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# Characterization of Hydrophobic Anti-Cancer Drug-Loaded Amphiphilic Peptides as a Gene Carrier

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## ABSTRACT

An amphiphilic peptide with a 3-arginine stretch and a 6-valine stretch was evaluated as a gene carrier. The short amphiphilic peptide, R3V6, not only formed micelles in aqueous solution, but was also able to deliver plasmid DNA (pDNA) into cells without toxicity. In this research, various amphiphilic peptides were synthesized with a 3-arginine stretch and a 6-valine, -alanine, -leucine, or -phenylalanine stretch. In vitro transfection assays in human embryonic kidney 293 cells showed that R3V6 and R3L6 peptides had higher transfection efficiencies than R3A6, R3F6, and poly-L-lysine (PLL). Since the peptide micelles had hydrophobic cores, a hydrophobic anti-cancer drug, bis-chloronitrosourea (BCNU),was able to be loaded into the cores of the micelles. The incorporation of the hydrophobic drug into the cores of the peptide micelles may stabilize the micelle structure and increase the transfection efficiency. The in vitro transfection assay with BCNU-loaded R3V6 (R3V6-BCNU) or R3L6 (R3L6-BCNU) showed that the BCNU-loaded peptide micelles had a higher transfection efficiency than the peptide micelles without BCNU. R3V6-BCNU and R3L6-BCNU had the highest transfection at a 0.8:1 weight ratio (BCNU:R3V6) and a 1.2:1 weight ratio (BCNU:R3L6), respectively. Furthermore, compared to simple diffusion, a more efficient delivery of the drug into cells may be facilitated by endocytosis of the micelles. R3L6-BCNU and R3V6-BCNU had higher cell toxicity to cells than BCNU alone. Therefore, the R3V6- and R3L6-BCNU may be useful for drug and gene combination cancer therapy. J. Cell. Biochem. 113: 1645–1653, 2012. 

**KEY WORDS:** PEPTIDE; MICELLE; DRUG DELIVERY; GENE DELIVERY; CANCER

Gene therapy, a method used to introduce genetic material into cells to treat disease, requires therapeutic genes and their carriers. Specifically, successful gene therapy depends on the development of an efficient and non-toxic gene delivery system. Gene delivery systems are divided into two groups, viral and non-viral carriers [Han et al., 2000; Kang et al., 2005]. It has been generally accepted that viral carriers have higher gene delivery efficiency than non-viral carriers. However, non-viral carriers do not integrate the delivered genes into the host chromosome, which eliminates the possibility of oncogenic recombination [Kang et al., 2005]. In addition, non-viral carriers have less immunogenicity and cytotoxicity than viral vectors [Kang et al., 2005]. Despite the higher biosafety of non-viral gene carriers, the transfection efficacy of non-viral carriers is not sufficient for therapeutic application. In addition, cytotoxicity of the non-viral carriers is another problem that limits their application to clinical settings. Polyethylenimine (25 kDa, PEI25k) and poly-L-lysine (PLL) are the most widely investigated polymeric gene carriers [Lee and Kim, 2002; Kang et al., 2005]. PEI25k and PLL have high cell toxicity and various derivatives of PEI25k and PLL have been developed to reduce the toxicity of the carriers [Lee and Kim, 2005].

The cytotoxicity of the cationic carriers is mainly due to high charge density [Fischer et al., 1999]. Therefore, shielding of the cationic charges using polyethylene glycol (PEG) is one strategy that has been used to reduce cytotoxicity [Lee and Kim, 2005]. Another important approach is the use of natural cationic peptides as gene carriers [Balicki et al., 2000; Puebla et al., 2003; Kaouass et al., 2006; Kim et al., 2006a, 2008; Wagstaff et al., 2007; Han et al., 2009; Shen et al., 2009]. Most natural cationic peptides originate from nuclear proteins. Histones and high mobility group box proteins have high

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arginine and lysine contents and can bind to DNA or RNA without sequence-specificity[Kaouass et al., 2006]. As a result, the nuclear proteins or their fragments have been evaluated as gene carriers [Kaouass et al., 2006]. Peptides have less charge density than PLL because the arginine and lysine content in histones or high mobility group box proteins is less than that in PLL [Kim et al., 2008; Han et al., 2009]. Therefore, natural peptides have low toxicity. The results from gene delivery studies show the potential of peptides as gene carriers. However, further improvements for higher transfection are required for clinical application.

Short cationic peptides are also useful for gene delivery. Oligoarginines have been used in the delivery of small interfering RNA (siRNA) [Kim et al., 2006b]. Although the short peptides delivered siRNA with high efficiency, they are not suitable for the delivery of high molecular weight plasmid DNA (pDNA). The short peptides can bind efficiently to siRNA; however, even if the peptides are able to bind to pDNA, they cannot condense it efficiently. Therefore, modification of the short peptides is required for pDNA delivery. In a previous study, an amphiphilic peptide composed of a 3-arginine stretch and a 6-valine stretch was evaluated as a pDNA carrier [Ryu et al., 2011]. In that study, the R3V6 peptides were found to form micelle structures in aqueous solution and it was suggested that they might behave like a high molecular weight cationic peptide. The R3V6 peptide also demonstrated higher pDNA delivery efficiency than PLL in human embryonic kidney 293 (HEK293) cells.

In the present study, R3V6, R3A6, R3L6, and R3F6 were evaluated as gene carriers. In order to stabilize the micellar structure, a stronger hydrophobic core was achieved by loading bis-chloronitrosourea (BCNU), a hydrophobic anti-cancer drug, into the cores of the peptide micelles. The incorporation of the hydrophobic drug into the core of the peptide micelles may render the micelles more stable by providing a tighter core, thereby increasing the transfection efficiency. Furthermore, compared to simple diffusion, a more efficient delivery of the drug into cells may be facilitated by endocytosis of the micelles. Therefore, a hydrophobic drug loaded micelle may be useful as a drug and gene combined delivery carrier. In order to assess the potential for this phenomenon, in vitro transfection assay and physical characterization of the BCNU-loaded peptide micelles were performed in this study. The results suggest that the BCNU-loaded peptide micelles may be useful for cancer drug and gene combined therapy.

