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■ RESEARCH ARTICLE

Development of Highly Efficient Cellular Uptake Cell-Penetrating Peptide for Novel Cancer Treatment

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ABSTRACT: Cell-penetrating peptides (CPPs) are short peptides, which can carry various types of molecules into cells. Therefore, CPPs have been predominantly used in preclinical and basic cancer research for more than 30 years. However, low cellular uptake of CPPs caused severe side effects for breast cancer treatment. CPPs are rich in positively charged amino acids such as arginine and lysine and can translocate over membranes and gain access to the cell interior. Therefore, we hypothesized that the addition of positive charge peptides on cell-penetrating peptides would enhance the cellular uptake of CPPs. In this study, we constructed three types of CPPs: BR2 (+7 charge), R9 (+9 charge), BR2-R9 (+16 charge). To localize the CPPs inside the cancer cells, we added Fluorescein isothiocyanate (FITC), which is a bright green fluorophore, on the C-terminal of each peptide. BR2-R9 showed much higher cellular uptake compared to BR2 and R9 on both human breast cancer cell lines (MCF-7 and MDA-MB-231). This result indicates that BR2-R9, which contains the most positive amino acids, can be applied for efficient drug delivery in cancer treatment. This study successfully develops a novel CPP for enhancing the cellular uptake in cancer cells and provides new insights into clinical applications of cancer treatment.

KEYWORDS: Biology; Cancer Biology; Cell-penetrating peptide; Cancer Treatment.

■ Introduction

Breast cancer occurs everywhere around the world, among women who are in the stages of puberty or later. In 2022, 2.3 million women were diagnosed with breast cancer, with about 680,000 deaths worldwide. However, near the end of 2022, 7.8 million women with breast cancer were still alive. This made breast cancer the most "prevalent" cancer globally because of its 90% 5-year survival rate.[1]

In breast cancer chemotherapy, drugs are used to target and destroy breast cancer cells. The drugs are usually injected into a vein or are also taken as pills. Chemotherapy is often used with several other treatments like surgery, radiotherapy, or hormone therapy. It helps increase the chances of a cure, reduce the risk of cancer recurrence, or lessen cancer symptoms. Importantly, if breast cancer has spread to other body parts, chemotherapy can be used as the primary treatment. However, it carries a risk of side effects such as hair loss, easy bruising, infection, and many more.[2]

In radiotherapy, high-energy X-rays, protons, or other particles are used to kill cancer cells, as cancer cells are more prone to the effects of radiation therapy than normal cells. The radiation for breast cancer may be delivered through external and internal radiation. It is an effective way to decrease breast cancer recurrence and ease the symptoms caused by cancer. Many patients also use radiotherapy if breast cancers are too big to remove through surgery or have inflammatory breast cancer. However, there may be side effects like fatigue, skin irritation, or breast swelling.[3]

Hormone therapy is used to block hormones from attaching to the receptors of cancer cells or even to reduce the body's production of hormones. This method is often used after surgery to decrease the risk of cancer recurrence. It may also be used to shrink a tumor before going into surgery. Hormone therapy is mainly used for hormone receptor-positive breast cancers, also called ER-positive or PR-positive by doctors. However, there can also be significant side effects such as nausea, vaginal irritation, muscle pain, etc. Apart from therapy, medications like tamoxifen or toremifene block hormones from attaching to cancer cells.[4]

Cell-penetrating peptides consist of peptides (short chains of amino acids) that allow entering endocytic pathways to transport the molecules across the cell membrane. Many of them are known to mediate intracellular delivery of nucleic acids, proteins, or nanoparticles.[5] The peptide sequences have a positive charge and are rich in lysine or arginine. Their physicochemical properties also classify CPPs into categories like cationic, amphipathic, and hydrophobic classes. Many approaches have been developed to enhance the permeability of therapeutic proteins by attaching them to a CPP.[6] The peptide-based delivery can increase the consumption of drugs in tumor cells and increase the effectiveness of certain treatments of either small molecule drugs or oligonucleotide-based therapeutics. Additionally, as CPP can transport cargoes into the cell, CPP-based delivery is a promising strategy for cancer drug delivery. CPP can be helpful to both chemotherapeutics and modern gene-based drugs for delivering them into tumor cells.[7]

In this study we hypothesized that the addition of positive charge peptides on cell-penetrating peptides would enhance the cellular uptake of CPPs. We constructed three types of CPPs: BR2 (+7 charge), R9 (+9 charge), BR2-R9 (+16 charge) and tested their efficacy.

