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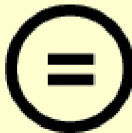

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The cyclized oligopeptide targeting LRP5/6-DKK1 interaction reduces the growth of tumor burden in a multiple myeloma mouse model

Bo Mi Park

Department of Medical Science

The Graduate School, Yonsei University

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YONSEI UNIVERSITY

The cyclized oligopeptide targeting LRP5/6-DKK1 interaction reduces the growth of tumor burden in a multiple myeloma mouse model

Directed by Professor Sung-Kil Lim

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Bo Mi Park

June 2016

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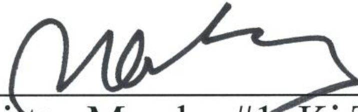
This certifies that the Master's Thesis of Bo Mi Park is approved.

Thesis Supervisor: Sung-Kil Lim



Thesis Committee Member#1: Ki Taek Nam

Thesis Committee Member#2: Jong In Yook

The Graduate School
Yonsei University

June 2016

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ABSTRACT

The cyclized oligopeptide targeting LRP5/6-DKK1 interaction reduces the growth of tumor burden in a multiple myeloma mouse model

Bo Mi Park

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Sung-Kil Lim)

DKK1 has been extensively investigated in mouse models of multiple myeloma where the disease develops osteolytic bone lesions in the advanced state. Myeloma patients have elevated DKK1 levels in the bone marrow plasma and serum, which are associated with focal bone lesions, and the elevated level of DKK1 inhibits the differentiation of osteoblast precursors. Present pharmaceutical approaches to target bone metastases are limited to antiresorptive agents such as bisphosphonates and denosumab. In this study, the cyclized oligopeptide against DKK1-LRP5/6 interaction was developed, and the effects of the oligopeptide on tumor burden were tested. The cyclized oligopeptide based on the DKK1-LRP5/6 interaction was synthesized chemically, and the cyclized oligopeptide containing NXI motif bound to the E1 domain of LRP5/6 effectively on SPR analysis. The NMR structure between the cyclized oligopeptide and the first propeller domain of LRP5 were assessed. The cyclized oligopeptide abrogated the Wnt–β-catenin signaling inhibited by DKK1 but not by sclerostin dose dependently. MOPC315.BM.Luc cells were injected into the tail vein and 5 days after injection, the cyclized oligopeptide was delivered subcutaneously 6 days a week for 4 weeks. Bioluminescence images showed that the treatment of cyclized oligopeptide reduced tumor burden compared to the vehicle

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group. RT-PCR and ALP staining showed increased expressions of osteoblast markers according to the treatment concentrations. In conclusion, the cyclized oligopeptide developed here can be another option for the treatment of tumor burden in multiple myeloma.

Key Words: DKK1, multiple myeloma, oligopeptide, tumor burden, Wnt signaling

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I. INTRODUCTION

Multiple myeloma (MM) is a disease of cancerous plasma cells expanding in bone marrow, causing symptoms such as anemia, hypercalcemia, and osteolytic bone lesions.[1] It is characterized by uncontrolled proliferation of a plasma cell clone with an accumulation of monoclonal immunoglobulins, particularly IgG and IgA.[2] Myeloma and stromal cells produce osteoclast activating factors such as IL-6, IL-3, receptor activator nuclear factor kappa B ligand (RANKL), TNF-α, and MIP1α, which are responsible for bone resorption.[3-7] Furthermore, the myeloma cells induce changes in the bone marrow microenvironment, thereby production of osteoblastic inhibitor factors such as TGF-β, TNF-α, and IL-3 is increased.[8,9] Accordingly an imbalance in osteoclast and osteoblast activity with the prevalence of bone resorption leads to marrow destruction.