## MATERIALS AND METHODS

### PEPTIDES

The peptides of a 3-arginine (R3) stretch combined with a 6-valine (V6), 6-leucine (L6), 6-alanine (A6), and 6-phenylalanine (F6) stretch were synthesized chemically and purified by C18 reverse-phase chromatography (Peptron Co., Daejeon, South Korea). The peptides were dissolved in water at 5 mg/ml and were stored at $-70^{\circ}$C.

### PLASMID DNA (pDNA)

Previously constructed pCMV-Luc [Han et al., 2001] was propagated in DH5a *E. coli* and purified using the Qiagen Maxiprep Kit (Invitrogen, Valencia, CA).

### IN VITRO TRANSFECTION ASSAYS

Human embryonic kidney 293 (HEK293), mouse neuroblastoma N2A, human glioblastoma HTB14, human hepatoma Hep3B, and human hepatoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Welgene, Seoul, Korea) containing 1% penicillin and 10% FBS at 37°C in 5% $CO_2$ atmosphere. HEK293 cells were seeded at a density of $1 \times 10^5$ cells/well in 12-well plates for luciferase assays and were incubated for 24 h at 37°C before transfection.

The pDNA/R3V6, R3L6, R3A6, and R3F6 complexes were prepared at a 1:20 weight ratio based on a previous report [Ryu et al., 2011]. Before transfection, the cell culture medium was replaced with serum-free DMEM. The transfection mixtures were added, and all of the cells were incubated for 4 h at 37°C in a 5% $CO_2$ atmosphere. Then, the medium was replaced with DMEM containing 1% penicillin and 10% FBS, the cells were subsequently incubated for an additional 24 h at 37°C in a 5% $CO_2$ atmosphere, and were harvested for luciferase assay.

### LUCIFERASE ASSAY

After transfection and incubation at 24 h, the cells were washed twice with 0.5 ml of PBS, and 120 μl of reporter lysis buffer (Promega, Madison, WI) was added to each well. The cell extracts were harvested and transferred to microcentrifuge tubes. The extracts were centrifuged at 13,000 rpm for 3 min to remove cell debris. Luciferase activities of the samples were measured at room temperature using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Protein concentrations in the extracts were measured using a BCA assay kit (Pierce, Iselin, NJ). The final luciferase activities were calculated as relative light units (RLU) per mg protein.

### PREPARATION OF THE BCNU-LOADED R3V6 OR R3L6 PEPTIDE MICELLES

BCNU was dissolved in water at 4 mg/ml (Sigma, St. Louis, MO). The BCNU solution was mixed with the R3V6 or R3L6 peptide solution. After sonication, the solution was incubated at room temperature for 30 min. To determine the optimal ratios of R3V6 or R3L6 to BCNU, R3V6-BCNU, and R3L6-BCNU were prepared at various weight ratios of peptide to BCNU. R3V6-BCNU or R3L6-BCNU was mixed with pDNA at a weight ratio of 1:20 (pDNA:peptide). To determine the optimal ratios of pDNA to R3V6-BCNU or R3L6-BCNU, the weight ratio between peptide and BCNU was fixed at 1:0.6 (peptide:BCNU) and the pDNA/R3V6-BCNU or pDNA/R3L6-BCNU was prepared at various weight ratios. In vitro transfection assays were performed as described above.

### SCANNING ELECTRON MICROSCOPY (SEM) IMAGE

A suspension of nanoparticles in deionized water was mounted on aluminum holders at room temperature, dried overnight, and then coated with platinum while under a vacuum. And then, the morphology and size of nanoparticles were investigated by scanning electron microscopy (S-4800 UHR FE-SEM, Hitachi, Japan).

### GEL RETARDATION ASSAY

Complex formation between plasmid DNA (pDNA) and the peptides was verified through a gel retardation assay. A fixed amount of pCMV-Luc (0.5 μg) was mixed with increasing amounts of the peptides. The mixtures were analyzed on a 1% agarose gel in the presence of ethidium bromide (EtBr). pDNA was visualized using a UV transilluminator.

### HEPARIN COMPETITION ASSAY

The stability of the pDNA/R3V6or R3L6 complexes was evaluated by heparin competition assay. The pDNA/R3V6 or/R3L6 and pDNA/PLL complexes were prepared at 1:20 (pDNA:peptide) and 1:2 (pDNA:PLL) weight ratios. A total of 0.5 μg of pDNA was mixed with 10 μg of R3V6 and R3L6 peptide or 1 μg of PLL to form complexes in 20 μl of 5% glucose solution. The weight ratios used were the optimal ratios for the highest transfection efficiency determined in this report (R3V6 and R3L6) and in the previous report (PLL) [Lee et al., 2001]. Increasing amounts of heparin (Sigma) were added to the pDNA/peptides complexes. The samples were analyzed on a 1% agarose gel containing EtBr. pDNA was visualized using a UV transilluminator.

### CYTOTOXICITY

Evaluation of cytotoxicity of the pDNA/R3V6-BCNUor R3L6-BCNU peptides complexes was performed by the MTT assay. The cells were seeded at a density of $5 \times 10^4$ cells/well in 24-well plates and incubated for 24 h before transfection. The pDNA/R3V6-BCNU and R3L6-BCNU complexes were prepared at their optimal ratios for transfection. The pDNA/PLL complex was prepared at a 1:2 weight ratio, based on the previous report [Lee et al., 2001]. The complexes were added to the cells in serum-free DMEM followed by incubation for 4 h. After incubation, the medium was replaced with 0.5 ml of fresh DMEM containing 1% penicillin and 10% FBS. The cells were incubated for an additional 24 h at 37° in a 5% $CO_2$ incubator. After transfection, 40 μl of 2 mg/ml MTT solution in PBS was added to the wells. The plates were then incubated for 4 h at 37°C. The MTT medium was removed, and 700 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals formed by the live cells. Cell survival was evaluated by measuring the absorbance at 570 nm. Cell viability (%) was calculated according to the following equation: Cell viability (%) = $(OD_{570\,nm(sample)}/OD_{570\,nm(control)}) \times 100$, where $OD_{570\,nm(sample)}$ represents the wells treated with plasmid/peptides complexes and $OD_{570\,nm(control)}$ represents the wells treated with 5% glucose solution.

