Novel Polymeric Nanoparticles for Intracellular Delivery of Peptide Cargos: Antitumor Efficacy of the BCL-2 Conversion Peptide NuBCP-9

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Abstract

The preclinical development of peptide drugs for cancer treatment is hampered by their poor pharmacologic properties and cell penetrative capabilities in vivo. In this study, we report a nanoparticle-based formulation that overcomes these limitations, illustrating their utility in studies of the anticancer peptide NuBCP-9, which converts BCL-2 from a cell protector to a cell killer. NuBCP-9 was encapsulated in polymeric nanoparticles composed of a polyethylene glycol (PEG)–modified polyactic acid (PLA) diblock copolymer (NuBCP-9/PLA–PEG) or PEG–polypolypropylene glycol-PEG-modified PLA–tetrablock copolymer (NuBCP-9/PLA–PEG–PPG–PEG). We found that peptide encapsulation was enhanced by increasing the PEG chain length in the block copolymers. NuBCP-9 release from the nanoparticles was controlled by both PEG chain length and the PLA molecular weight, permitting time-release over sustained periods. Treatment of human cancer cells with these nanoparticles in vitro triggered apoptosis by NuBCP-9–mediated mechanism, with a potency similar to NuBCP-9 linked to a cell-penetrating poly-Arg peptide. Strikingly, in vivo administration of NuBCP-9/nanoparticles triggered complete regressions in the Ehrlich syngeneic mouse model of solid tumor. Our results illustrate an effective method for sustained delivery of anticancer peptides, highlighting the superior qualities of the novel PLA–PEG–PPG–PEG tetrablock copolymer formulation as a tool to target intracellular proteins. Cancer Res; 74(12); 3271–81. ©2014 AACR.

Introduction

BCL-2 family proteins are important regulators of the mitochondrial outer membrane potential (MOMP) and thereby the induction of apoptosis (1, 2). The BCL-2 family is divided into proapoptotic and antiapoptotic members that share BCL-2 homology (BH) domains. The proapoptotic BCL-2 family members BAX and BAK contain multiple BH domains and control the MOMP. Other proapoptotic members that possess only the BH3 domain (BAD, BIM, BID, PUMA, and NOXA) either directly activate BAX/BAK, or inhibit the antiapoptotic activity of BCL-2 and BCL-xL to promote BAX/BAK activation. The antiapoptotic activities of the BCL-2 and BCL-xL proteins have been targeted with peptides derived from the BH3 domain and with small molecule inhibitors, such as ABT-737 and navitoclax, that mimic the BH3 \(\alpha\)-helix (3–5). BCL-2 has also been targeted with a Nur77-derived peptide of 9 amino acids (NuBCP-9; ref. 6). Nur77 is an orphan nuclear receptor that interacts with the BCL-2 N-terminal loop region and induces a BCL-2 conformational change (7). Binding of Nur77 exposes the BCL-2 BH3 domain and converts BCL-2 from an antiapoptotic protein to an inducer of apoptosis (7). Based on these findings, the D-amino acid NuBCP-9 peptide corresponding to the Nur77 region that interacts with BCL-2 was conjugated to the cell-penetrating D-Arg octamer (r8). Significantly, NuBCP-9-r8 was shown to induce apoptosis of cancer, but not normal, cells by a BCL-2–dependent mechanism (6). As predicted from Nur77 studies, NuBCP-9 binding was associated with a BCL-2 conformational change and thereby neutralization of BCL-2–mediated inhibition of BAX (6). NuBCP-9 binding to BCL-2 also exposes the BCL-2 BH3 domain and inhibits the survival function of BCL-xL (6). These findings and the demonstration that NuBCP-9-r8 induces apoptosis of cancer cells in vitro and in animal models supported the development of NuBCP-9-r8 as a selective anticancer agent.

Intracellular cancer targets that are devoid of an ATP-binding pocket are often undruggable with small chemical inhibitors. Cell-penetrating therapeutic peptides have thus emerged as promising agents because of their potential for targeting intracellular proteins in cancer cells with high specificity and limited off-target toxicity (8, 9). However, delivery of anticancer peptides presents a challenge as a result of the potential degradation and immunogenicity of these agents. In...
addition, small therapeutic peptides generally have short circulating half-lives and require frequent administration for sustained inhibition of their target proteins (10). Therapeutic peptides also frequently require a protein transduction domain (PTD) for cell membrane penetration and intracellular localization (11–13). Cell-penetrating peptides (CPP) that have been widely used for cargo delivery include, among others, the TAT peptide, penetratin, and oligoarginines (11). The selection of a certain CPP is of importance in that the CPP can in a cargo-dependent manner contribute to decreased serum stability, inefficient transit from the endosome to the cytosol, and unanticipated toxicities (14). Indeed, for NuBCP-9, the cell-penetrating R8 used for intracellular delivery acts in synergy with the N-terminal phenylalanine of NuBCP-9 to cause membrane blebbing and cell necrosis that are independent of BCL-2 expression (15). These findings have supported the study of alternative CPPs for NuBCP-9 or other formulations that preclude the use of a CPP for intracellular delivery.