The Wnt signaling pathway plays important role in the process of osteogenic differentiation and the maintenance of mesenchymal stem cells[10] and in embryonic development.[11] Abnormally activating and deactivating the canonical Wnt signaling pathway in osteogenesis cause bone diseases such as sclerosteosis and osteoporosis in humans and mice.[12] Several attempts have been made to target the Wnt signaling pathway by inhibiting endogenous antagonists and by regulating intracellular

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mediators for the treatment of osteogenic disorders.[12] Antibodies against endogenous Wnt antagonists, such as sclerostin, have shown bone-forming and fracture healing effects.[13] Dickkopf 1 (DKK1) is another important endogenous inhibitor of the canonical Wnt signaling pathway, which is a member of DKK family proteins.[14] It binds to low density lipoprotein receptor-related protein (LRP) 5 and 6 with Kremen receptor together inhibiting the canonical Wnt signaling pathway.[15] Most MM cells express DKK1, which is detected in MM patients' serum samples,[16] and the elevated DKK1 levels of MM patient serum and bone marrow plasma correlate with the presence of focal bone lesions.[17]

DKK1 has been extensively investigated in the murine model of MM where osteolytic lesions are a characteristic feature of advanced disease. Many studies have confirmed that the interaction of MM cells with the bone marrow microenvironment causes the bone lesions of myeloma.[18] MM cancer cells secrete DKK1, which disrupts the balance of osteoblastogenesis and osteoclastogenesis.[19] DKK1 disrupts the differentiation of mesenchymal stem cells to osteoblast lineage cells and this results a shift of the RANKL/osteoprotegerin (OPG) ratio leading to the excessive bone resorption and marrow destruction.[20] Enhanced bone resorption is not followed by neo-matrix deposition due to the inhibition of further differentiation of osteoblast precursors by DKK1.[21] Therefore, DKK1 has been a potential therapeutic target for the treatment of MM.

However, presently approved medications that target bone diseases are limited to anti-resorptive agents (e.g., bisphosphonates, denosumab) and bone-anabolic agents (e.g., full-length parathyroid hormone, teriparatide).[22] However, bone-protecting antiresorptive agents failed to confer significant antitumor activities in clinical studies.[23] There are no bone anabolic agents approved for the treatment of MM bone lesions.[22] As an inhibitor of the canonical Wnt signaling pathway, DKK1 binds to LRP5/6 receptor and Kremen and inhibits the osteoblast differentiation and proliferation.[24] A small number of papers have reported that the treatment of antibodies against DKK1 not only reduces the bone lesions but also reduces the tumor

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burden of the disease.[25,26] However, the exact mechanisms remain under investigation. I hypothesized that this DKK1-inhibiting oligopeptide would bind in the place of DKK1 of the Wnt signaling pathway, reducing the binding of the overexpressed DKK1 protein by MM cells. The present study aimed to develop a cyclized oligopeptide containing the NXI motif already known in DKK1, and to test the effects of the oligopeptide on tumor burden.

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II. MATERIALS AND METHODS

1. Synthesis and purification of peptides

Peptides were synthesized via Fmoc SPPS (solid phase peptide synthesis) using ASP48S (Peptron Inc) and purified via the reverse phase high-performance liquid chromatography using a Vydac Everest C18 column (250 mm × 22 mm, 10 μm). Elution was carried out with a water-acetonitrile linear gradient (3%–40% [v/v] of acetonitrile) containing 0.1% (v/v) trifluoroacetic acid. Molecular weights of the purified peptide were confirmed using Liquid Chromatography/Mass Spectrometry (Agilent HP1100 series).

2. Wnt 3a, Wnt7a, sclerostin and DKK1 preparation

Wnt3a conditioned medium was obtained from an L-Wnt-3a stable cell line (CRL-2647). Wnt7a conditioned medium was obtained from a Wnt7a stable cell line. Cells were grown in Dulbecco modified Eagle medium (DMEM) (Welgene, Korea) in 5% CO_2 at 37 °C. Media were collected 96 hours after the first media and 72 hours after the second media. Media were collected twice and mixed together into a conditioned media containing Wnt7a protein. DKK1 and sclerostin medium was obtained from an SF9 insect cell line. SF9 cells were inoculated with baculovirus containing the genes of interest: *DKK1* and sclerostin (*SOST*). After the cells were grown at 27 °C, the cultures were obtained and filtered (0.22 μm) to only extract the media with the proteins.