### CASPASE 3/7 ASSAY

Apoptosis of the transfected cells by BCNU was measured using Caspase-Glo 3/7 Assay reagent (Promega). For transfection, the neuroblastoma N2A cells were seeded at a density of $1 \times 10^4$ cells/well in 96-well flat-bottomed plates and incubated for 24 h before transfection. The pDNA/R3V6-BCNU and R3L6-BCNU complexes were prepared at their optimal ratios for transfection. Before transfection, the medium was replaced fresh medium without FBS. Then, the pDNA/R3V6-BCNU and R3L6-BCNU complexes were added to the cells. The amount of plasmid DNA was fixed at a 0.3 μg/well. The cells were incubated for 4 h at 37°C in a 5% $CO_2$ incubator. After 4 h, the transfection mixtures were removed, and replaced fresh medium with FBS. The cells were incubated for an additional 24 h at 37°C in a 5% $CO_2$ incubator. After incubation, 50 μl of Caspase-Gloreagent was added to each well and samples are incubated at room temperature for 1 hr. The luminescence of each sample was measured in terms of RLU using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany).

### ANNEXIN V ASSAY

N2A cells were seeded at a density of $1 \times 10^5$ cells/well in six-well plates (BD Biosciences, Franklin Lakes, NJ) 24 h before transfection. Carrier/pDNA complexes were prepared at optimized weight ratios. Transfection was performed as described above. After transfection, the cells were harvested to fresh tubes. The cells were washed twice with clod PBS and then apoptosis was determined by annexin V staining according to the instructions of the manufacturer (BD Biosciences, Franklin Lakes, NJ). Flow cytometry was performed using the BD FACSCalibur™ (BD Biosciences Immunocytometry Systems, San Jose, CA).

### STATISTICAL ANALYSIS

Statistical analysis was conducted by ANOVA. All data are presented as the mean ± SEM, and $P$ values less than 0.05 were considered statistically significant.

## RESULTS

### TRANSFECTION EFFICIENCIES OF R3V6, R3L6, R3A6, AND R3F6

In a previous report, it was shown that the R3V6 peptide had a higher transfection efficiency than PLL in HEK293 cells [Ryu et al., 2011]. In the present study, the hydrophobic block was optimized with different hydrophobic amino acids in order to improve the

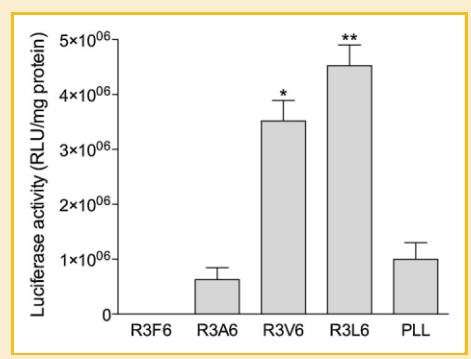


Fig. 1. In vitro transfection efficiencies of R3F6, R3A6, R3V6, and R3L6. The pCMV-Luc/peptide complexes were prepared at a 1:20 weight ratio (pDNA:-peptide) and subsequently transfected into HEK293 cells. The pCMV-Luc/PLL complex was used as a control. Transfection efficiency was measured by luciferase assay. The data are presented as the mean ± standard error of the quadruplicated experiments. $^*P < 0.05$ as compared with R3F6, R3A6, R3L6, and PLL. $^{**}P < 0.05$ as compared with R3F6, R3A6, and PLL.

transfection efficiency of the amphiphilic peptides. The synthesized amphiphilic peptides were evaluated in terms of the transfection efficiency to HEK293 cells (Fig. 1). The weight ratios between pDNA and peptide were fixed at 1:20, which was the ratio that yielded the highest transfection efficiency of the R3V6 peptide in a previous report [Ryu et al., 2011]. The transfection results showed that the transfection efficiencies of R3V6 and R3L6 were higher than that of PLL (Fig. 1). However, R3A6 and R3F6 had much lower transfection efficiencies than PLL (Fig. 1). Therefore, R3A6 and R3F6 were excluded in the following experiments.

## TRANSFECTION EFFICIENCIES OF THE BCNU-LOADED PEPTIDE MICELLES

BCNU and R3V6 or R3L6 were mixed with each other at various weight ratios to produce the BCNU-loaded R3V6 (R3V6-BCNU) and R3L6 (R3L6-BCNU). The transfection assays were performed with the pDNA/R3V6-BCNU or pDNA/R3L6-BCNU complex. The weight ratio between pDNA and R3V6-BCNU or R3L6-BCNU was fixed at 1:20 (pDNA:peptide). The highest transfection efficiency of R3V6-BCNU was obtained at a R3V6:BCNU weight ratio of 1:0.8 (Fig. 2A). On the contrary, R3L6-BCNU showed its highest transfection with at a R3L6:BCNU weight ratio of 1:1.2 (Fig. 2B). Therefore, R3V6-BCNU and R3L6-BCNU were prepared at 1:0.8 and 1:1.2 weight ratios (peptide:BCNU), respectively, in the following experiments.