■ Methods

Cell culture and maintenance:

Human breast cancer cell line MCF7 and MDA-MB-231 were purchased from Korea Cell Line Bank. RPMI 1640

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DOI: 10.36838/v4i5.2

(Gibco) with 10% fetal bovine serum and 1% penicillin and streptomycin was used to culture cancer cell lines. The cells were maintained in a 37 ˚C CO_2 cell incubator.

CPP stock solution preparation:

The weight of the CPP powder was measured to prepare the stock solution for each CPP (BR2, R9, and BR2-R9). The stock concentration of 1 mM of each CPP was prepared. 1.2 mg of BR2 powder was measured, and 535.7 mL DMSO was added. 1.7 mg of R9 powder was measured, and 882.7 mL DMSO was added. 0.8 mg of BR2-R9 powder was measured, and 204.1 mL DMSO was added.

CPP delivery optimization test:

Two glass slides were prepared to test the CPP delivery efficiency. Each glass slide has eight sections. One slide was used for MCF7, and the other slide was used for MDA-MB-231. Both of the slides consisted of two no-treatment sections, and the other sections contained 5 mM and 10mM concentrations for BR2, R9, and BR2-R9 (Figure 1).

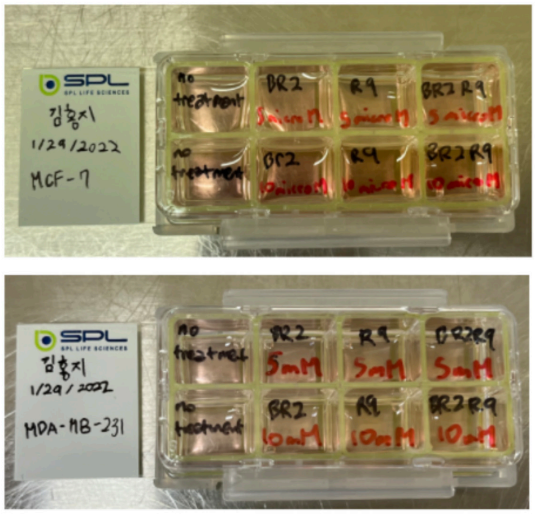

Figure 1: Cell containing slide for testing CPP delivery efficiency.

CPP delivered cell fixation and fluorescence imaging:

300 µL of methanol was added to each slide after removing all the cell media from the cell slide. The methanol was removed after 10 minutes of incubation. 15 µL of DAPI-containing mounting solution (VECTASHIELD) was added in the middle of each sample. The glass slide was covered on top of the cell containing the slide. Then, the slide was placed on the fluorescence microscope (Nikon), and the image of the cells was photographed.

Quantification of green fluorescence cells:

After the cell media was removed, we added the Trypsin-EDTA and incubated the cells for five minutes. 400µL RPMI media, cells, and the CPPs (BR2, R9, BR2R9) were added to each tube. The tubes were incubated for 10 minutes. 15 µl of the cell suspension was added into a PhotonSlide (Logos Biosystem). The LUNA-FL, a cell counting device, was used to count the green-fluorescent cells.

Results and Discussion

We used herein to design several CPP with cancer cell specificity. BR2, a motif of an anticancer peptide Buforin IIB, is a 17-amino acid peptide that was found to have cancer-specificity without toxicity to normal cells (Table 1). BR2 enters the cancer cells after targeting them with gangliosides through lipid-mediated micropinocytosis. BR2 also showed a higher membrane translocation efficiency than the other CPPs.[8]

Table 1: The CPPs used in this study and charge number.

Name	Sequence	Charge
BR2	RAGLQFPVGRLLRRLLR (17 aa)	+ 7
R9	RRRRRRRRR (9 aa)	+ 9
BR2-R9	RAGLQFPVGRLLRRLLRRLLR-RRRRRRRRR (30 aa)	+ 16

Arg[9], a synthetic homophily-arginine nonapeptide (R9), plays an essential role in cellular uptake. R9 is a cell-penetrating peptide (CPP) with a cationic guanidinium group that forms electrostatic interactions with anionic cell membrane components like phospholipids and sulfated proteoglycans.[9] The interaction can also trigger intracellular signaling and internalization in many pathways. Since R9 has a simple peptide structure with positive charges, R9 has been extensively studied with many peptide modifications for specific drug delivery.[10]

A high concentration of BR2 is needed for cancer cell delivery since BR2 shows relatively low cell delivery in cancer cells. We combined BR2 and R9 to synthesize the BR2-R9 fusion peptide to solve this problem. BR2 has a charge of +7, and R9 has a charge of +9, making BR2-R9 have a charge of +16 (Table 1). The positive charge will increase the electrostatic interaction, and this property will allow more cellular uptake in cancer cells.