3. LRP5 E1 domain construction

The LRP5 E1 domain was inserted into the vector. The vector was maintained in the SF9 insect cell line at 27 °C. Cells were grown in T75 flasks and inoculated with the vector. LRP5 E1 proteins were expressed as secreted proteins. At 48 hours after inoculation, the media were collected and spun down at 3000 rpm. The supernatant containing the proteins was filtered (0.22 μm).

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4. Cell culture

MOPC315.BM.Luc cells[27] were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. ST2 cells were cultured in DMEM medium with 10% FBS and 1% penicillin-streptomycin (100 U/mL). Luciferase transfected MC3T3-E1 cells were cultured in alpha-minimum essential medium (α-MEM) with 10% FBS and 1% penicillin-streptomycin (100 U/mL) (Welgene, Korea). Cells were maintained in 5% CO_2 at 37 °C. For maintenance, cell media were changed every two days and cells were subcultured when cells reached 80%–85% confluence.

5. NMR spectroscopy

NMR spectra were acquired at 283K on a Bruker DRX-500 spectrometer equipped with a triple resonance probe with an x, y, and z-shielded pulsed-field gradient coil. Two-dimensional (2D) NMR spectra were recorded in phase-sensitive mode using time proportional phase increment (TPPI) for quadrature detection in the t_1 domain. Total correlation spectroscopy (TOCSY)[28] using a dipsi-2 spin-lock pulse sequence with a mixing time of 70ms and nuclear Overhauser enhancement spectroscopy (NOESY)[29] with mixing times of 250–600ms were performed. All NMR spectra were acquired with 2048 complex data points in t_2 and 256 increments in the t_1 dimension, with 64 scans per each increment. All NMR data were processed using nmrPipe/nmrDraw or XWIN-NMR software (Bruker Instruments, Karlsruhe, Germany) and analyzed using the Sparky 3.95 program.

6. Structure calculations

Distance restraints were derived from the NOESY spectra in 100% deuterated DMSO solution. The solution structures were calculated using the hybrid distance geometry and dynamical simulated annealing (SA) protocol

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using the CNS 1.1 program on a Linux workstation. Final structures were analyzed using the PROCHECK20 and MOLMOL programs.[30]

7. Surface plasmon resonance (SPR) assay

An SPR36 assay was performed by the Gyeonggi Bio Center (Suwon, Korea), using a ProteOn XPR36 instrument (Bio-Rad Laboratories, CA, USA). The SPR assay involved the LRP5 E1 domain being tethered (immobilized) onto a biosensor chip surface; then, five different concentrations (1 μM, 3 μM, 10 μM, 30 μM, 100 μM) of the oligopeptide were passed over the LRP5 E1 domain in order for binding interactions to occur. The Langmuir model, a simple 1:1 bimolecular interaction kinetic fir model, of the ProteOn Manager™ program (Bio-Rad Laboratory) was used for SPR data analysis.

8. Transfected MC3T3-E1 cell luciferase reporter assay

In the Wnt signaling study, MC3T3-E1 cells were transfected as follows.[31] Briefly, MC3T3-E1 cells were seeded at 4.5×10^4 cells/well in 24 well plates and were transfected with super8xTopflash (S-Top) plasmid (Clontech, USA) and maintained in α-MEM media containing G418 (500 ng/ml) to obtain MC3T3-E1-S-Top stable cells. These stable cells were seeded at 5×10^4 cells/well in 24 well plates and maintained overnight. Then, cells were treated in different concentrations (50 nM, 500 nM, 5 μM) of oligopeptide along with Wnt7a and DKK1 for 24 hours. Cells were collected after lysis in 1X passive lysis buffer (Promega, USA) and measured for luciferase reporter activity following the Promega protocol for the firefly luciferase assay system.

