

# Anticancer Evaluation of Methoxy Poly(Ethylene Glycol)-*b*-Poly(Caprolactone) Polymeric Micelles Encapsulating Fenbendazole and Rapamycin in Ovarian Cancer

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**Purpose:** We aimed to inhibit ovarian cancer (OC) development by interfering with microtubule polymerization and inhibiting mTOR signaling. To achieve this, previously developed micelles containing fenbendazole and rapamycin were applied.

**Methods:** Herein, we prepared micelles for drug delivery using fenbendazole and rapamycin at a 1:2 molar ratio and methoxy poly(ethylene glycol)-*b*-poly(caprolactone)(mPEG-*b*-PCL) via freeze-drying. We revealed their long-term storage capacity of up to 120 days. Furthermore, a cytotoxicity test was performed on the OC cell line HeyA8, and an orthotopic model was established for evaluating in vivo antitumor efficacy.

**Results:** Fenbendazole/rapamycin-loaded mPEG-*b*-PCL micelle (M-FR) had an average particle size of  $37.2 \pm 1.10$  nm, a zeta potential of  $-0.07 \pm 0.09$  mV, and a polydispersity index of  $0.20 \pm 0.02$ . Additionally, the average encapsulation efficiency of fenbendazole was  $75.7 \pm 4.61\%$  and that of rapamycin was  $98.0 \pm 1.97\%$ . In the clonogenic assay, M-FR was 6.9 times more effective than that free fenbendazole/rapamycin. The in vitro drug release profile showed slower release in the combination formulation than in the single formulation.

**Conclusion:** There was no toxicity, and tumor growth was suppressed substantially by our formulation compared with that seen with the control. The findings of our study lay a foundation for using fenbendazole and rapamycin for OC treatment.

**Keywords:** ovarian cancer treatment, combination therapy, drug delivery, nanoformulation

## Introduction

Ovarian cancer (OC) is the deadliest gynecological cancer,<sup>1,2</sup> distinguished into epithelial ovarian cancer (EOC) and non-epithelial ovarian cancer.<sup>3</sup> Although several treatments, such as targeted therapy, chemotherapy, and surgery, have been developed to treatment of EOC, side effects and sequelae still occur.<sup>4-7</sup> To overcome this challenge, drug delivery using nanocarriers is a promising treatment method to maximize the efficacy and stability of drugs.<sup>8-11</sup> Nanocarriers deliver poorly soluble drugs,<sup>12,13</sup> and increase the residence time in the body,<sup>9,14-16</sup> thereby maximizing the therapeutic efficacy.<sup>17-21</sup> In particular, the drug encapsulated in nanocarriers can be designed to accumulate adequately in the tumor using the enhanced permeability and retention (EPR) effect.<sup>22-25</sup> Solvents as such as polysorbate 80 (Tween 80<sup>®</sup>), Cremophor EL<sup>®</sup> and ethanol are used to dissolve poorly soluble drugs in intravenous formulations, but these have side effects and toxicity due to the toxicity of the solvent.<sup>26,27</sup> These disadvantages can also be overcome with nanocarriers.

Therefore, we designed micelles using methoxy poly(ethylene glycol)-*b*-poly(caprolactone) (mPEG-*b*-PCL) polymer in a simple way. Poly(caprolactone) (PCL) is a biodegradable, biocompatible, and semi-crystalline polymer with a very low glass transition temperature,<sup>28</sup> which has been used as nanoparticles encapsulated with anticancer agents.<sup>28,29</sup> Poly(ethylene glycol) (PEG) is hydrophilic, non-toxic, and non-immunogenic.<sup>30</sup> PEGylation of hydrophobic nanoparticles offers advantages such as amphiphilicity, improved biocompatibility, and controllable degradation rates. As such, micelles formed using mPEG-*b*-PCL show biodegradability, biocompatibility, amphiphilicity, blood stability, non-toxicity, non-immunogenicity, and non-inflammatory properties,<sup>31</sup> and have been used as an excellent drug delivery system.<sup>32,33</sup>

Combination therapy using two or more drugs has many benefits. By administering a combination of two drugs acting via different molecular mechanisms, individual compound doses can be reduced to minimize or lower drug toxicity.<sup>34,35</sup> In addition, it can reduce the likelihood of drug resistance arising from continued treatment with a single drug.<sup>36</sup> Finally, if the formulation is designed using a rational drug combination, it can further help inhibit cancer cells.<sup>37</sup>

Owing to its low toxicity and side effects and high safety in several experimental animal models, fenbendazole is a broad-spectrum benzimidazole anthelmintic approved for use in many animals.<sup>38–41</sup> Currently, widely used anticancer drugs such as paclitaxel and docetaxel exhibit antitumor effects by interfering with microtubule polymerization and formation.<sup>42,43</sup> Fenbendazole interferes with microtubule polymerization and blocks cell cycle progression.<sup>44,45</sup> In addition, the mechanism of anticancer activity of fenbendazole occurs through increased p53 protein stability and apoptosis induction.<sup>46</sup> While developing new drugs is time-consuming and expensive, repurposing drugs is a promising efficient and economical alternative.<sup>47</sup> Fenbendazole, an antiparasitic drug with potential for anticancer activity,<sup>48</sup> such as apoptosis, microtubule inhibition, and inhibition of cancer cell invasion and migration, can be a promising anticancer agent.<sup>49–52</sup> However, fenbendazole has low permeability and water solubility; hence, its bioavailability is low.<sup>53,54</sup>

Rapamycin is a well-known potent immunosuppressant which inhibits the function of the mammalian target of rapamycin (mTOR)<sup>55</sup> and angiogenesis.<sup>56,57</sup> Hence, rapamycin can be expected to have excellent anticancer effects.<sup>58,59</sup> The mTOR signaling pathway plays an important role in cell survival, growth, proliferation, and angiogenesis.<sup>60,61</sup> Furthermore, abnormalities in the mTOR pathway cause tumorigenesis, such as OC.<sup>62,63</sup> Several preclinical models have been developed to demonstrate the role of the mTOR signaling pathway in OC.<sup>64</sup> Another study demonstrated the efficacy of an OC model for preclinical testing of inhibitors of mTOR signaling.<sup>65</sup> When mTOR is targeted, it significantly inhibits OC cell proliferation and migration.<sup>66</sup> The combination of a drug that interferes with microtubule polymerization and an mTOR inhibitor has a synergistic effect.<sup>67</sup> Despite being efficient, rapamycin has the disadvantage of low bioavailability and water solubility (2.6 µg/mL).<sup>68,69</sup>

To increase the bioavailability of poorly soluble drugs, a method of encapsulating the drug in a polymer and delivering it into the body is widely used.<sup>70,71</sup> Many studies have developed drug delivery systems that can control the release time of rapamycin and prolong its residence time in the body.<sup>72</sup> Moreover, successful delivery via intravenous (IV) injection of these formulations has been reported.<sup>73</sup> To improve the bioavailability of fenbendazole and rapamycin and maximize their anticancer potential, we demonstrated an IV formulation in micelles.