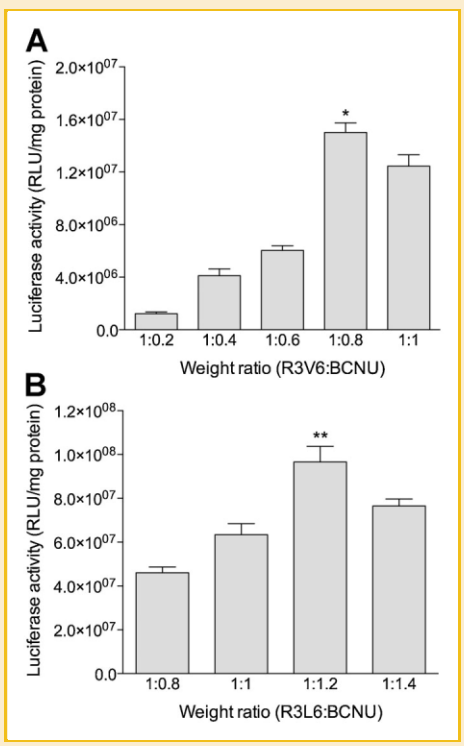


Fig. 2. Transfection efficiencies of R3V6-BCNU and R3L6-BCNU bypeptide:BCNU weight ratio. R3V6-BCNU and R3L6-BCNU were prepared at various peptide:BCNU weight ratios. The R3V6-BCNU and R3L6-BCNU were mixed with pCMV-Luc at a 1:20 weight ratio (pDNA:peptide) to prepare pCMV-Luc/R3V6-BCNU and pCMV-Luc/R3L6-BCNU complexes. The pDNA/peptide-BCNU complexes were transfected into HEK293 cells. Transfection efficiency was measured by luciferase assay. The data are presented as the mean ± standard error of the quadruplicated experiments. A: R3V6-BCNU and (B) R3L6-BCNU. $^{*}P < 0.05$ as compared with 1:0.2, 1:0.4, 1:0.6, and 1:1. $^{**}P < 0.05$ as compared with 1:0.8, 1:1, and 1:1.4.

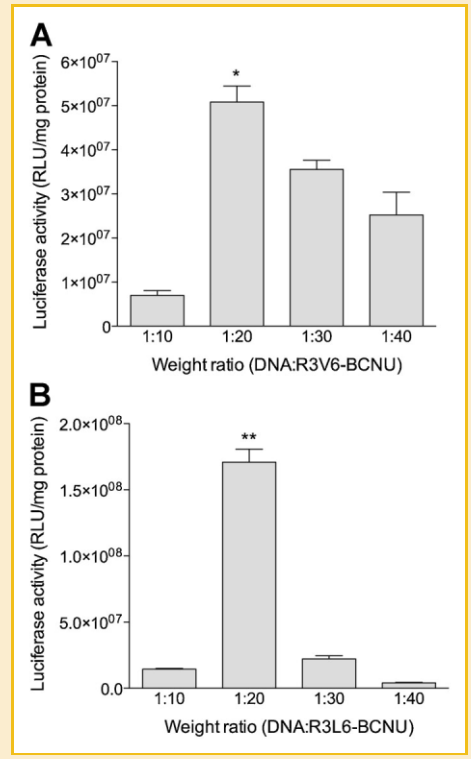


Fig. 3. Transfection efficiencies of R3V6-BCNU and R3L6-BCNU by pDNA:R3V6-BCNU or pDNA:R3L6-BCNU weight ratio. R3V6-BCNU and R3L6-BCNU were prepared at 1:0.8 and 1:1.2 weight ratios (peptide:BCNU), respectively. The pCMV-Luc/R3V6-BCNU and pCMV-Luc/R3L6-BCNU complexes were prepared at various weight ratios (pDNA:peptide) and subsequently transfected into HEK293 cells. Transfection efficiency was measured by luciferase assay. The data are presented as the mean ± standard error of the quadruplicated experiments. A: R3V6-BCNU and (B) R3L6-BCNU. $^{*}P < 0.05$ as compared with 1:10, 1:30, and 1:40. $^{**}P < 0.01$ as compared with 1:10, 1:30, and 1:40.

In order to optimize the pDNA:peptide-BCNU weight ratios, the pDNA/R3V6-BCNU and pDNA/R3L6-BCNU complexes were prepared at various weight ratios (pDNA/peptide) and transfected into HEK293 cells. Figure 3A shows that R3V6-BCNU had the highest transfection efficiency at a 1:20 weight ratio (pDNA:R3V6-BCNU). Similarly, R3L6-BCNU had the highest transfection efficiency at a 1:20 weight ratio (pDNA:R3L6-BCNU) (Fig. 3B). At this weight ratio, the transfection efficiencies of R3V6-BCNU and R3L6-BCNU were compared with those of R3V6, R3L6, and PLL. The results showed that R3V6-BCNU and R3L6-BCNU had higher transfection efficiency than PLL by approximately seven- and ninefold, respectively (Fig. 4A). In addition, R3V6-BCNU had about fourfold higher transfection efficiency than R3V6, and R3L6-BCNU had about fivefold higher transfection efficiency than R3L6. These results