Nanoparticle delivery systems for anticancer agents, particularly small molecules such as doxorubicin and paclitaxel, have been developed to improve pharmacokinetic parameters and therapeutic index (16). In this context, tumor microenvironments that are subject to hypoxia and acidosis can limit the effectiveness of small molecule anticancer agents (16). By extension and through what is referred to as the enhanced permeation retention (EPR) effect, nanoparticle delivery of anticancer agents can overcome limitations imposed by the tumor microenvironment and sustain drug exposure (16, 17). Nanoparticles can also be decorated with ligands to selectively target the surface of tumor cells (18). Polymeric nanoparticles represent one class that has been widely studied and shown to be nontoxic, biocompatible, and biodegradable (19, 20). Specifically, PLA-PEG block copolymer nanoparticles have been used as carriers for anticancer drugs in sustained/controlled release and targeted delivery systems to enhance efficacy and circumvent drug resistance (19, 20). For example, Genexol-PM is a paclitaxel-loaded PLA-PEG polymeric nanoparticle that is approved for use in Korea and is undergoing phase II evaluation in the United States for metastatic cancers (19, 21). BIND Biosciences also has under development docetaxel-encapsulated PLGA-PEG nanoparticles for the treatment of solid tumors (19). Curiously, polymeric nanoparticles have not been fully evaluated for the delivery of anticancer peptide drugs.

The present studies have focused on the encapsulation of NuBCP-9 into PLA-PEG nanoparticles or a novel PLA-PEG-PPG-PPEG triblock nanoparticle system to assess delivery of this anticancer peptide to malignant cells in vitro and in vivo. The results obtained provide the experimental basis for the further development of NuBCP-9/nanoparticles and for the potential delivery of other anticancer peptide drugs.

Materials and Methods

Synthesis and characterization of PLA-PEG copolymers

PLA-PEG diblock copolymers were synthesized using 72-kDa PLA (NatureWorks) or ~12-kDa PLA (Purac Chemicals). PEG was used at 1, 2, or 4 kDa (CDH, India) for coupling to PLA. PEG-PPG-PPEG (12.5 kDa; Poloxamer-F127, Sigma-Aldrich) was also used for the synthesis of PLA block copolymers. In a standard experiment, 0.014 mmol PLA and PEG or PEG-PPG-PPEG were dissolved in 100 mL dichloromethane (CH2Cl2) and stirred at 0°C to 2°C. To these solutions, 5 mL of 1% N,N-dicyclohexylcarbodiimide (DCC) was added slowly, followed by the addition of 2 mL of 0.1% 4-dimethylaminopyridine (DMP) and stirring for 16 hours. The resulting PLA-PEG and PLA-PEG-PPEG-PPEG block copolymers were precipitated with a 1:1 mixture of diethyl ether and methanol to remove unreacted PEG and PEG-PPEG-PPEG. Gel permeation chromatography (GPC) analysis was performed at room temperature using a Visotec GPC system with tetrahydrofuran as the mobile phase. The synthesized PLA block copolymers were dried under vacuum and stored at −20°C until use. 1HNMR of PLA-PEG or PLA-PEG-PPEG-PPEG was performed in CDCl3 at 300 Hz (Bruker).

Preparation of rhodamine B- and NuBCP-9–loaded nanoparticles

Loading of the PLA-PEG and PLA-PEG-PPEG-PEG nanoparticles was performed using a double emulsion solvent evaporation method. PLA-PEG or PLA-PEG-PPEG-PPEG copolymers (100 mg) were dissolved in 5 mL acetonitrile. Rhodamine B (1 mg) or l-amino acid NuBCP-9 (10 mg; Bioconcept, India) was added to the solution with brief sonication. The resulting primary emulsion was then added dropwise into a 20 mL aqueous phase composed of Poloxamer-F127 (PEG-PPEG-PPEG) in distilled water and stirred at room temperature for 6 to 8 hours to facilitate solvent evaporation and nanoparticle stabilization. Nanoparticles were loaded with NuBCP-9 (FSRSLHSLL) peptide (7) and filtered through an Amikon 10-kDa ultrafilter (Millipore). The nanoparticles were lyophilized and stored at −20°C until use. The filtrate was collected and analyzed for free NuBCP-9 peptide using a Micro-BCA Kit (Pierce Chemicals). Encapsulation efficiency of NuBCP-9 peptide was determined using the following formula:

$$EE(%) = \left( \frac{(\text{Peptide} \times \text{Total} - \text{Peptide} \times \text{Filtrate})}{\text{Peptide} \times \text{Total}} \right) \times 100$$

Morphology and particle size of the nanoparticles was determined using a Zeiss EVO 50 Series scanning electron microscope (SEM). Potential of the nanoparticles was assessed by nanoparticle tracking analysis (NanoSight NS500).

Assessment of NuBCP-9 release from nanoparticles

The in vitro release kinetics of NuBCP-9 from nanoparticles were determined by the ultrafiltration method. Briefly, samples of freeze-dried nanoparticles (10 mg) were suspended in PBS and incubated at 37°C with gentle shaking at 150 to 160 rpm. At predetermined time points of up to 60 days, the samples were removed from the incubator and ultrafiltered through 10-kDa Amikon filters (Millipore). The filtrates were collected for analysis and fresh buffer was added to the respective tubes. Peptide concentration in the filtrates was determined by micro-BCA assay.

Cell culture

Human MCF-7 breast cancer and HepG2 hepatocellular carcinoma cells were grown in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 g/mL streptomycin. Human
umbilical vein endothelial cells (HUVEC) were cultured in EBM-2 medium with endothelial cell growth supplement (Lonza). Proliferation was assessed by the XTT-Based In Vitro Assay Kit (Cayman). To assess uptake of nanoparticles, MCF-7 cells were seeded on coverslips and grown for 24 hours. After incubation with rhodamine B loaded nanoparticles, the coverslips were removed, washed with PBS, and fixed with 4% paraformaldehyde. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) and visualized under a confocal laser scanning microscope (CLSM; Olympus, Fluoview FV1000 Microscope).

