal of improved patient outcomes.

In this study, we demonstrated the synthesis and evaluation of NP[carf+dox] as an effective means to deliver carfilzomib and doxorubicin to multiple myeloma tumor cells at their optimal synergistic ratio for improved therapeutic effect. Our results demonstrated that NP[carf+dox] had improved efficacy *in vitro* as well as *in vivo* compared with the free drug combination, highlighting the significance of using nanotechnology as a delivery vehicle for combination drugs in achieving better outcomes in multiple myeloma. Hence, this study, for the first time, demonstrated the synergy between carfilzomib and doxorubicin and their incorporation into nanoparticles for improved therapeutic effect.

In our design, liposomes were selected due to the advantages they possess over other nanoparticle types (9, 12). Furthermore, using our novel synthetic method, we are able to incorporate the lipids and the drug molecules with stoichiometric precision, which enabled the incorporation and delivery of the therapeutics at their optimal ratio. NP[carf+dox] exhibited high stability and reproducibility and efficient drug loading at 1 mol%. On the basis of literature reports, the drug loading of liposomes can be enhanced to 5% to 10% mol, depending on lipid packing, particle size, and the particular therapeutics (9, 35). Ongoing studies are being conducted in our laboratory to increase the loading of carfilzomib and doxorubicin with even higher efficiency to increase the effectiveness of each and every nanoparticle reaching the tumor site.

By combining the enhanced drug delivery capabilities of nanoparticles with successful combination therapies, significant advances in medicine could be made that may have a profound positive impact in the clinic. Our study demonstrated for the first time, the synergy between carfilzomib and doxorubicin and their incorporation into nanoparticles for improved therapeutic effect. Taken together, this study demonstrates the therapeutic potential of these first-generation carfilzomib and doxorubicin dual drug–loaded liposomal nanoparticles, and provides the preclinical rationale for clinical development and evaluation of NP[carf+dox] for improved patient outcomes in multiple myeloma.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** J.D. Ashley, T. Kiziltepe, B. Bilgicer

**Development of methodology:** J.D. Ashley, M.A. Suckow, T. Kiziltepe, B. Bilgicer

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.D. Ashley, C.J. Quinlan, V.A. Schroeder, V.J. Pizzuti

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.D. Ashley, C.J. Quinlan, T. Kiziltepe
**Writing, review, and/or revision of the manuscript:** J.D. Ashley, C.J. Quinlan, M.A. Suckow, T. Kiziltepe, B. Bilgicer
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.D. Ashley, V.A. Schroeder, B. Bilgicer
**Study supervision:** J.D. Ashley, M.A. Suckow

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