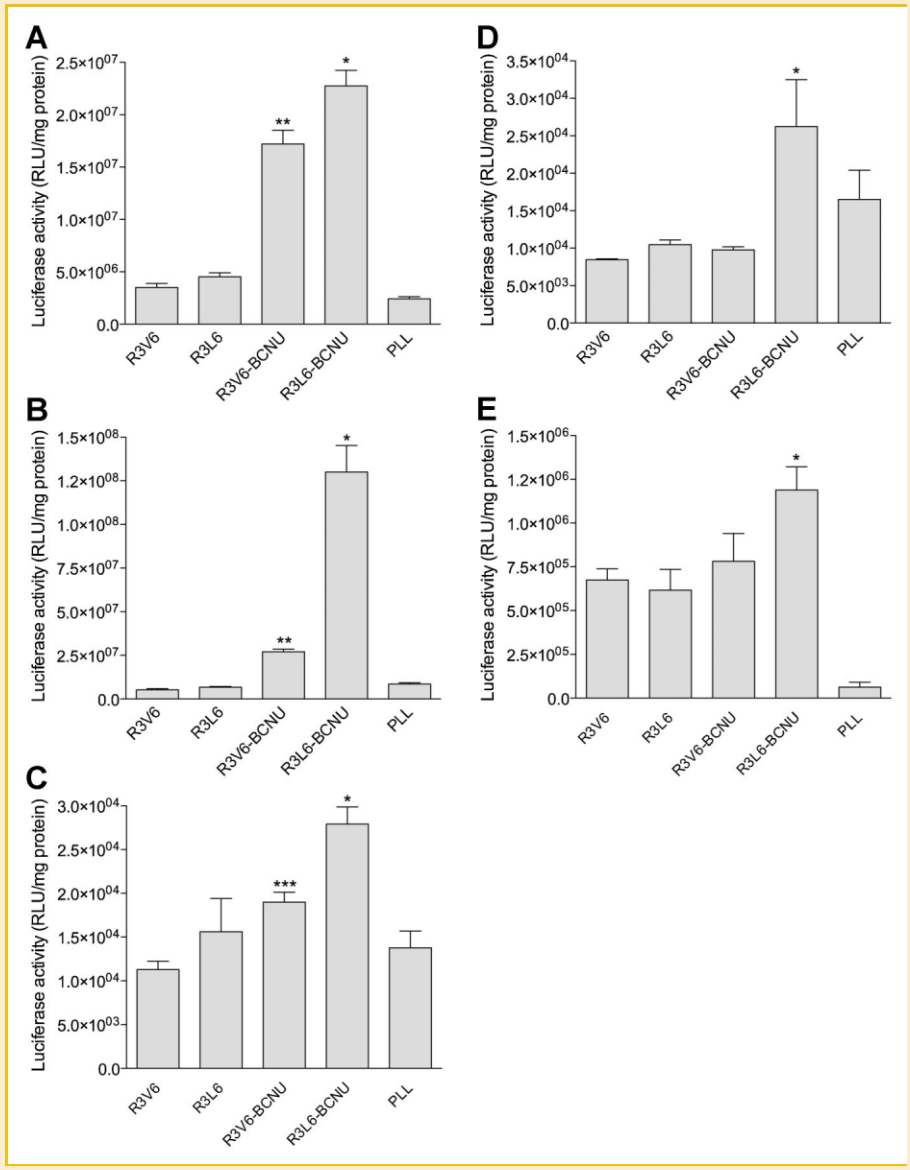


Fig. 4. Comparison of transfection efficiencies of R3V6, R3L6, R3V6-BCNU, R3L6-BCNU, and PLL. The pCMV-Luc/peptide complexes were prepared at their optimal ratios, and subsequently transfected into (A) HEK293, (B) N2A, (C) HTB14, (D) HepG2, and (E) Hep3B cells. Transfection efficiency was measured by luciferase assay. The data are presented as the mean ± standard error of the quadruplicated experiments. $^{*}P < 0.05$ as compared with R3V6, R3L6, R3L6-BCNU, and PLL. $^{**}P < 0.05$ as compared with R3V6, R3L6, and PLL. $^{***}P < 0.05$ as compared with R3V6 and PLL.

suggest that the pDNA/peptide complexes were stabilized by the addition of BCNU into the cores of the micelles and that the transfection efficiencies of the micelles were enhanced by the addition of BCNU. The transfection assays were also performed in N2A cells. In N2A cells, R3V6-BCNU and R3L6-BCNU had higher transfection efficiencies than PLL (Fig. 4B). Also, R3V6-BCNU and R3L6-BCNU had higher transfection efficiencies than R3V6 and R3L6 by 5- and 13-fold, respectively (Fig. 4B). Similarly, in HTB14 cells, R3V6-BCNU and R3L6-BCNU had higher transfection efficiencies than R3V6 and R3L6 (Fig. 4C). In HepG2 or Hep3B cells, R3L6-BCNU had higher transfection efficiency than R3L6. However, R3V6-BCNU had similar transfection efficiency to R3V6 (Fig. 5D,E). This result suggests that R3V6-BCNU may not enhance transfection efficiency in hepatocytes. R3L6-BCNU had higher transfection efficiencies than PLL overall.

## PHYSICAL CHARACTERIZATION OF pDNA/R3V6-BCNU AND pDNA/R3L6-BCNU COMPLEXES

The morphology of the BCNU-loaded micelles was studied by scanning electron microscopy (SEM). pDNA/peptide complexes were prepared at their optimal ratios for transfection and observed

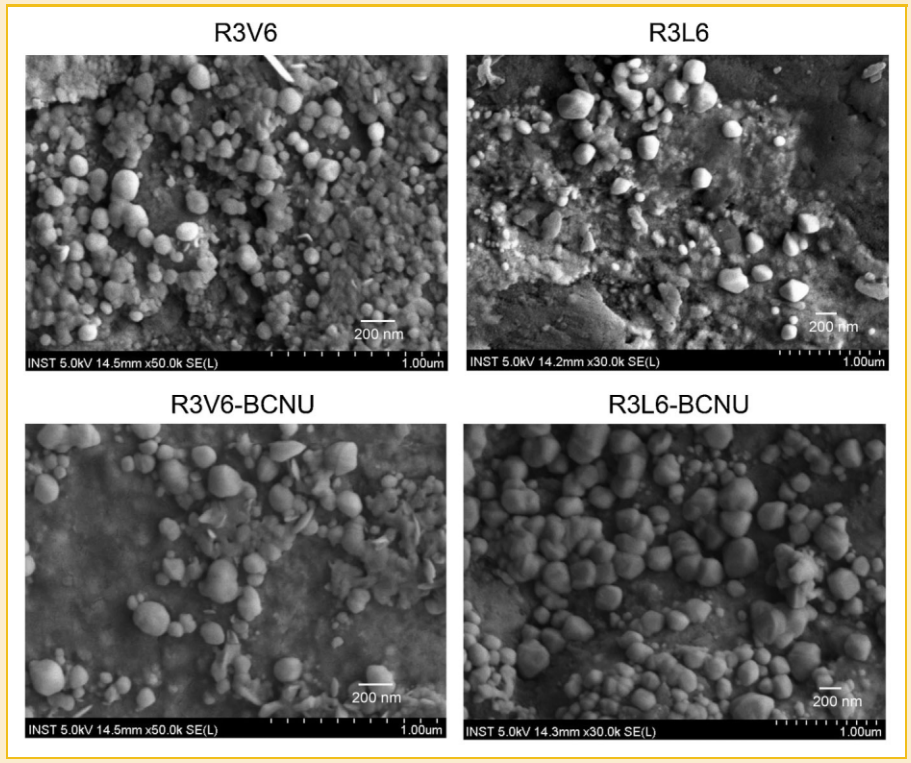


Fig. 5. Morphology of pDNA/R3V6-BCNU and pDNA/R3L6-BCNU complexes. The pCMV-Luc/R3V6-BCNU and pCMV-Luc/R3L6-BCNU complexes were prepared at their optimal ratios. The pCMV-Luc/R3V6 and/R3L6 complexes were also prepared as controls. The morphology of the complexes was observed by scanning electron microscopy.