Assessment of apoptosis
Cells were stained using the Annexin V-Alexa Fluor 488/PI Apoptosis Assay Kit (Invitrogen). Quantification of apoptosis/necrosis was performed using Cellometer Vision (Nexcelom Bioscience LLC). Cells were also imaged using the CLSM microscope.

Immunoblot analysis
Cell lysates were prepared with M-PER reagent (Pierce Chemicals) and analyzed by immunoblotting with anti-BCL-2, anti-caspase-3 (Santa Cruz Biotechnology) and anti-β-actin (Sigma).

Analysis of antitumor activity
Mouse Ehrlich tumor cells were injected subcutaneously in the hind limb of syngeneic Balb/c mice (17−22 g). Mice-bearing tumors (~400 mm³) were divided into 9 groups (10 mice/group) and treated intraperitoneally (i.p.) or intratumorally (i.t.) with different agents using two schedules for 21 days (Supplementary Table S1). Tumor volume was determined by calipers and calculated using the formula ($A \times B^2 / 2$), where $A$ and $B$ are the longest and shortest tumor diameters, respectively. From each group, 1 mouse was sacrificed on day 7, 14, and 21 for harvesting of tumor for histopathologic examination. Statistical analysis of tumor volumes was performed by one-way ANOVA and the Dunnett test using Origin 8.0 (Origin Lab). Survival of the mice was determined by the Kaplan–Meier method using Prism 4.0 software (GraphPad Software). One mouse from each of the control and NuBCP-9/nanoparticle-treated (i.p. once/week) groups was sacrificed on day 14 for tumor excision. The tumors were fixed in 10% formalin/saline and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin.

Results
Preparation and characterization of NuBCP-9−loaded polymeric nanoparticles
PLA-PEG nanoparticles have been commonly synthesized using PLA of ~20 kDa or less (19−21). In this work, we studied the development of polymeric nanoparticles that incorporate PLA of ~72 kDa to regulate the release of peptide drugs. PLA-PEG and PLA-PEG-PPG-PEG block copolymers were synthesized by the DCC/DMAP conjugation method. As determined by GPC, the molecular weight of PLA was 72,487 Da and it increased directly upon conjugation with increasing block length of PEG (1, 2, or 4 kDa) or with PEG-PPG-PEG (Table 1). *HNMR of

### Table 1. Characterization and physicochemical properties of PLA-PEG block copolymers and nanoparticles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mn</th>
<th>Mw</th>
<th>Mw/Mn</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential after peptide loading (mV)</th>
<th>Particle size after peptide loading (nm)</th>
<th>Peptide/polymer ratio (w/w)</th>
<th>EE%</th>
<th>Zeta potential after peptide loading (mV)</th>
<th>Particle size after peptide loading (nm)</th>
<th>Peptide/polymer ratio (w/w)</th>
<th>EE%</th>
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<tbody>
<tr>
<td>PLA</td>
<td>59,745</td>
<td>72,487</td>
<td>1.213</td>
<td>1.03</td>
<td>-15.8 ± 1.45</td>
<td>1:10</td>
<td>42.93</td>
<td>15.8</td>
<td>-15.8 ± 1.45</td>
<td>1:10</td>
<td>42.93</td>
<td>15.8</td>
</tr>
<tr>
<td>PLA-PEG 1K</td>
<td>67,716</td>
<td>72,806</td>
<td>1.072</td>
<td>1.03</td>
<td>12.5 ± 3.7</td>
<td>1:10</td>
<td>50.09</td>
<td>10.2</td>
<td>12.5 ± 3.7</td>
<td>1:10</td>
<td>50.09</td>
<td>10.2</td>
</tr>
<tr>
<td>PLA-PEG 2K</td>
<td>70,802</td>
<td>73,486</td>
<td>1.040</td>
<td>1.03</td>
<td>12.0 ± 2.5</td>
<td>1:10</td>
<td>58.96</td>
<td>11.9</td>
<td>12.0 ± 2.5</td>
<td>1:10</td>
<td>58.96</td>
<td>11.9</td>
</tr>
<tr>
<td>PLA-PEG 3K</td>
<td>75,879</td>
<td>74,436</td>
<td>1.082</td>
<td>1.03</td>
<td>11.1 ± 3.9</td>
<td>1:10</td>
<td>65.50</td>
<td>11.1</td>
<td>11.1 ± 3.9</td>
<td>1:10</td>
<td>65.50</td>
<td>11.1</td>
</tr>
<tr>
<td>PLA-PEG 4K</td>
<td>80,876</td>
<td>78,416</td>
<td>1.066</td>
<td>1.03</td>
<td>12.0 ± 2.3</td>
<td>1:10</td>
<td>64.04</td>
<td>12.0</td>
<td>12.0 ± 2.3</td>
<td>1:10</td>
<td>64.04</td>
<td>12.0</td>
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<tr>
<td>PLA-PEG-PPG-PEG 10K</td>
<td>85,356</td>
<td>85,678</td>
<td>1.023</td>
<td>1.03</td>
<td>11.2 ± 2.5</td>
<td>1:10</td>
<td>56.12</td>
<td>11.2</td>
<td>11.2 ± 2.5</td>
<td>1:10</td>
<td>56.12</td>
<td>11.2</td>
</tr>
<tr>
<td>PLA-PEG-PPG-PEG 15K</td>
<td>90,356</td>
<td>85,678</td>
<td>1.066</td>
<td>1.03</td>
<td>12.0 ± 2.3</td>
<td>1:10</td>
<td>56.12</td>
<td>12.0</td>
<td>12.0 ± 2.3</td>
<td>1:10</td>
<td>56.12</td>
<td>12.0</td>
</tr>
<tr>
<td>PLA-PEG-PPG-PEG 20K</td>
<td>95,356</td>
<td>85,678</td>
<td>1.023</td>
<td>1.03</td>
<td>11.2 ± 2.5</td>
<td>1:10</td>
<td>56.12</td>
<td>11.2</td>
<td>11.2 ± 2.5</td>
<td>1:10</td>
<td>56.12</td>
<td>11.2</td>
</tr>
</tbody>
</table>