In this study, fenbendazole/rapamycin-loaded micelles (M-FR) were evaluated for EOC treatment. We evaluated the synergism of the two drugs in OC, their formulation stability, and long-term storage capacity. We estimated their cytotoxicity using EOC cell line HeyA8, performed an *in vitro* release assay, and antitumor efficacy *in vivo*. The anticancer effect of mPEG-*b*-PCL micelles in combination with fenbendazole and rapamycin has been demonstrated in lung cancer.<sup>74</sup> Here, we evaluated the anticancer ability of this formulation in OC and established an orthotopic model to further observe its anticancer activity in the body. We believe that this study will be of great help in the development of OC treatments.

## Materials and Methods

### Materials and Reagents

Acetonitrile (ACN) and Hanks' Balanced Salt Solution (HBSS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Distilled water (DW) was purchased from Tedia (Fairfield, OH, USA). Rapamycin was purchased

from LC Laboratories<sup>®</sup> (Woburn, MA, USA). Thiazolyl blue tetrazolium bromide (MTT), *tert*-Butyl alcohol, dimethyl sulfoxide (DMSO), and fenbendazole were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The mPEG-*b*-PCL (MW~2000:2000 Da) was purchased from PolySciTech<sup>®</sup> (West Lafayette, IN, USA). All other reagents were of analytical reagent grade.

## High-Performance Liquid Chromatography (HPLC) Analysis

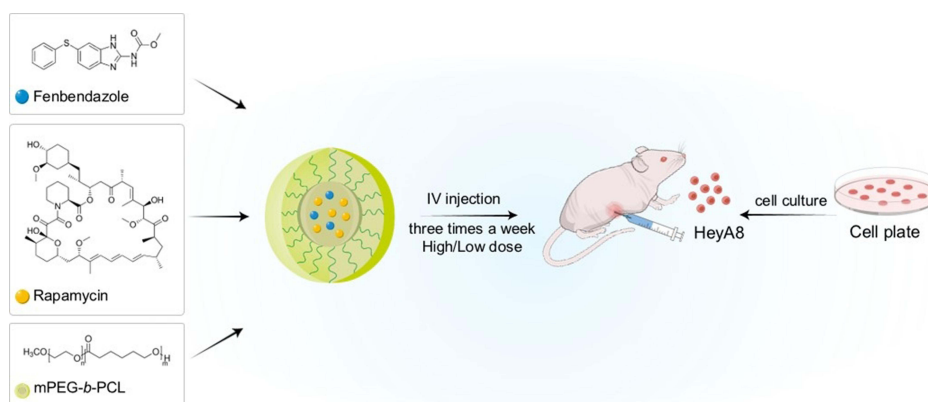
The concentrations of fenbendazole and rapamycin in the samples were analyzed using a Waters HPLC system (Milford, MA, USA) consisting of a 2695 separation module and a 2996 photodiode array detector. A Fortis C18 chromatography column (5  $\mu$ m, 4.6 $\times$ 250 mm) was used at 30°C for analysis. Fenbendazole and rapamycin were eluted in the isocratic mode; the injection volume was 10  $\mu$ L, and the flow rate of the mobile phase consisting of ACN/DW (70:30, v/v) was 1.0 mL/min. Fenbendazole was detected at 295 nm, and the retention time was 5 min. Rapamycin was detected at 277 nm, with a retention time of 20 min. The concentration of each drug was calculated by comparing the calibration curve to the peak area.

## Cell Lines and Cell Culture

Human OC (HeyA8\_RRID: CVCL8878) cells were a gift from Dr. Anil K. Sood, Department of Cancer Biology, University of Texas M.D. Anderson Cancer Center, TX, USA. Penicillin streptomycin solution, fetal bovine serum (FBS), Roswell Park Memorial Institute 1640 (RPMI 1640) medium, Dulbecco's phosphate-buffered saline (DPBS) and trypsin were purchased from Corning Inc. (Corning, NY, USA). HeyA8 cells were cultured in the RPMI 1640 medium supplemented with 1% (w/v) penicillin streptomycin solution and 10% (v/v) FBS. Cells were grown at 37°C under a 5% CO<sub>2</sub> atmosphere.

## Preparation of Micelles

M-FR were prepared using the freeze-drying method (Figure 1).<sup>74</sup> Briefly, the polymer and various ratios of fenbendazole and rapamycin were weighed in a scintillation vial and dissolved in 1 mL *tert*-butanol at 60°C. After adding 1 mL DW at 60°C, the solution was vortexed. The samples were stored in a -70°C freezer for 1 h and then lyophilized (Advantage Pro; SP Scientific, Warminster, PA, USA) at -20°C for 24 h. For reconstitution, 1 mL of 60°C DW was added to the freeze-dried cake, and vortexed. After centrifugation (Hanil Science Inc., Gimpo, South Korea) at 4°C and 13,000 rpm for 5 min, unencapsulated drugs and polymers were removed, and the centrifuged micelle supernatant was filtered with a 0.2  $\mu$ m-pore size filter (Corning, NY, USA).



**Figure 1** Schematic diagram of cell injection for orthotopic model establishment and M-FR IV injection for OC treatment.

## Physicochemical Characterization of the Micelles

All particle size, zeta potential, and polydispersity index (PDI) measured using a dynamic light scattering (DLS) device (Litesizer 500, Anton Paar, Graz, Austria). All samples were diluted 10 times with DW before measurement.

The encapsulation efficiency (EE, %) and drug loading (DL, %) of fenbendazole and rapamycin in this study were measured using HPLC and calculated as follows:<sup>75,76</sup>

$$EE(\%) = \frac{\text{Weight of drug in micelles}}{\text{Weight of feeding drug}} \times 100$$

$$DL(\%) = \frac{\text{Weight of drug in micelles}}{\text{Weight of feeding drug and polymer}} \times 100$$

The results of each micelle sample analysis were expressed as the mean  $\pm$  SD of three separate experiments.