by SEM. The pDNA/peptide complexes formed spherical nanoparticles (Fig. 5).

The complex formation between pDNA and R3V6-BCNU or R3L6-BCNU was confirmed by gel retardation assays. In Figure 6, it is apparent that R3V6-BCNU retarded pDNA completely at a 1:1.6 weight ratio (pDNA:peptide), while R3V6 retarded pDNA at a 1:1.8 weight ratio. R3L6-BCNU also retarded pDNA at a 1:1.8 weight ratio (Fig. 6). However, R3L6 retarded pDNA at a 1:2 weight ratio. This suggests that the BCNU-loaded micelles formed complexes with pDNA more efficiently than the micelles without BCNU.

The stabilities of the complexes were evaluated by heparin competition assays. Increasing amounts of heparin were added to the pDNA/peptide solution and dissociation of the complex was measured by gel electrophoresis. R3V6, R3L6, and R3V6-BCNU released pDNA as a result of the addition of 5 μg of heparin, while R3L6-BCNU released pDNA by the addition of 7.5 μg of heparin (Fig. 7). The results suggest that the stability of pDNA/R3L6-BCNU complex was more stable than pDNA/R3V6, pDNA/R3L6 and pDNA/R3V6-BCNU complexes. In addition, the pDNA/PLL complex was less stable than the peptide or peptide-BCNU (Fig. 7).

### CYTOTOXICITIES OF PEPTIDE-BCNU

The toxicities of R3V6-BCNU and R3L6-BCNU were measured by MTT assays. BCNU is an anti-cancer drug that has been used for brain cancer therapy. Therefore, BCNU is effective in killing the cells. The transfections were performed in HEK293, N2A, and HepG2 cells and the toxicities were evaluated. In Figure 8A, it is evident that BCNU was more toxic to cells than R3V6, R3L6, and PLL in HEK293 cells, confirming that BCNU is an effective anti-cancer drug for the treatment of tumors. The cytotoxicity of R3V6-BCNU or R3L6-BCNU was higher than BCNU alone, suggesting that the R3V6 or R3L6 peptide micelles may be useful carriers of BCNU (Fig. 8A). However, R3V6 and R3L6 were less toxic than PLL, suggesting that the cytotoxicity of R3V6-BCNU or R3L6-BCNU was not due to the peptides (Fig. 8A). The same results were observed in N2A, HTB14, HepG2, and Hep3B cells (Fig. 8B,C,D,E).

Caspase 3/7 assay was performed to measure the apoptosis level by BCNU in N2A cells. The results showed that R3V6-BCNU and R3L6-BCNU induced higher caspase 3/7 level, compared to BCNU only, suggesting that BCNU was delivered more efficiently by R3V6-BCNU and R3L6-BCNU than BCNU only (Fig. 9A). In Annexin V assay, the apoptosis level by R3L6-BCNU was higher than that by BCNU only (Fig. 9B).

## DISCUSSION

In this research, we have shown that BCNU can be loaded into the cores of R3V6 and R3L6 peptides. In addition, R3V6-BCNU and

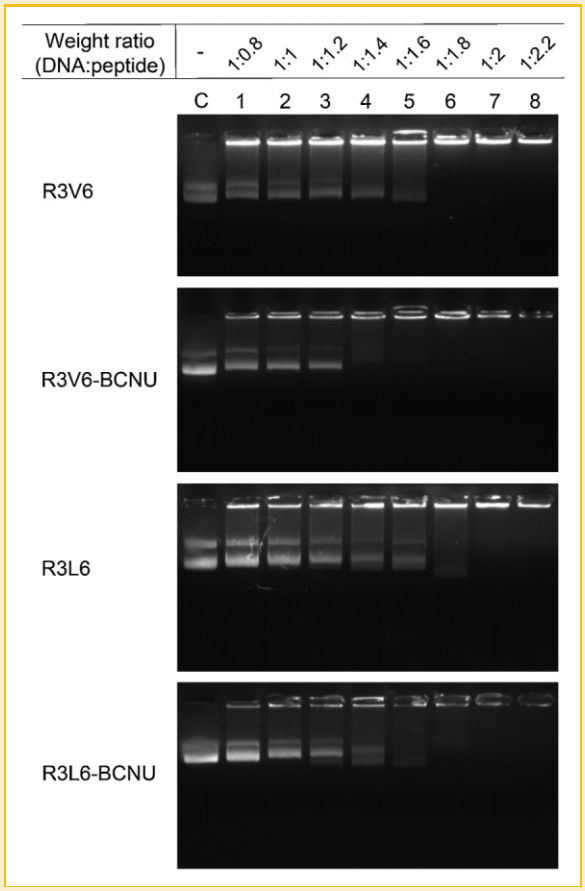


Fig. 6. Gel retardation assay. pCMV-Luc was mixed with increasing amounts of R3V6-BCNU, R3L6-BCNU, R3V6, and R3L6. After 30 min of incubation at room temperature, the samples were analyzed on a 1% agarose gel.