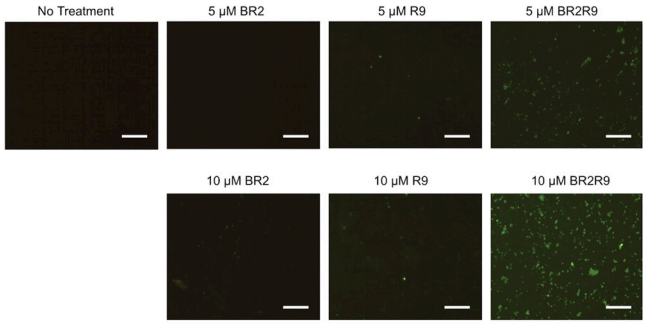

Figure 2: BR2-R9 showed the most efficient cell penetration on MCF7 breast cancer cell line. MCF7 cells were incubated with either 5 µM and 10 µM of BR2, R9, or BR2-R9 for 48 hours. The green fluorescence cells indicate the CPP uptake cells. Scale bar = 200 µm.

We aimed to analyze the cellular uptake level of BR2, R9, and BR2-R9 on MCF7 breast cancer cells. Either 5 µM and 10 µM of BR2, R9, or BR2-R9 were incubated with MCF7 cells for 48 hours. Then, we photographed the green-fluorescent positive cells using a fluorescence microscope. The green-fluorescent cells indicate the CPP cellular uptake (Figure 2). Both 5 µM and 10 µM of BR2 treatment conditions showed the lowest number of cells with green fluorescence. The treatment conditions of 5 µM and 10 µM of R9 showed

DOI: 10.36838/v4i5.2

an increased number of green-fluorescent cells compared to BR2 treatment conditions (Figure 2). This result indicates that the cellular uptake of R9 is higher than BR2. As expected, both 5 μM and 10 μM of BR2R9 treatment conditions exhibited the highest number of green-fluorescent cells than BR2 and R9 (Figure 2). We speculate that this result is due to the positive charges of BR2 and R9 fusion peptides. In conclusion, BR2R9 fusion peptides show the most efficient cellular uptake on MCF7 breast cancer cells.

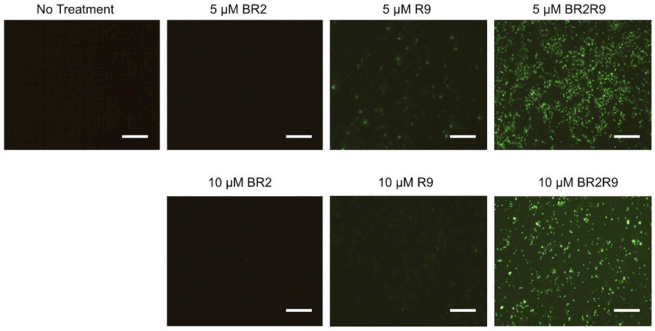

Figure 3: BR2-R9 showed the most efficient cell penetration on the MDA-MB-231 breast cancer cell line· MDA-MB-231 cells were incubated with either 5 μM and 10 μM of BR2, R9, or BR2-R9 for 48 hours. The green fluorescence cells indicate the CPP uptake cells. Scale bar = 200 μm.

Next, we analyzed the cellular uptake of CPP on different breast cancer cell lines to confirm the result in Figure 2. MDA-MB-231, which is known to have more invasive characteristics than MCF7 was used to test the cellular uptake of BR2, R9, and BR2-R9. MDA-MB-231 was incubated with 5 μM and 10 μM of BR2, R9, or BR2-R9 for 48 hours. We took photos through a fluorescence microscope to check the results of the green fluorescent positive cells. The green fluorescent cells and their CPP cellular uptake are shown (Figure 3). The 5 μM and 10 μM of BR2 treatment conditions showed the lowest number of cells with green fluorescence (Figure 3). The treatment conditions of 5 μM and 10 μM of R9 showed a greater increase in green fluorescent cells than BR2 treatment conditions (Figure 3). This result indicates that the cellular uptake of R9 is higher than BR2 in both MCF7 and MDA-MB-231. As hypothesized, both 5 μM and 10 μM of BR2-R9 treatment conditions appeared to have the highest number of green fluorescent cells than BR2 and R9 on MDA-MB-231 cells (Figure 3). Additionally, there was more cellular uptake in MDA-MB-231 than MCF7, as we can see that there are more green fluorescent cells present in MDA-MB-231 than MCF7 (Figure 3). BR2R9 fusion peptides showed the highest cellular uptake on MDA-MB-231 (Figure 3).