**Abbreviation:** N.D., not done.

*GPC of PLA-PEG block copolymers at room temperature using Viscotech GPC system with tetrahydrofuran as mobile phase.

**Encapsulation efficiency expressed as a percentage mean of three determinants ± SD of NuBCP-9 recovered in nanoparticles compared with theoretical load.
PLA72K-PEG showed peaks at 5.2 ppm \([\text{OCH(\text{CH}_2\text{CH}_3})\text{O}\text{]}\times\text{O-CH}_2\text{CH}_3\text{O}]\), 3.7 ppm \([\text{OCH(\text{CH}_2\text{CH}_3})\text{O}\text{]}\times\text{O-CH}_2\text{CH}_3\text{O}]\), and 1.6 ppm \([\text{OCH(\text{CH}_2\text{CH}_3})\text{O}\text{]}\times\text{O-CH}_2\text{CH}_3\text{O}]\). An additional peak at 2.1 ppm was observed for PLA72K-PEG-PPG-PEG because of the \(-\text{OCH}_3\) proton of PPG. These results confirmed coupling of PLA72K with PEG or PEG-PPG-PEG.

The PLA-PEG block copolymer consists of a bilayer structure with the PLA hydrophobic core and the PEG hydrophilic shell interfacing with the aqueous medium. By contrast, conjugation of PEG-PPG-PEG with PLA results in multilayered nanoparticles as a result of PPG extending the hydrophobic structure (22). SEM studies of both the PLA72K-PEG4K and PLA72K-PEG-PPG-PEG nanoparticles showed that the particles are spherical with uniform sizes ranging from 40 to 50 nm in diameter (Fig. 1A, left and right). The nanoparticles were then loaded with rhodamine B to assess their uptake in cells. Incubation of the rhodamine B/PLA72K-PEG4K nanoparticles with MCF-7 breast cancer cells demonstrated uptake over 3 to 12 hours (Fig. 1B). The results further showed intracellular fluorescence of the rhodamine B nanoparticles diffusely throughout the cytosol (Fig. 1B). Similar results were obtained with the rhodamine B/PLA72K-PEG-PPG-PEG nanoparticles (data not shown). Uptake of PLA-based nanoparticles is through endocytosis and is associated with surface charge reversal (anionic to cationic) in the acidic pH of the endosomes. This charge reversal facilitates interaction of the nanoparticles with vesicular membranes, leading to transient and localized membrane destabilization, and thereby escape of the nanoparticles into the cytosol (23). Alternatively, rhodamine B may have been released from the nanoparticles. However, extensive washing of the nanoparticles after rhodamine B loading and the short 12-hour duration of the experiment support a mechanism other than release.

Our results further demonstrate that the hydrophilic PEG segment of the block copolymers facilitated the encapsulation of the \(\) amino acid NuBCP-9 peptide into the core of the PLA72K-PEG and PLA72K-PEG-PPG-PEG nanoparticles. For example, when the feeding ratio of NuBCP-9/block copolymers (w/w) was 1:10, the encapsulation efficiency of the different polymeric nanoparticles ranged from 43% to 66% (Table 1). The encapsulation efficiency of NuBCP-9 in PLA72K-PEG nanoparticles was higher than that obtained with PLA72K nanoparticles (Table 1). In concert with those findings, increases in PEG chain length resulted in improved encapsulation of the hydrophilic NuBCP-9 peptide (Table 1) and maximum encapsulation was obtained with PLA72K-PEG4K. Moreover, the encapsulation efficiency of the PLA72K-PEG-PPG-PEG nanoparticles (64%) was similar to that obtained with the PLA72K-PEG4K nanoparticles (66%; Table 1). Final loading of NuBCP-9 into these PLA-based nanoparticles was approximately 0.065 mg peptide/mg polymer and this concentration was used throughout the following in vitro and in vivo studies.

Nanoparticle tracking analysis measurements of the nanoparticles documented a hydrodynamic diameter ranging from 114 to 125 nm, which did not change significantly after NuBCP-9 loading (Table 1). In addition, the hydrodynamic diameter of the nanoparticles was not significantly altered by coupling to the different PEG molecular weights (1, 2, and 4 kDa), consistent with the greater mass of PLA as compared with PEG (Table 1). By contrast, the \(\) potential of the nanoparticles increased with increasing PEG block length (Table 1), a finding in concert with the demonstration that the \(\) potential of nanoparticles approaches neutral with increases in hydrophilicity because of PEG coupling (24). Notably, the \(\) potential of the PLA72K-PEG4K nanoparticles was similar to that for PLA72K-PEG-PPG-PEG nanoparticles before and after NuBCP-9 loading (Table 1). We also found that NuBCP-9 loading is associated with a marked decrease in \(\) potential as compared with that obtained with the unloaded nanoparticles (Table 1). The precise reason for this decrease in \(\) potential is not clear; however, it is plausible that because of the interaction of the positively charged adsorbed peptide with the negatively charged PLA, the peptide carboxyl groups, which have a negative charge,

![Figure 1](image-url)
are exposed on the surface of the nanoparticles. Studies have shown that negatively charged nanoparticles are taken up less efficiently by cells in vitro as compared with positively charged nanoparticles (25). However, negatively charged nanoparticles are not cleared as rapidly from the circulation as positively charged nanoparticles (25). Thus, the decrease in ζ potential of the NuBCP-9–loaded nanoparticles could affect their behavior in vitro and in vivo.