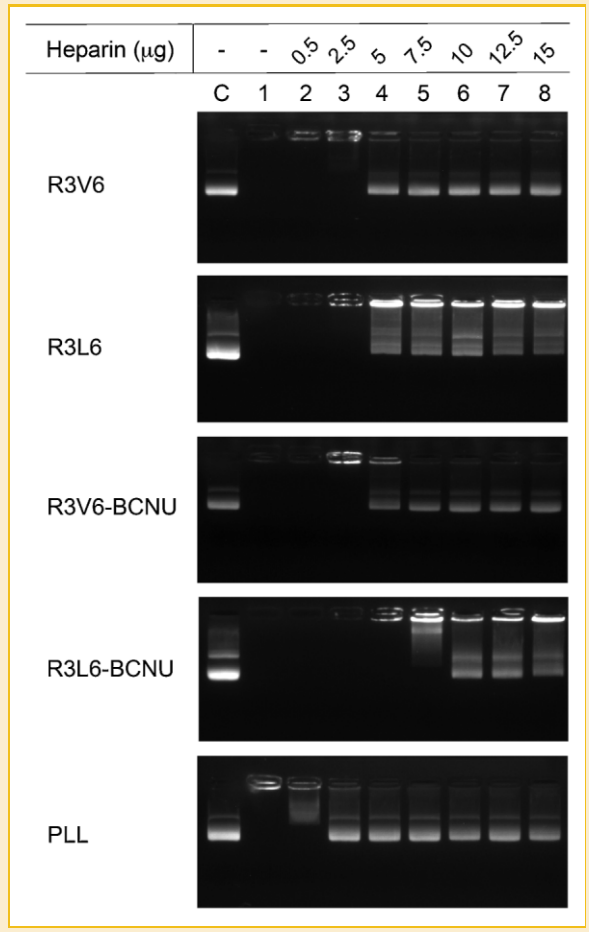


Fig. 7. Heparin competition assay. The pCMV-Luc/peptide complexes were prepared at their optimal transfection ratios as described in Materials and Methods. Increasing amounts of heparin were added to the pCMV-Luc/carrier complexes. After incubation at room temperature, the complexes were analyzed on a 1% agarose gel.

R3L6-BCNU had higher transfection efficiencies than R3V6 and R3L6. The ideal delivery vehicle should be non-toxic and efficient in gene delivery. The R3V6 peptide was previously evaluated as a pDNA carrier, was found to have about twice the transfection efficiency as PLL in 293 cells, and was non-toxic to target cells [Ryu et al., 2011]. Conversely, the transfection efficiency of PLL has been shown to be insufficient for clinical application [Kang et al., 2005]. Based on these previous findings, the aim of this research was to improve transfection efficiency by modifying the R3V6 peptide. Valines were replaced with other hydrophobic amino acids, and the resulting amphiphilic peptides with alanines, leucines, and phenylalanines were evaluated in terms of transfection efficiency. The results showed that only R3V6 and R3L6 had higher efficiency than PLL (Fig. 1). Since the hydrophobic stretch of R3V6 and R3L6 peptides was short, making the micelles with R3V6 and R3L6 potentially unstable in aqueous solution, a hydrophobic anti-cancer drug, BCNU, was loaded into the micelles in order to increase the hydrophobicity of the micelle cores. The results showed that the loading of BCNU into the micelles increased the transfection efficiency (Fig. 4).

BCNU is widely used for the treatment of cancers. Loading BCNU into the cores of the micelles increased its cytotoxicity (Fig. 8). R3V6-BCNU and R3L6-BCNU had higher toxicity than BCNU alone. Nanoparticles with R3V6 or R3L6 and BCNU may be more efficient than BCNU alone in terms of delivery. BCNU enters cells by simple diffusion. However, nanoparticles with R3V6 or R3L6 and BCNU might be readily taken up by endocytosis. These findings suggest that the peptide micelles enhance the delivery efficiency of BCNU and that R3V6-BCNU and R3L6-BCNU may be useful for gene and drug combinational therapy of brain tumors. However, we cannot exclude the possibility that R3V6-BCNU and R3L6-BCNU had higher toxicity than BCNU alone due to the toxicities of R3V6 and R3L6. However, considering the low toxicity of R3V6 and R3L6 (Fig. 8), it is likely that peptide micelles may only increase the efficiency of BCNU delivery.

In the heparin competition assay, pDNA was released from the pDNA/PLL complex more easily than from the pDNA/peptide-BCNU complex. The lower stability of the pDNA/PLL might be because the