After the treatment of BR2, R9, and BR2-R9 on both MCF7 and MDA-MB-231, we used a cell counter device to calculate the percentage of green-fluorescent cells. We used three CPP treatment concentrations to quantify the green-fluorescent cells on MCF7 and MDA-MB-231. Each CPP was incubated with the cells for only 10 minutes. After the incubation, the cells were inserted into cell counting slides. Finally, the cell counter device quantified the percentage of green-fluorescent cells. The 0 μM CPP concentration showed no green-fluorescent cells, indicating there were no green-fluorescent cells without CPP treatment (Table 2). The 5 μM of

Table 2: · Quantification of green fluorescence cells using cell counter device.

CPP concentration	Type of CPP	Cell type	
		MCF7	MDA-MB-231
0 μM	-	0 %	0 %
5 μM	BR2	0 %	11.2 %
	R9	21.7%	4.1%
	BR2-R9	73.8%	97.6%
10 μM	BR2	0%	0 %
	R9	5.5%	4.5%
	BR2-R9	56.1%	84.1%

BR2 shows 0% green-fluorescent cells on MCF7 and 11.2% on MDA-MB-231 (Table 2). The 5 μM of R9 shows 21.7% green-fluorescent cells on MCF7 and 4.1% on MDA-MB-231 (Table 2). The 5 μM of BR2-R9 shows 73.8% green-fluorescent cells on MCF7 and 97.6% on MDA-MB-231 (Table 2). The 10 μM of BR2 shows 0% green-fluorescent cells on MCF7 and 0% on MDA-MB-231 (Table 2). The 10 μM of BR2 shows 5.5% green-fluorescent cells on MCF7 and 4.5% on MDA-MB-231 (Table 2). The 10 μM of BR2 shows 56.1% green-fluorescent cells on MCF7 and 84.1% on MDA-MB-231 (Table 2). In conclusion, BR2-R9 had the most cellular uptake in MCF7 and MDA-MB-231.

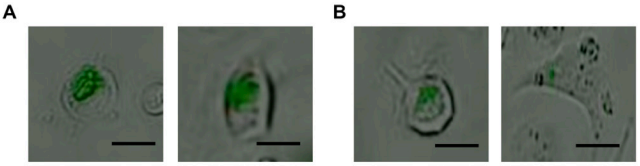

Figure 4: BR2-R9 penetrates the cell membrane and localizes inside the breast cancer cell lines. (A) Green fluorescence of BR2-R9 inside MDA-MB-231 cells. (B) Green fluorescence of BR2-R9 inside MCF7 cells. Scale bar= 10 μm.

Next, we used a 200x magnification microscope to observe BR2-R9 localization inside the breast cancer cells in higher resolution. Since BR2-R9 contains a FITC tag, which emits a green-fluorescent signal, we hypothesized that a green fluorescence signal would be detected inside the cancer cells. After we incubated MCF7 and MDA-MB-231 cells with BR2-R9 for 48 hours, the image was captured to analyze the internalization of BR2-R9. We detected that BR2-R9 was localized inside the cells as the green-fluorescent signal appears to cover about 60% of the cells. This indicates that BR2-R9 covers more than half of the cell components. We can also see that BR2-R9 CPPs were aggregated towards a certain cell membrane rather than being present in all parts of the MDA-MB-231 cells (Figure 4A). We detected that MCF7 was present inside the cells, but the green-fluorescent signal covers less than MDA-MB-231. The BR2-R9 CPPs in MCF7 were aggregated towards a certain side of the cell, similar to MDA-MB-231 (Figure 4B). In conclusion, we can see that BR2-R9 successfully penetrates the cell membrane of both MCF7 and MDA-MB-231 cells. When BR2-R9

enters the cells, they are aggregated in specific regions of the cells. Even after 48 hours of incubation, the BR2-R9 remained inside the cells. This indicates that this fusion peptide is stable in breast cancer cells for at least 48 hours.

Conclusion

The fusion peptides that we created can be used to make better drug delivery or breast cancer treatment. We can also use low concentrations of CPP, which can also lower the cost of the treatment strategies. Using low concentrations will reduce the side effects as well. The cellular cytotoxicity of CPPs can be a limitation because it was not investigated in this study. Additionally, more human cancer cells can be tested because this study only used two models. In the future, we can widen our field and experiment with other types of cancer cells and use mouse models for our study and test the CPP delivery in these different kinds of cancer cells. More investigation on how the CPP enters the cells should be studied in the future.

Acknowledgements

I would like to thank my teacher Woo Rin Lee for helping me with this project. He allowed me to work on this project in detail. Along with that, I would also like to thank my parents for supporting me.

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DOI: 10.36838/v4i5.2