The morphology of M-FR was observed by using a JEM-2100 transmission electron microscope (TEM, JEOL LTD., Tokyo, Japan). In the sample preparation procedure for TEM measurement, the diluted micelle solution was dropped on a copper grid, dried at room temperature for 2 days, and examined at accelerating voltage of 200 Kv.

## Analysis of the Combination Index (CI) Between Drugs

CI was analyzed using Chou's method to analyze interactions between drugs.<sup>77</sup> The CI value between fenbendazole and rapamycin was calculated using the following equation:

$$\text{Combination Index (CI)} = \frac{(D)_1}{(D_x)_2} + \frac{(D)_2}{(D_x)_1}$$

$(D_x)_1$  and  $(D_x)_2$  in this equation are the inhibitory concentrations of drugs 1 and 2, respectively.  $(D)_1$  and  $(D)_2$  are the inhibitory concentrations of each drug in combinations.  $CI < 1$  indicated synergism,  $CI > 1$  indicated antagonism, and  $CI = 1$  indicated an additive effect.

## M-FR Stability Test

M-FR stability was tested in a refrigerator at 4°C and in a water bath 37°C. On days 0, 1, 2, 4, 7, 10, and 14, 100  $\mu$ L of each sample was collected and dissolved in 900  $\mu$ L DW for measurement. The size and PDI of M-FR were measured using a DLS device. The experiments were conducted in triplicate.

## Storage Stability Test After Freeze-Drying

All M-FR required for the experiment were prepared at once on the same day, and the freeze-dried M-FR were stored in a  $-20^\circ\text{C}$  freezer until testing. M-FR were hydrated on days 1, 7, 14, 30, 60 and 120 to measured EE (%), size, PDI and zeta potential. Based on this, the possibility of long-term storage use was evaluated. This experiment was performed three times.

## In vitro Cytotoxicity Assay

Cytotoxicity was performed by MTT assay.<sup>78</sup> HeyA8 cells were seeded at 4000 cells/well in a 96-well plate, and incubated at 37°C and 5%  $\text{CO}_2$  conditions for 24 h. Then, the medium was replaced with a fresh medium with varying molar ratios of free fenbendazole/rapamycin, free fenbendazole, free rapamycin or M-FR each. A medium without any drug was used as the control (CON). The cells treated with various drug combinations and M-FR were incubated for 48 h. The treated high concentrations of free fenbendazole and free rapamycin were 170,000 nM and 54,705 nM, respectively, which were diluted 10-fold. The medium was removed, and 100  $\mu$ L of MTT solution (0.5 mg/mL) was added and incubated for 4 h. The MTT solution was removed and 100  $\mu$ L of DMSO was added, followed by shaking for 10 min using an orbital shaker (NB-101S; N-BIOTEK, Bucheon, South Korea) at 200 rpm for complete solubilization of DMSO. The absorbance was measured at 540 nm using a microplate reader (Spectra Max ID3; Molecular Devices, San Jose, CA, USA). All data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

## In vitro Clonogenic Assay

HeyA8 cells were seeded in 6-well plates at a density of 200 cells per well and incubated at 37°C for 24 h. After cell adhesion, cells were treated with free fenbendazole, fenbendazole-loaded micelles (M-F), free rapamycin, rapamycin-loaded micelles (M-R), free fenbendazole/rapamycin, or M-FR. The high concentrations treated were 500 nM for free fenbendazole and M-F, 521 nM for free rapamycin and M-R, and 9910 nM for free fenbendazole/rapamycin and M-FR, respectively. After 2 weeks, the medium was removed, and 1 mL of crystal violet (0.5% w/v) was added to each well to stain the colonies. After incubation for 30 min, the 6-well plates were rinsed with clean water. The number of colonies with more than 50 cells was counted. The colony formation percentage was calculated using the following equation below:

$$\text{Colony formation(\%)} = \frac{\text{Number of colonies after treatment}}{\text{Number of colonies of control}} \times 100$$

## In vitro Drug Release Assay from Micelles

The release of fenbendazole and rapamycin in micelles was assessed using the dialysis method.<sup>79</sup> A dialysis membrane bag (MWCO 20 kD) containing M-F, M-R, or M-FR was immersed in 2.0 L of phosphate-buffered saline (PBS, pH 7.4). The release medium was stirred at 200 rpm using a magnetic bar at 37°C. Each sample (20 µL) was collected at 0, 2, 4, 6, 24, 48, 72, 168, 240, and 336 h, diluted in 180 µL of ACN, and measured using HPLC. The PBS was replaced with a fresh medium to maintain sink conditions at 8, 24, 72, 168, and 240 h. A curve fitting of % drug release was performed based on the first-order association using GraphPad Prism 8.4.2.<sup>80</sup> All experiments were performed in triplicate.

## Animal Care and Development of in vivo Models

This experiment was reviewed and approved by the institutional animal care and use committee (IACUC) of Samsung Biomedical Research Institute (protocol No. H-A9-003), which is an accredited facility by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and abides by the Institute of Laboratory Animal Resources (ILAR) guideline. All female BALB/c nude mice used in the experiments were purchased from Orient Bio Inc (Seong-nam, Korea). To establish an orthotopic model, HeyA8 ( $2.5 \times 10^5$  cells/0.1 mL HBSS) cells were injected into the peritoneal cavities of the female BALB/c nude mice.<sup>81</sup> The mice were randomly divided into four groups, and the experiment was conducted at two doses: (i) CON, M-F (2.5 mg/kg), M-R (15 mg/kg), and M-FR (2.5 mg/kg for fenbendazole and 15 mg/kg for rapamycin), (ii) CON, M-F (5 mg/kg), M-R (30 mg/kg), and M-FR (5 mg/kg for fenbendazole and 30 mg/kg for rapamycin). All treatment were IV injected via the tail vein every two days. The mice (n = 10 per group) were monitored daily for tumor development, and sacrificed on days 28–35 after the injection of cancer cells. Body weight, tumor weight, and the number of tumor nodules were recorded. Tumors were fixed in formalin and embedded in paraffin or snap frozen in an optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) in liquid nitrogen.

## Statistical Analysis

Statistical analysis of all data identified significant differences using analysis of variance (ANOVA) with of GraphPad Prism v 5.0. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  indicate statistical significance.