Release of NuBCP-9 from nanoparticles in vitro

The release profiles (percent cumulative release and percent release/day) of NuBCP-9 from the nanoparticles was assessed at pH 7.4 for up to 60 days (Fig. 2A–D and Supplementary Fig. S1A–S1D). PLA72k and PLA72k-PEG nanoparticles with PEG chain lengths of 1, 2, and 4 kDa exhibited cumulative releases of 62%, 78%, 85%, and 92%, respectively (Fig. 2A and B and Supplementary Fig. S1A and S1B). Maximum NuBCP-9 release at pH 7.4 was thus observed with the PLA72k-PEG4k nanoparticles, consistent with an increase in flexibility and hydrophilicity, and thereby hydration of the nanoparticles with increases in PEG chain length. At pH 5.0, the initial release of NuBCP-9 from PLA72k-PEG nanoparticles was similar to that found at pH 7.4 (Supplementary Fig. S2A and S2B). However, NuBCP-9 release at pH 5.0 was slower after day 7 and was only 68% as compared with 92% at pH 7.4 (Supplementary Fig. S2A and S2B). At pH 5.0, PLA chains remain in a coiled state and are thereby less susceptible to hydrolytic degradation (26, 27). By contrast, at pH 7.4, the PLA chains uncoil because of ionization, which is associated with an increase in nanoparticle degradation and release of NuBCP-9. The slower release at pH 5.0 could be a favorable characteristic in that there would be less NuBCP-9 release in the lysosome, a compartment with acidic pH, and thereby less degradation of the peptide. In addition, as compared with PLA72k-PEG4k nanoparticles, release of NuBCP-9 from PLA72k-PEG-PEG-PEG nanoparticles was slower at pH 7.4 (Fig. 2A–D), which is likely because of the hydrophobic nature of the PEG in the PEG-PEG-PEG block. In this regard, the recent synthesis of PLA-PEG-PEG-PEG copolymers by ring-opening polymerization of ε-lactide has demonstrated that the hydrophilic–hydrophobic balance is not sufficient for the formation of bilayer vesicles and that the assembly of an onion-like vesicle is responsible for sustained insulin release (22).

Lower molecular weight PLA (12 kDa) was also used to synthesize PLA-PEG block copolymers to assess the effect on NuBCP-9 loading and release. The encapsulation efficiency of NuBCP-9 into PLA 12K-PEG-PPG-PEG was similar to that obtained with PLA 72K-PEG-PPG-PEG (Table 1). However, release of NuBCP-9 from PLA12K-PEG-PPG-PEG was sustained for only 10 days as compared with 60 days for PLA72K-PEG-PPG-PEG. After 10 days, peptide was no longer detectable in the filtrate, a finding probably due in part to the degradation and bio-solubilization of low molecular weight PLA, which interferes with peptide detection in the assays (Fig. 2A–D).

Based on these results, the NuBCP-9–encapsulated PLA72K-PEG4K and PLA72K-PEG-PPG-PEG nanoparticles were further studied for biologic activity in vitro and in vivo.

Effects of NuBCP-9–encapsulated nanoparticles on cancer cell growth and survival in vitro

To assess activity of the NuBCP-9–loaded nanoparticles, we studied their effects on growth of BCL-2–expressing MCF-7 (28) and HepG2 (29) cells and, as a control, primary HUVEC cells. Notably, NuBCP-9 is ineffective in inhibiting cancer cell proliferation in the absence of a CPP, such as r8 (6), and these
Observations were confirmed when NuBCP-9 was tested against MCF-7 and HepG2 cells (Fig. 3A and B). However, as expected from previous studies (6), the L-amino acid NuBCP-9-R8 (R denotes L-amino acid; r denotes D-amino) completely blocked growth of these cells after treatment at 15 μmol/L for 48 hours (Fig. 3A and B), confirming that R8 is necessary for cell penetration and BCL-2 targeting. Notably, the NuBCP-9 that has been encapsulated in nanoparticles in this study is devoid of R8 and therefore would be expected to be inactive unless effectively delivered by the nanoparticles. Indeed, the NuBCP-9-encapsulated nanoparticles were highly effective in inhibiting growth of MCF-7 and HepG2 cells (Fig. 3A and B). By contrast, empty nanoparticles not encapsulated with NuBCP-9 had little if any effect on growth (data not shown). Other studies were performed to assess the effects of using different concentrations of NuBCP-9 nanoparticles on cell viability. As expected, NuBCP-9-R8 induced death of MCF-7 cells in a concentration-dependent manner (Fig. 3C). In addition, the NuBCP-9 nanoparticles (PLA72K-PEG4K and PLA72K-PEG-PPG-PEG) were effective in killing MCF-7 cells (Fig. 3C). Similar results were obtained when HepG2 cells were treated with different concentrations of PLA-PEG nanoparticles (Fig. 3D).
Delivery of NuBCP in Polymeric Nanoparticles