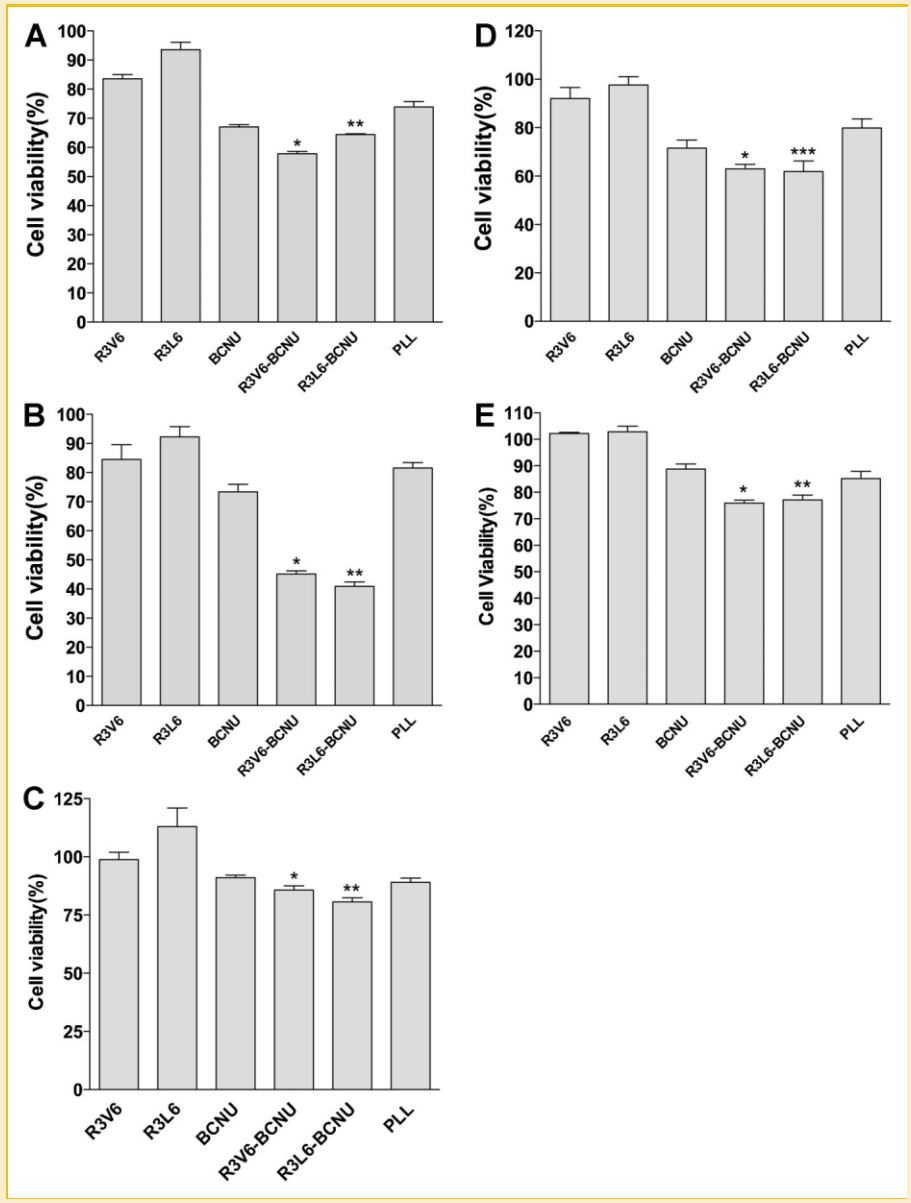


Fig. 8. Cytotoxicity. The pCMV-Luc/peptide complexes were prepared at their optimal transfection ratios as described in Materials and Methods. The complexes were transfected into (A) HEK293, (B) N2A, (C) HTB14, (D) HepG2, and (E) Hep3B cells. After 24 h, cytotoxicity was measured by MTT assay. The data are presented as the mean ± standard error of the quadruplicated experiments. $^{*,**}P < 0.05$ as compared with R3V6, R3L6, BCNU, and PLL. $^{***}P < 0.05$ as compared with R3V6, R3L6, and PLL, but not significant as compared with BCNU.

pDNA/PLL complex was prepared at a lower weight ratio than pDNA/peptide and pDNA/peptide-BCNU complexes. In the heparin competition assay, the pDNA/PLL complex was prepared at a 1:2 weight ratio (pDNA:PLL) since the pDNA/PLL complex had the highest transfection efficiency at that ratio in a previous report [Lee et al., 2001]. The pDNA/peptide or pDNA/peptide-BCNU complexes were prepared at a 1:20 weight ratio (pDNA:peptide) since they had the highest transfection efficiency at that ratio. pDNA/peptide and pDNA/peptide-BCNU complexes were prepared with more peptide than the pDNA/PLL complex and therefore might be more stable than the pDNA/PLL complex.

In summary, the hydrophobic anti-cancer drug, BCNU, was loaded into R3V6 and R3L6 peptide micelles. R3V6-BCNU and R3L6-BCNU had higher transfection efficiencies than R3V6 and R3L6. In addition, R3V6-BCNU and R3L6-BCNU had higher cytotoxicity to cancer cells. Therefore, R3V6-BCNU and R3L6-BCNU may be useful for the development of combinational therapy to treat cancer.

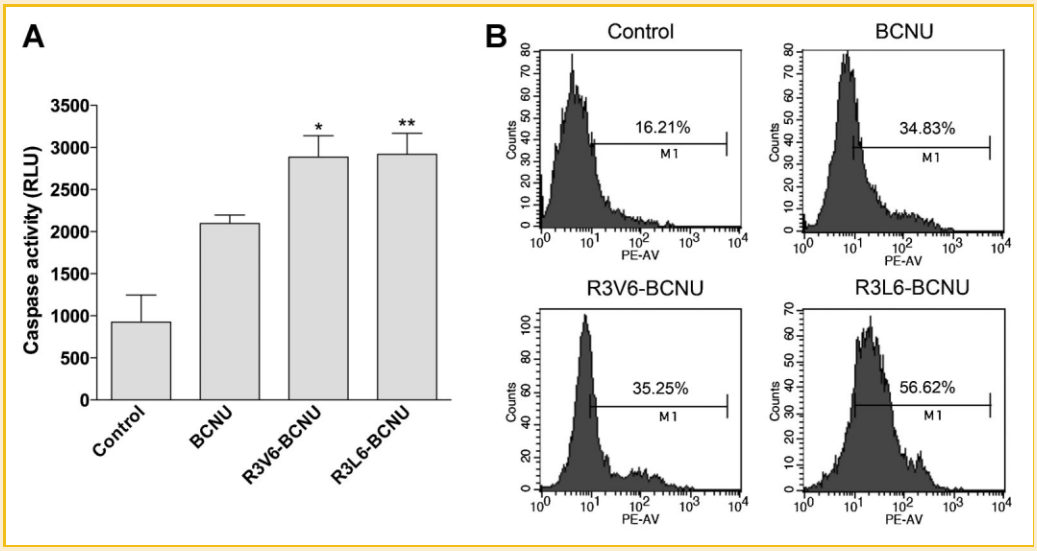


Fig. 9. Apoptosis assay. The pCMV-Luc/R3V6-BCNU and pCMV-Luc/R3L6-BCNU complexes were prepared at their optimal ratios for transfection. Apoptosis level was measured by (A) caspase 3/7 assay and (B) Annexin V assay at 24 h after transfection. The data are presented as the mean ± standard error of the quadruplicated experiments. *,**$P < 0.05$ as compared with control and BCNU.

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