## Results

### Evaluation of the Synergistic Effects of Fenbendazole and Rapamycin in HeyA8 Cells

The optimal ratio of fenbendazole and rapamycin was determined by measuring the 50% inhibition concentration (IC<sub>50</sub>) values of the two drugs in HeyA8 and analyzing the CI values.<sup>82</sup> IC<sub>50</sub> values were measured at various molar ratios of fenbendazole and rapamycin (1:1, 1:2, 2:1, 1:4, and 4:1), and the CI values were indicated. The ratio of fenbendazole and rapamycin showing a synergistic effect in HeyA8 cell was confirmed, which became a potential basis for determining the final ratio of the formulation (Table 1 and Figure S1). The inhibitory effect varied depending on the combination ratio that determines the optimal ratio. When the molar ratio was 1:4, CI value > 1, that is, antagonism was observed. All other

**Table 1** IC<sub>50</sub> and CI Values of Fenbendazole and Rapamycin at Various Molar Ratios (n = 3)

Molar Ratio (Fendazole:Rapamycin)	IC <sub>50</sub> (nM)		CI value
	Fenbendazole	Rapamycin	
1:1	0.10	0.10	0.20
1:2	0.04	0.07	0.11
1:4	0.37	1.46	1.83
2:1	0.57	0.29	0.86
4:1	0.17	0.04	0.21

Abbreviation: CI, combination index.

ratios (1:1, 1:2, 2:1, and 4:1) had CI values of < 1, indicating a synergistic effect. A ratio of 1:2 showed the lowest CI value (Figure S2).

## Preparation of M-FR

A micelle was prepared and evaluated, except for the molar ratio of fenbendazole:rapamycin=1:4, in which antagonism appeared. DL (%), EE (%), zeta potential, PDI, and size of M-FR at different ratios (1:1, 1:2, 2:1, and 4:1) were assessed. The PDI and size are listed in Table 2. In the molar ratio of fenbendazole and rapamycin of 1:2, EE (%) of fenbendazole and rapamycin was  $75.7 \pm 4.61$  and  $98.0 \pm 1.97$ , respectively. At the molar ratio of 1:1, EE (%) of fenbendazole and rapamycin was  $81.5 \pm 2.45$  and  $76.2 \pm 8.36$ , respectively. In contrast, in the ratio of 2:1 and 4:1, the EE (%) of fenbendazole was  $40.3 \pm 5.17$  and  $19.5 \pm 0.49$ , respectively, indicating low EE (%). Therefore, considering EE (%), a 1:2 ratio using 100 mg of the mPEG-*b*-PCL polymer was selected as an optimal ratio. The size distribution graph in Figure 2A shows that the vehicle was 30.07 nm, and M-F, M-R, and M-FR were < 30 nm. Also, TEM images showed that the micelles were uniformly spherical (Figure 2B).

## Stability Test

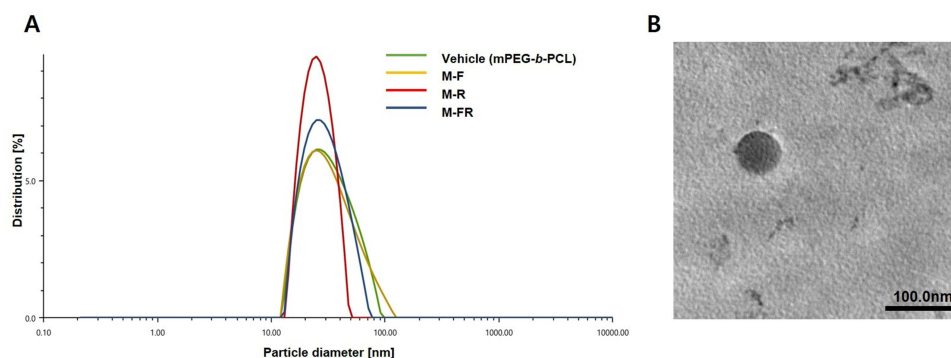
The mean size and PDI of M-FR were measured at 4°C and 37°C for 14 days. At 4°C, the mean size was < 50 nm on day 7, and < 80 nm by day 14. During the measurement period, the PDI was < 0.3. At 37°C, the mean size increased to  $\geq 200$  nm after day 2, and the PDI remained < 0.3 (Figure 3).

## Storage Stability Test After Freeze-Drying

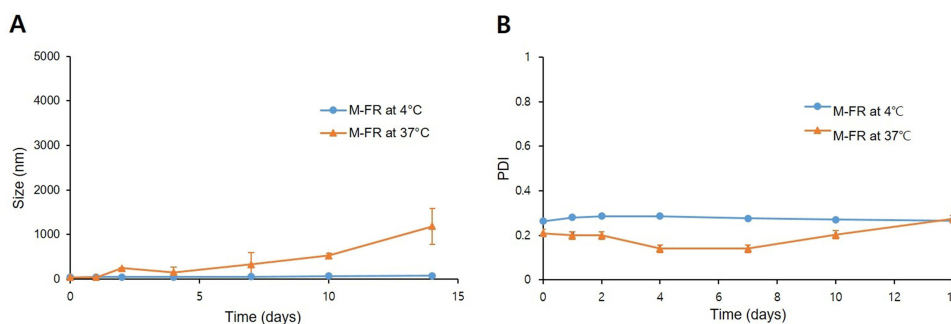
Simultaneously encapsulated micelles containing fenbendazole and rapamycin were evaluated for 120 days after freeze-drying. The evaluated physicochemical properties are shown in Table 3. After 1 day after of preparation, the size and PDI of M-FR was  $37.5 \pm 1.37$  nm and  $0.23 \pm 0.02$ , respectively. The size and PDI of M-FR after 120 days were  $37.4 \pm 0.82$  nm and  $0.21 \pm 0.01$ , respectively. The EE (%) of fenbendazole on days 1 and 120 were  $69.7 \pm 4.72\%$  and  $76.4 \pm 12.5\%$ ,

**Table 2** Physicochemical Properties of M-FR Prepared with Synergistic Drug Ratio (n = 3, Mean  $\pm$  SD)

Molar Ratio (Fenbendazole: Rapamycin)	Polymer Amount Used (mg)	Encapsulation Efficiency (EE %)		Drug Loading (DL %)		Particle Size (nm)	PolyDispersity Index (PDI)	Zeta Potential (mV)
		Fenbendazole	Rapamycin	Fenbendazole	Rapamycin			
1:1	100	$81.5 \pm 2.45$	$76.2 \pm 8.36$	0.99	3.01	$36.9 \pm 0.04$	$0.27 \pm 0.01$	$-1.63 \pm 0.59$
1:2	100	$75.7 \pm 4.61$	$98.0 \pm 1.97$	0.99	5.66	$37.2 \pm 1.10$	$0.20 \pm 0.02$	$-0.07 \pm 0.09$
2:1	100	$40.3 \pm 5.17$	$61.9 \pm 4.86$	1.96	3.01	$37.1 \pm 0.41$	$0.27 \pm 0.00$	$-1.10 \pm 1.06$
4:1	100	$19.5 \pm 0.49$	$59.2 \pm 3.83$	3.85	3.01	$36.5 \pm 0.26$	$0.27 \pm 0.01$	$-0.80 \pm 1.00$



**Figure 2 (A)** Representative size distribution of vehicle, M-F, M-R, and M-FR. **(B)** Transmission electron microscopy (TEM) images of M-FR.  
**Abbreviations:** TEM, Transmission electron microscopy; M-F, fenbendazole-loaded micelle; M-R, rapamycin-loaded micelle; M-FR, fenbendazole/rapamycin-loaded micelle.