**Table 2. IC\textsubscript{50} of NuBCP-9 encapsulated PLA-PEG nanoparticles**

<table>
<thead>
<tr>
<th>IC\textsubscript{50} (\mu mol/L)</th>
<th>MCF-7</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NuBCP-9-PLA</td>
<td>2.58</td>
<td>4.18</td>
</tr>
<tr>
<td>NuBCP-9-PLA-PEG\textsuperscript{1K}</td>
<td>2.31</td>
<td>3.90</td>
</tr>
<tr>
<td>NuBCP-9-PLA-PEG\textsuperscript{2K}</td>
<td>1.90</td>
<td>2.39</td>
</tr>
<tr>
<td>NuBCP-9-PLA-PEG-PPG-PEG\textsuperscript{12,3K}</td>
<td>2.15</td>
<td>2.03</td>
</tr>
<tr>
<td>NuBCP-9-R8</td>
<td>2.00</td>
<td>ND</td>
</tr>
<tr>
<td>NuBCP-9-PLA-PEG-PPG-PEG\textsuperscript{12,3K}</td>
<td>7.11</td>
<td>9.10</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

The IC\textsubscript{50} values for the NuBCP-9 nanoparticles ranged from 1.9 to 4.2 \mu mol/L, as compared with 7.1 to 9.1 \mu mol/L for NuBCP-9 R8 (Table 2). In addition and in concert with the lack of NuBCP-9 activity against normal cells (6), NuBCP-9-encapsulated nanoparticles had no apparent effect on HUVEC cell growth (Fig. 3E). These results indicate that NuBCP-9 can be delivered intracellularly in an active form by polymeric nanoparticles.

**NuBCP-9 nanoparticles induce apoptosis of cancer cells**

NuBCP-9-R8 is a selective inducer of cancer cell apoptosis by targeting Bcl-2 (6). To assess the effects of NuBCP-9 nanoparticles on the apoptotic response, MCF-7 cells were treated with NuBCP-9-PLA\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles and monitored for externalization of phosphatidylserine at the cell membrane. Confocal images of MCF-7 cells stained with Annexin V-Alexa flour 488/PI demonstrated that treatment with NuBCP-9 nanoparticles is associated with the induction of an apoptotic response (Fig. 4A). By contrast, treatment with empty nanoparticles had no apparent effect (Fig. 4A). Quantitation of Annexin V and PI staining by Cellometer Vision confirmed that NuBCP-9-PLA\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles and NuBCP-9 PL\textsuperscript{72K}-PEG-PPG-PEG nanoparticles are as effective as NuBCP-9-R8 in inducing apoptosis of MCF-7 cells at 48 hours (Fig. 4B). As controls, empty PLA\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles and NuBCP-9 devoid of R8 had little, if any effect on MCF-7 cell apoptosis (Fig. 4B). As reported (28, 29), immunoblot analysis of MCF-7 and HePG2 cell lysates confirmed the expression of BCL-2 (Fig. 4C). Moreover, and in concert with the effects of NuBCP-9 on BCL-2 (6), treatment of MCF-7 and HePG2 cells with NuBCP-9 nanoparticles was associated with activation of caspase-3 (Fig. 4D). These findings thus demonstrate that, like NuBCP-9-R8, encapsulation of NuBCP-9 in nanoparticles results in the induction of apoptosis (Fig. 4D).

**NuBCP-9 nanoparticles are effective in inducing complete tumor regressions**

To assess the antitumor effects of NuBCP-9 nanoparticles in vivo, we treated Balb/c mice bearing established (~400 mm\textsuperscript{3}) subcutaneous BCL-2-positive Ehrlich syngeneic tumors (30). In this widely used model to assess the effects of anticancer agents, accurate administration of the viscous suspensions of nanoparticles was problematic in the narrow tail vein. As such, we used the i.p. route of administration, which allows nanoparticles to enter the systemic circulation through mesenteric vessels and the portal vein (31). Moreover, these studies were performed with NuBCP-9 (devoid of R8) because (i) NuBCP-9-R8 is active in vivo when administered i.t. (6), and (ii) the NuBCP-9 should be inactive in the absence of a CPP. Intraperitoneal injection of NuBCP-9 on a twice weekly schedule for 21 days had no significant effect on tumor growth as compared with that obtained with the saline control (PBS; Fig. 5A and Supplementary Table S1). Significantly, i.p. treatment with NuBCP-9 PL\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles at a dose of 20 mg/kg (NuBCP-9 peptide dose) on the same schedule was associated with complete and prolonged tumor regressions (Fig. 5A). Moreover, in contrast to the empty PL\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles, i.e. injection of NuBCP-9 PL\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles on a weekly schedule for 21 days resulted in similar anti-tumor responses (Fig. 5B). For assessment of direct delivery of NuBCP-9 nanoparticles into the tumors, we also administered NuBCP-9 PL\textsuperscript{72K}-PEG-PPG-PEG nanoparticles i.t. on a twice weekly schedule for 21 days and compared the effects of intraperitoneal administration of PL\textsuperscript{72K}-PEG-PPG-PEG on tumor growth. The results demonstrate that regression of the tumors was similar when the NuBCP-9 nanoparticles were delivered i.t. as compared with i.p. (Fig. 5C). However, i.p. treatment was more effective than i.t. in maintaining these antitumor responses after completing treatment (Fig. 5C). Similar results were obtained when NuBCP-9 PL\textsuperscript{72K}-PEG-PPG nanoparticles were administered i.t. weekly or twice weekly (Supplementary Fig. S3). Analysis of survival as determined by Kaplan–Meier plots further demonstrated that mice treated with NuBCP-9 nanoparticles (PLA\textsuperscript{72K}-PEG\textsuperscript{4K} or PL\textsuperscript{72K}-PEG-PPG-PEG) weekly or twice weekly survived significantly longer than the saline, empty nanoparticles, or NuBCP-9 controls (Fig. 5D and E). Moreover, the NuBCP-9 nanoparticles–treated mice survived longer when administration was i.p. as compared with the i.t. route (Fig. 5D and E). Significantly, there was no weight loss or other overt toxicities observed in the NuBCP-9/nanoparticle-treated mice (data not shown). Analysis of tissue sections demonstrated that tumor cells from the control mice exhibited mitosis and little, if any, evidence of necrosis (Fig. 5F, left). By contrast, tumor cells from NuBCP-9 PL\textsuperscript{72K}-PEG\textsuperscript{4K} nanoparticles-treated mice had decreased mitotic activity and considerably larger regions of necrosis (Fig. 5F, right), consistent with the antitumor activity of NuBCP-9.