**Figure 3 (A)** Size and **(B)** PDI change in M-FR at 4°C and 37°C (n = 3).  
**Abbreviations:** M-FR, fenbendazole/rapamycin-loaded micelle; PDI, polydispersity index.

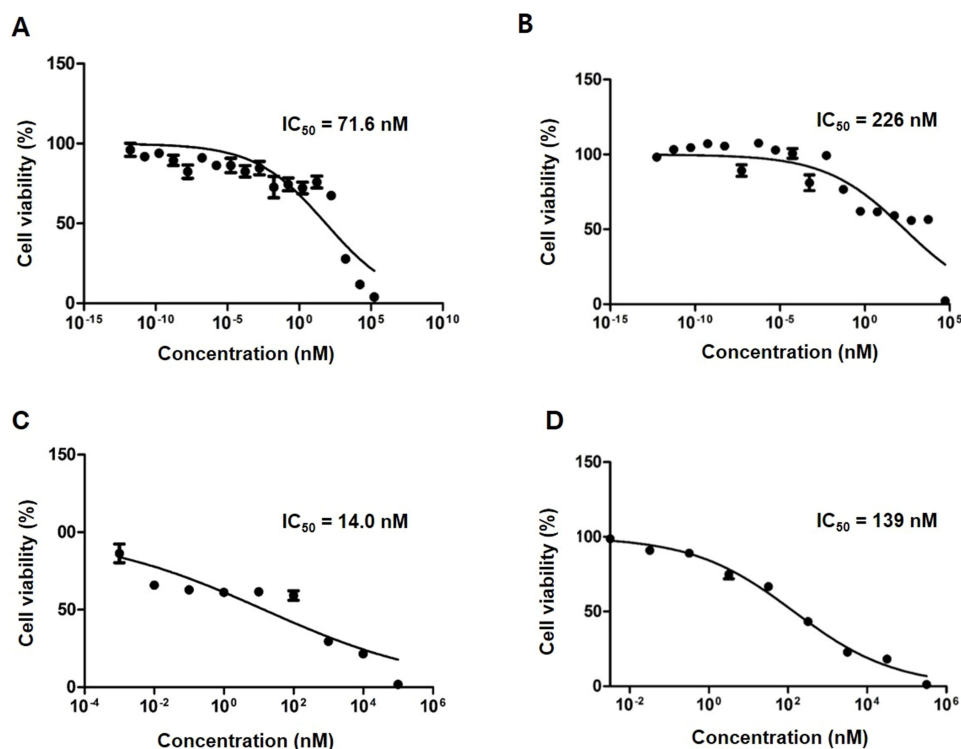
respectively. The EE (%) of rapamycin on days 1 and 120 were  $96.9 \pm 1.65\%$  and  $91.6 \pm 1.08\%$ , respectively. The zeta potential affects several properties of nano-drug delivery systems.<sup>83</sup> Therefore the zeta potential of M-FR was evaluated, and our formulation was measured to be  $-0.23 \pm 0.13$  on day 1 and  $0.13 \pm 0.19$  on day 120. There was no considerable change in the EE (%), size, and zeta potential of M-FR until the last day of evaluation.

## Cytotoxicity of M-FR in HeyA8 Cells

Figure 4 shows the cytotoxicity of free fenbendazole, free rapamycin, the final ratio of free fenbendazole/rapamycin, and M-FR. The  $IC_{50}$  value of free fenbendazole was 71.6 nM and free rapamycin was 226 nM (Figure 4A and B). The  $IC_{50}$  value of free fenbendazole/rapamycin was 14.0 nM, and of the M-FR was 139 nM (Figure 4C and 4D).

**Table 3** Long-Term Storage Stability Study of M-FR (n = 3 ± SD)

Days After Freeze-Drying	Encapsulation Efficiency (EE %)		Particle Size (nm)	PolyDispersity Index (PDI)	Zeta Potential (mV)
	Fenbendazole	Rapamycin			
1	69.7 ± 4.72	96.9 ± 1.65	37.5 ± 1.37	0.23 ± 0.02	-0.23 ± 0.13
7	68.1 ± 3.86	97.6 ± 1.97	37.3 ± 0.25	0.24 ± 0.02	-1.10 ± 0.51
14	65.1 ± 8.60	99.3 ± 0.39	42.4 ± 1.35	0.26 ± 0.01	-0.30 ± 0.43
30	65.7 ± 1.09	99.9 ± 0.02	37.6 ± 0.34	0.25 ± 0.01	-1.07 ± 1.44
60	75.7 ± 4.60	96.3 ± 0.75	32.8 ± 0.08	0.15 ± 0.01	-1.13 ± 0.71
120	76.4 ± 12.5	91.6 ± 1.08	37.4 ± 0.82	0.21 ± 0.01	0.13 ± 0.19



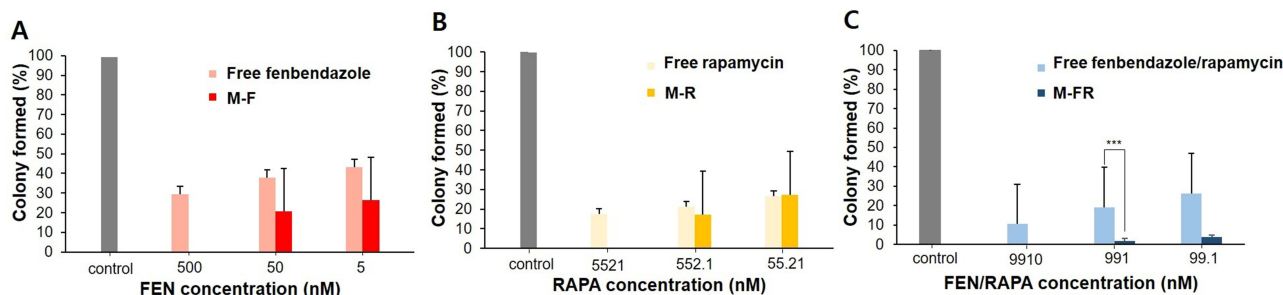
**Figure 4** In vitro cytotoxicity assay of HeyA8 cells treated with (A) Free fenbendazole, (B) Free rapamycin, (C) Free fenbendazole/rapamycin, and (D) M-FR. **Abbreviations:** IC<sub>50</sub>, 50% inhibition concentration; M-FR, fenbendazole/rapamycin-loaded micelle.

## Clonogenic Assay

Clonogenic assays were performed to evaluate the long-term anticancer efficacy of drugs and micelles. Colony formation was compared after 2 weeks of treatment with free fenbendazole, free rapamycin, free fenbendazole/rapamycin, M-F, M-R, or M-FR in HeyA8 cells. For M-F, no colonies were found after 500 nM treatment. Cell viability in the 50 nM and 5 nM treatments was 20.7% and 26.5%, respectively, indicating 1.8-fold and 1.6-fold greater inhibition than free fenbendazole (Figure 5A). For M-R, no colonies were found after 5521 nM treatment, and the colony formation inhibition rate was 1.2 times higher than free rapamycin at 552.1 nM (Figure 5B). For M-FR, no colonies were found after 9910 nM treatment. Treatments with 991 nM and 99.1 nM showed 10-fold and 6.9-fold higher colony formation inhibition than free fenbendazole/rapamycin, respectively, which was statistically significant ( $***p < 0.001$ ) (Figure 5C).

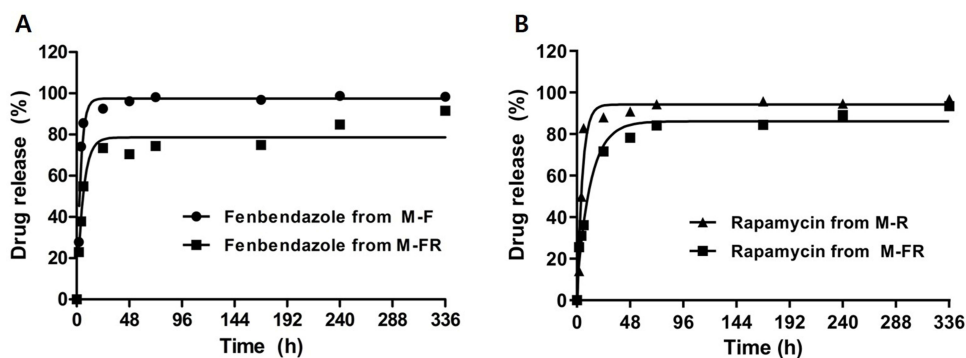
## In vitro Drug Release Assay

The release profiles of M-F, M-R and M-FR were evaluated and are shown in Figure 6. The goodness of fit ( $R^2$ ) and rate constant ( $k$ ,  $h^{-1}$ ) based on the first-order are also analyzed. The  $R^2$  and  $k$  of fenbendazole calculated from the release



**Figure 5** In vitro clonogenic assay results after the treatment of HeyA8 cells with (A) Free fenbendazole and M-F, (B) Free rapamycin and M-R, and (C) Free fenbendazole/rapamycin and M-FR ( $n = 3$ ). **Abbreviations:** M-F, fenbendazole-loaded micelle; M-R, rapamycin-loaded micelle; M-FR, fenbendazole/rapamycin-loaded micelle.





**Figure 6** Single and combined drug-release profiles of fenbendazole and rapamycin in micelles. (A) Fenbendazole release in M-F and M-FR, and (B) Rapamycin release in M-R and M-FR ( $n = 3$ ).

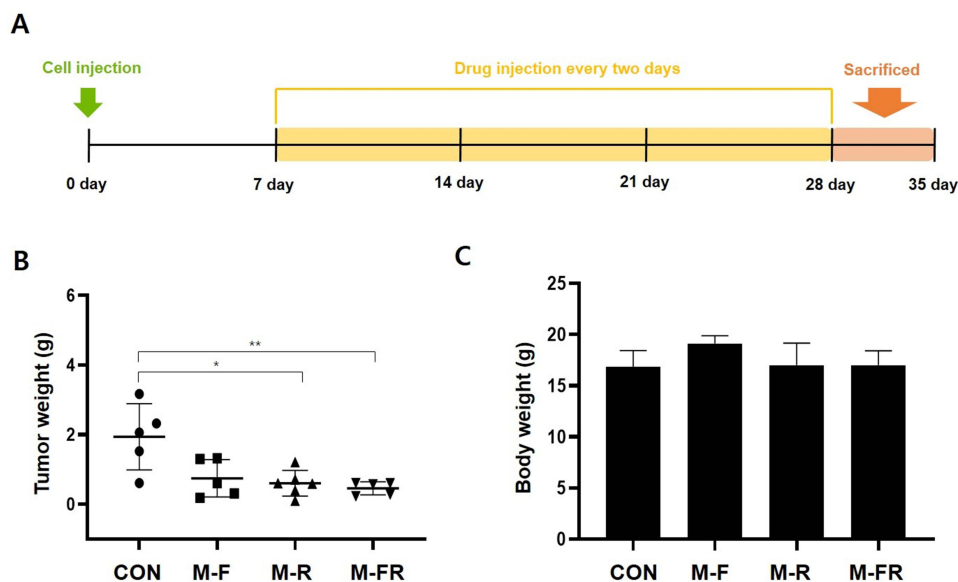
**Abbreviations:** M-F, fenbendazole-loaded micelle; M-R, rapamycin-loaded micelle; M-FR, fenbendazole/rapamycin-loaded micelle.

profile were  $R^2 = 0.9711$ ,  $k = 0.2960 \text{ h}^{-1}$  and  $R^2 = 0.9568$ ,  $k = 0.1795 \text{ h}^{-1}$  in M-F and M-FR, respectively. The  $R^2$  and  $k$  of rapamycin were  $R^2 = 0.9517$ ,  $k = 0.2212 \text{ h}^{-1}$  and  $R^2 = 0.9778$ ,  $k = 0.0796 \text{ h}^{-1}$  in M-R and M-FR, respectively. For fenbendazole, the release rate was  $96.2 \pm 1.49\%$  in M-F and  $70.4 \pm 14.0\%$  in M-FR after 48 h ( $*p < 0.05$ ). Also,  $98.8 \pm 0.47\%$  and  $84.9 \pm 6.29\%$  were released at 240 h, respectively ( $*p < 0.05$ ). For rapamycin, more than 90% drug was released after 48 h in M-R, but  $78.2 \pm 7.56\%$  was released in M-FR. Also,  $96.7 \pm 3.20\%$  and  $89.2 \pm 7.31\%$  were released at 240 h, respectively.