**Discussion**

Peptide drugs have considerable potential for targeting intracellular oncoproteins that lack hydrophobic pockets and are thereby not eminently druggable with small molecules (32). In this context, there are increasing reports of peptide drugs under development that target non-kinase proteins such as survivin (33), HDM2 (34), NOTCH (35), MUC1 (36), and β-catenin (37), among others. The BCL-2 family proteins have also been the focus of peptide inhibitor development, specifically in one strategy using stabilized α-helices of BCL-2 domains (SAHB; ref. 38). BCL-2 has also been converted from an anti-apoptotic protein to an inducer of cell death by the Nur77-
derived NuBCP peptide (6, 7). With the exception of SAHBs, which penetrate cell membranes, delivery of peptide drugs has in general required the addition of a PTD for intracellular delivery (11). In this way, NuBCP was linked to the CPP D-Arg octamer (r8; ref. 6). NuBCP-9-r8 is a potent and selective inducer of cancer cell apoptosis \textit{in vitro} (6). NuBCP-9-r8 is...
also effective in inducing apoptosis in vivo when injected directly into tumors (6). Indeed, a challenge for the development of many peptide drugs, like NuBPC-9, is the potential for systemic delivery, which can be limited by pharmacologic parameters, such as short circulating half-lives, that prevent prolonged drug exposure. For example, systemic delivery of MUC1-targeted peptides has necessitated frequent daily dosing for effective antitumor activity in xenograft tumor models (36). The present studies, using NuBPC-9 as a model, were therefore performed to assess the delivery and release of an anticancer peptide drug in nanoparticles.

Nanoparticles are being widely used for the effective delivery of small molecules (19–21). However, the collective experience with encapsulation of peptides into nanoparticles is limited. A polycationic peptide targeting BCL-2 has been incorporated into cationic liposomes as a potential anticancer agent (39, 40). Other cytoxic peptides have been developed in cyclodextrin polymers with greater delivery efficacy (36). The present studies, using NuBPC-9 as a model, were therefore performed to assess the delivery and release of an anticancer peptide drug in nanoparticles.

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Figure 5. Antitumor activity of NuBPC-9 nanoparticles. A, Ehrlich tumor-bearing mice were treated with PBS (i.p., closed squares, twice weekly), 20 mg/kg NuBPC-9 (i.p., closed triangles, twice weekly), or 20 mg/kg NuBPC-9 PLA72K-PEG4K nanoparticles (i.p., closed circles, twice weekly) for a 21-day cycle. Tumor measurements were performed on the indicated days. The results are expressed as tumor volumes (mean ± SD). B, Ehrlich tumor-bearing mice were treated with empty PLA72K-PEG4K nanoparticles (i.p., closed diamonds, twice weekly), 20 mg/kg NuBPC-9 PLA72K-PEG4K nanoparticles (i.p., open squares, once weekly), or 20 mg/kg NuBPC-9 PLA72K-PEG4K nanoparticles (i.p., twice weekly). Tumor measurements were performed on the indicated days. The results are expressed as tumor volumes (mean ± SD). C, Ehrlich tumor-bearing mice were treated with empty PLA72K-PEG4K nanoparticles (i.p., closed diamonds, twice weekly), 20 mg/kg NuBPC-9 PLA72K-PEG4K nanoparticles (i.p., open diamonds, twice weekly), or 20 mg/kg NuBPC-9 PLA72K-PEG4K nanoparticles (i.p., open circles, twice weekly) for 21-day cycle. Tumor measurements were performed on the indicated days. The results are expressed as tumor volumes (mean ± SD). D, empty PLA72K-PEG4K nanoparticles (i.p., closed triangles, twice weekly), NuBPC-9 (i.p., closed triangles), NuBPC-9 PLA72K-PEG4K nanoparticles (i.p., closed circles, twice weekly), and NuBPC-9 PLA72K-PEG4K nanoparticles (open squares, once weekly). The statistical analysis was performed between the vehicle control and the NuBPC-9 PLA-PEG nanoparticle group and the NuBPC-9 PLA-PEG-PPG-PEG nanoparticle group (P < 0.001). E, the results are expressed as the percentage survival as determined by Kaplan–Meier analysis. F, histopathology of tumor tissues obtained from mice treated with the control (left) or NuBPC-9 PLA72K-PEG4K nanoparticles (right) for 14 days and stained with hematoxylin and eosin (×400).