## In vivo Antitumor Efficacy

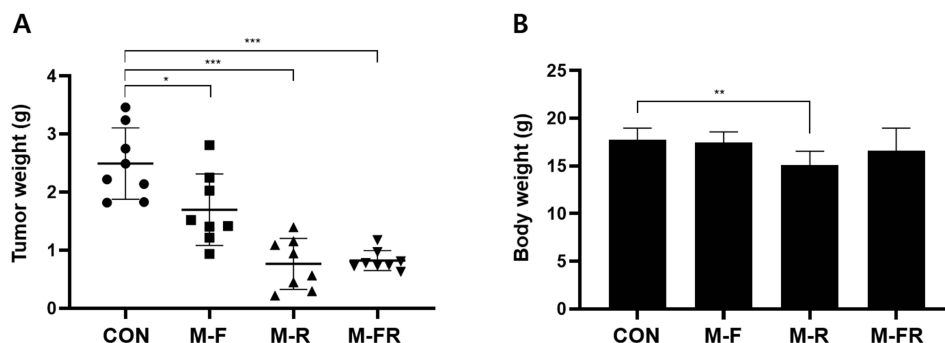
This experiment was performed twice in the female BALB/c nude mice bearing tumors. Both experiments were divided into the same group (CON, M-F, M-R, and M-FR), and only the dosage was different. Mice from both experiments were observed and injected for 3 weeks, and the sacrifice date and administration interval were also the same (Figure 7A).

In the first experiment, fenbendazole (2.5 mg/kg) and rapamycin (15 mg/kg) were evaluated and expressed as tumor and body weights (Figure 7B and C). The tumor weights of CON and M-FR were  $2.09 \pm 0.30 \text{ g}$  and  $0.46 \pm 0.17 \text{ g}$ , respectively, which were statistically significant ( $**p < 0.01$ ). In addition, no toxicity was observed in any of the groups.



**Figure 7** (A) Time schedule of antitumor efficacy using a mouse injected with HeyA8 cells, (B) Tumor weight when 2.5 mg/kg of fenbendazole and 15 mg/kg of rapamycin were administered, and (C) Body weight for toxicity ( $n = 10$ ). ( $*p < 0.05$  and  $**p < 0.01$ ).

**Abbreviations:** CON, control; M-F, fenbendazole-loaded micelle; M-R, rapamycin-loaded micelle; M-FR, fenbendazole/rapamycin-loaded micelle.



**Figure 8 (A)** Tumor weight when 5 mg/kg of fenbendazole and 30 mg/kg of rapamycin were administered, and **(B)** body weight for toxicity ( $n = 10$ ). (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ).

**Abbreviations:** CON, control; M-F, fenbendazole-loaded micelle; M-R, rapamycin-loaded micelle; M-FR, fenbendazole/rapamycin-loaded micelle.

In the second experiment, fenbendazole (5 mg/kg) and rapamycin (30 mg/kg) were evaluated and expressed as tumor and body weight (Figure 8A and B). The tumor weight of CON was  $2.49 \pm 0.58$  g, M-R was  $0.77 \pm 0.41$  g, and M-FR was  $0.82 \pm 0.16$  g. Compared with CON, M-R significantly inhibited the tumor (\*\* $p < 0.001$ ) but showed toxicity (\*\* $p < 0.01$ ). However, M-FR showed a significant antitumor effect (\*\* $p < 0.001$ ) and no toxicity.

## Discussion

Interfering with microtubule polymerization and inhibiting mTOR signaling are important for OC suppression.<sup>84,85</sup> Therefore, we hypothesized that fenbendazole, which exhibits anticancer activity by interfering with microtubule synthesis, and rapamycin, a mTOR inhibitor, would be effective in OC treatment. Fenbendazole, an anthelmintic drug, has been changed to an anticancer agent and applied to the treatment of OC,<sup>86</sup> and the anticancer potential of mPEG-*b*-PCL micelles encapsulated with fenbendazole and rapamycin has been reported.<sup>74</sup> Therefore, we evaluated the optimal ratio of the two drugs required for exhibiting a synergistic effect by obtaining CI values in HeyA8 cells, an OC cell line.<sup>77,87</sup> Although synergism was observed at various molar ratios of fenbendazole and rapamycin, the EE (%) of fenbendazole was significantly lower as <50% at 2:1 and 4:1. The EE (%) of 1:1 and 1:2 were >70%,<sup>88</sup> and the size and PDI of the two ratios were < 40 nm and 0.3, respectively. Therefore, M-FR can be expected to accumulate more in tumors than in normal tissues through the EPR effect.<sup>89–91</sup> Fabricating micelles using a 1:2 molar ratio of fenbendazole: rapamycin with high EE (%) and small size and synergistic effect is the most suitable.

In this study, we evaluated whether micelles could be stored for a long time. Freeze-drying is a suitable technique for improving the long-term stability of colloidal nanoparticles.<sup>92</sup> Our formulation was stored at  $-20^{\circ}\text{C}$  and evaluated for up to 120 days. No significant difference in physicochemical properties was observed from day 1. Therefore, we recommend long-term storage of the formulation at  $-20^{\circ}\text{C}$ . In addition, M-FR in an aqueous solution were observed at  $4^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  for 2 weeks. At  $4^{\circ}\text{C}$ , day 7 showed  $51.9 \pm 3.36$  nm, and <90 nm was maintained until day 14. Therefore, it is recommended to store at  $4^{\circ}\text{C}$  to maintain the stability of M-FR in a liquid state. On the other hand, the size gradually increased from the day 1 at  $37^{\circ}\text{C}$ , and the PDI was maintained at <0.3 until 14 days.<sup>93</sup>

In the evaluation of in vitro cytotoxicity by the MTT assay, the  $\text{IC}_{50}$  value of free fenbendazole/rapamycin was lesser than of M-FR, possibly because the micelles delay drug release.<sup>15</sup> Also, the drug encapsulated in micelles was released more slowly than 48 h (drug treatment time in MTT assay), indicating the cytotoxic effect of the drug may be lower than that of the free drug.<sup>94</sup> In the clonogenic assay, the colony formation inhibition rate of fenbendazole and rapamycin was evaluated for 14 days. The clonogenic assay evaluates the rate of colonial formation inhibition over a longer period than the MTT assay.<sup>95</sup> As a result, the cell viability treated with 991 nM M-FR was 1.91%, showing a significant difference by inhibiting colonies by 6.9 times compared to the treatment with free fenbendazole/rapamycin at the same concentration (\*\* $p < 0.001$ ).