Delivery of NuBPC in Polymeric Nanoparticles

In addition, the membrane-disrupting cytolytic peptide, melitin, has been incorporated into the outer lipid monolayer of a perfluorocarbon nanoemulsion vehicle to target tumors (41). Other cytoxic peptides have been developed in cyclodextrin polymerized nanoparticles, glycolide nanoparticles, and poly (propylacrylic acid) nanoparticles (42, 43). To our knowledge, none of these peptide nanoformulations have been further developed for clinical evaluation. In this context, a significant consideration for the clinical development of nanoparticles is the concentration of emulsifier, which at high levels can be toxic to kidneys. In this study, the PLA-PEG and PLA-PEG-PPG-PEG nanoparticles were prepared using a double emulsion solvent evaporation method (44). Our results show that an advantage of using PEG-PPG-PEG as a block copolymer in preparation of PLA nanoparticles is that emulsifier concentrations can be markedly reduced for PLA nanoparticle stabilization at pH 7.4. Indeed, for PLA-PEG nanoparticles, the final emulsifier concentration was 0.4%, however, we were able to further reduce that concentration by 5-fold to 0.08% for the PLA-PEG-PPG-PEG nanoparticles. These findings are therefore of potential significance for the clinical development of these polymeric nanoparticles.

Based on the results obtained with our double emulsion method, we studied loading of NuBPC-9 in the PLA-PEG and PLA-PEG-PPG-PEG nanoparticles. Previous work had demonstrated that increasing PEG chain length from 1 to 4 kDa significantly improves the hydrophilicity and flexibility of block copolymers (44). Consequently, we analyzed the effects of different lengths of the PEG block and found that increases in PEG chain length improves NuBPC-9 loading. Moreover, our results demonstrate that release of NuBPC-9 over time is enhanced by increases in PEG chain length, a finding in concert with greater flexibility, hydrophilicity and hydration of the nanoparticle. In turn, the rate of diffusion increases, as does release of the peptide from the nanoparticle matrix (20, 45–48).

Thus, for NuBPC-9 and potentially other peptide drugs, these
findings indicate that PEG chain length is of importance for both loading in and release from polymeric nanoparticles. Another aspect of our studies worth highlighting is the use of a high molecular weight PLA (72 kDa) in the NuBCP-9 encapsulated nanoparticles. Previous work on polymeric nanoparticles has generally involved lower molecular weight PLA, for example, using 20 kDa or less (49, 50). Our results indicate that in vitro release of NuBCP-9 from our nanoparticles was sustained to more than 60 days when using ~72-kDa PLA as compared with release of the peptide more than 10 days with ~12-kDa PLA. These results can be explained, at least in part, by the hydrolysis of PLA and thereby NuBCP-9 diffusion. At pH 5.0, PLA remains coiled and is less susceptible to hydrolysis; whereas at pH 7.4, the PLA chains open with an increased propensity for hydrolysis. The uncoiling of PLA is thus associated with an increase in its degradation and release of NuBCP-9 (26, 27). These findings collectively indicate that peptide release from polymeric nanoparticles can be controlled by varying the sizes of the PEG and PLA blocks.

NuBCP-9 is a highly promising anticancer peptide that selectively induces apoptosis of cancer cells by exposing the BCL-2 BH3 domain and blocking the BCL-xL survival function (6). NuBCP-9 was linked to the D-Arg octamer r8 for intracellular delivery, a modification that has been reported to decrease selectivity by inducing BCL-2–independent cell killing involving membrane disruption (15). In this work, delivery of NuBCP-9 into cancer cells by the polymeric nanoparticles was achieved without the need for the R8 PTD. In addition, the NuBCP-9–encapsulated nanoparticles were by comparison more potent in inducing apoptosis than NuBCP-9-R8. The NuBCP-9/nanoparticles also maintained the reported selectivity of NuBCP-9 for cancer cells as evidenced by their absence of HUVEC killing (6). Previous studies of the antitumor effects of NuBCP-9 in vitro were performed by direct injection of NuBCP-9-r8 into MDA-MB-435 breast cancer xenografts growing in SCID mice (6). Under these experimental conditions, NuBCP-9-r8 treatment was associated with partial MDA-MB-435 tumor regressions (6). In this studies using the subcutaneous Ehrlich tumor model in syngeneic mice, we compared the effectiveness of NuBCP-9/nanoparticles administered i.p. and i.t.. Significantly, both routes of NuBCP-9/nanoparticle administration were effective in inducing complete regressions of the Ehrlich tumors. Moreover and interestingly, i.p. administration was more effective than i.t. delivery in maintaining prolonged tumor regressions. The basis for this distinction is presently not clear, but is likely related to pharmacokinetics and pharmacodynamics of the NuBCP-9/nanoparticles, which will be the focus of subsequent studies. Of further importance, administration of the NuBCP-9/nanoparticles was well tolerated with no evidence of weight loss or overt toxicities. These results thus provide support for the effective delivery of L-amino acid NuBCP-9 by PLA-PEG-PPG-PEG nanoparticles and may be applicable to other anticancer peptides. In summary, our findings describe a novel nanoparticle-based approach for delivery of L-amino acid peptides without a cell-penetrating domain that (i) requires reduced levels of emulsifier, (ii) incorporates higher molecular weights of PLA for sustained and prolonged peptide release, (iii) induces effective anticancer activity in vitro and in vivo, and (iv) requires less frequent dosing (once/week) compared with the daily injections that were necessary for partial antitumor activity of the n-amino acid NuBCP-9-r8 peptide in vivo (6).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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Novel Polymeric Nanoparticles for Intracellular Delivery of Peptide Cargos: Antitumor Efficacy of the BCL-2 Conversion Peptide NuBCP-9

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