The release rate of fenbendazole was compared in M-F and M-FR, and the release of rapamycin in M-R and M-FR. At 6 h, rapamycin was released > 80% in M-R but  $36.2 \pm 16.6\%$  in M-FR. In addition, rapamycin release of more than 90% was observed at 48 h in M-R and 336 h in M-FR. At 48 h, fenbendazole was released by  $96.2 \pm 1.49\%$  and  $70.4 \pm$

14.0% in M-F and M-FR, respectively, indicating a statistically significant difference ( $*p < 0.05$ ). In addition,  $98.8 \pm 0.47\%$  and  $84.9 \pm 6.29\%$  of fenbendazole in M-F and M-FR at 240 h showed a significant difference in release ( $*p < 0.05$ ). Comparing the release of fenbendazole from combination micelles and single micelles, a significant difference was found at the two time points, suggesting that hydrophobic drug-drug interactions probably occurred in the micelle core.<sup>96</sup> No explosive release initially in M-FR, indicates the possibility of preventing the explosive release of high-dose drugs,<sup>97</sup> which, in turn, can prevent toxicity in the body.

The first evaluation of in vivo antitumor efficacy (2.5 mg/kg fenbendazole, 15 mg/kg rapamycin), showed that M-FR had a significant antitumor effect compared to the CON ( $**p < 0.01$ ). However, when M-F and M-R were compared with M-FR, there was no significant difference. We doubled the dose of each drug in the second step considering no toxicity was found in any group. In the second evaluation, only M-R showed toxicity ( $**p < 0.01$ ), and no toxicity observed in M-F. These results are attributed to the selective toxicity of fenbendazole to tumor cells and its high stability in vivo.<sup>98,99</sup> Nevertheless, in vivo anticancer evaluation indicates that M-FR does not significantly inhibit cancer cells compared with M-FR, probably due to the loss of carrier or drug after injection, as in vivo anticancer evaluations are performed in complex structures, unlike in vitro cytotoxicity tests.<sup>100,101</sup> Therefore, M-FR may not have reached the tumor or M-FR may not have reached the tumor at this designated ratio. However, in the two in vivo antitumor efficacy evaluations, M-FR showed a significant antitumor effect compared with CON ( $**p < 0.01$ ,  $***p < 0.001$ ) and no toxicity was observed.

Compare with rapamycin, fenbendazole did not enhance the anticancer effect of M-FR compared to rapamycin. This result may be due to insufficient fenbendazole dose; however, owing to its low solubility,<sup>102</sup> encapsulating a high dose of fenbendazole was not possible. Further studies are required to increase and improve the capacity of fenbendazole in other systems to make it a useful combination.

## Conclusions

We evaluated the ability of micelles encapsulated with fenbendazole and the mTOR inhibitor, rapamycin, to prevent microtubule polymerization in OC. Various ratios of drug combinations were evaluated in OC cells, and synergistic effects were observed in many combination ratios. After evaluating the physicochemical properties of the formulation prepared at a ratio showing a synergistic effect, an optimal formulation ratio of 1:2 was obtained. In an in vitro study, M-FR showed higher colony inhibition than the free drug and released the drug relatively slower than M-F and M-R. Formulations designed for the two doses were evaluated by establishing a HeyA8 orthotopic model for in vivo antitumor efficacy. M-FR significantly inhibited OC compared to CON, and M-FR was not toxic at either dose tested. Therefore, we believe that the application of other polymers may result in a better formulation. We hope that this study will help future research on fenbendazole and rapamycin researchers.

## Abbreviations

OC, ovarian cancer; EOC, epithelial ovarian cancer; EPR, enhanced permeability and retention; mPEG-*b*-PCL, methoxy poly(ethylene glycol)-*b*-poly(caprolactone); mTOR, mammalian target of rapamycin; IV, intravenous; ACN, acetonitrile; HBSS, Hank's balanced salt solution; DW, distilled water; MTT, thiazolyl blue tetrazolium bromide; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; RPMI, Roswell Park Memorial Institute; DPBS, Dulbecco's phosphate-buffered saline; M-FR, fenbendazole/rapamycin-loaded mPEG-*b*-PCL micelle; PDI, polydispersity index; DLS, dynamic light scattering; EE, encapsulation efficiency; DL, drug loading; CI, combination index; M-F, fenbendazole-loaded micelle; M-R, rapamycin-loaded micelle; PBS, phosphate-buffered saline; IACUC, Institutional Animal Care and Use Committee; AAALAC, Association for Assessment and Accreditation of Laboratory Resources; ILAR, Institute of Laboratory Animal Resources; IC<sub>50</sub>, 50% inhibition concentration.

## Data Sharing Statement

All the data contained in the article is available upon request.

## Ethics Approval

This experiment was reviewed and approved by the institutional animal care and use committee (IACUC) of Samsung Biomedical Research Institute (protocol No. H-A9-003), which is an accredited facility by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and abides by the Institute of Laboratory Animal Resources (ILAR) guideline.

## Author Contributions

Conceptualization, Y.B.S., D.H.S., and J.W.L.; methodology, Y.B.S. and J.Y.C.; software, Y.B.S.; validation, Y.B.S. and D.H.S.; formal analysis, Y.B.S. and J.Y.C.; investigation, Y.B.S. and J.Y.C.; resources, D.H.S. and J.W.L.; data curation, Y.B.S., J.Y.C., D.H.S., and J.W.L.; interpretation, Y.B.S., J.Y.C., D.H.S., and J.W.L.; writing-original draft preparation, Y.B.S.; writing-review and editing, Y.B.S., J.Y.C., D.H.S., and J.W.L.; visualization, Y.B.S., super-vision, D.H.S. and J.W.L.; project administration, Y.B.S., J.Y.C., D.H.S., and J.W.L. All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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## Disclosure

Yu Been Shin and Ju-Yeon Choi are co-first authors for this study. The authors report no conflicts of interest in this work.

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