9. Reverse transcription – polymerase chain reaction (RT-PCR)

MOPC315.BM.Luc cells (1.5×10^5 cells/well) and ST2 cells (2×10^5 cells/well) were seeded in six well plates and maintained overnight. Then,

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cells were treated with Wnt7a, DKK1, and different concentrations of oligopeptide (10 nM, 100 nM, 50 μM). After 48 hours, cells were washed with PBS and total RNA was extracted with Trizol Reagent (Life Technologies, USA) using a standard protocol. cDNA were synthesized with a LaboPassTM cDNA synthesis kit (Cosmogenetech, Korea) and PCR was proceeded with primers ALP, OPG, DKK1, RANKL and β-actin as the housekeeping gene. Reverse transcriptase PCR was performed in the following cycles with GoTaq® DNA polymerase (Promega, USA): ALP, 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 1 minute for 30 cycles; OPG, 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 1 minute for 30 cycles; DKK1, 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 1 minute for 34 cycles; RANKL, 95 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 1 minute for 38 cycles; and β-actin, 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 1 minute for 30 cycles. Primer sequences were as follows: ALP, forward (F) 5'-GAGCGACACGGACAAGAA-3' and reverse (R) 5'-CAGAACAGGGTGCGTAGGG-3'; OPG, F 5'-ACCCAGAAACTGGTCATCAGC-3' and R 5'-CTGCAATACACACACTCATCACT-3'; DKK1, F 5'-AGACACTTCTGGTCCAAGATC-3' and R 5'-ACAGGTAAGTGCCACACTGAG-3'; RANKL, F 5'-GTCTGTAGGTACGCTTCCCG-3' and R 5'-CATTTGCACACCTCACCATCAAT-3'; and β-actin, F 5'-GCTACAGCTTCACCACCACAG-3' and R 5'-GGTCTTTACGGATGTCAACGTC-3'. PCR products were resolved by electrophoresis on 2% agarose gel stained with SeeNA II Nucleic Acid Stain (Messenger biotech, Korea).

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10. ST2 cell alkaline phosphatase staining assay (ALP staining)

ST2 cells were seeded with 2×10^4 cells/well in 24 well plates and treated with Wnt7a, DKK1, and different concentrations of oligopeptide: 50 nM, 500 nM, 5 μM, and 50 μM. After 48 hours of treatment, an ALP staining assay was performed using an alkaline phosphatase kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. The intensity of the stained color indicated the level of ALP expression. Images were taken using an Olympus IX73 inverted microscope at ×40 objectives.

11. Mice and injections

Five-week-old BALB/c female mice were purchased (Orientbio, Korea) and housed with five mice per cage in Yonsei Biomedical Research Institute. Mice were maintained for 1 week to allow them to adapt to the new environment. A total of 30 mice were divided into three groups (ten mice each): a negative control, a positive control, and an experimental group. The negative control group received 150 μL PBS via tail vein injection while all other 20 mice received 2×10^5 MOPC315.BM.Luc cells in 150 μL PBS via tail vein injection. Five days after the cell injection, oligopeptide (75 μg/150 μL/mouse) and vehicle (150 μL of phosphate-buffered saline/mouse) treatments were administered via subcutaneous injection, 6 days per week, for 4 weeks.

12. Bioluminescence imaging (BLI)

All mice underwent bioluminescence imaging using an IVIS Spectrum (Caliper-Xenogen, USA) 5 days after the tail vein cell injection, and 4 weeks after oligopeptide treatment. D-luciferin (VivoGlo Luciferin, Promega, USA) was injected (150 mg/kg) with an anesthesia mixture of tiletamine + zolazepam (30 mg/kg) and xylazine (10 mg/kg) 10 minutes prior to taking the image via intraperitoneal injection. Bioluminescence was measured from the

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dorsal and ventral sides of mice. Images were evaluated with Living Image® 4.4 (Caliper-Xenogen, USA).

13. MOPC315.BM.Luc cell proliferation assay

MOPC315.BM.Luc cells proliferation was measured using a WST-based cell viability / cytotoxicity assay kit (EZ-Cytox, DoGen, Korea). Cells were cultured with 1,000 cells/well in 96 well plates at 37 °C for 24, 48, and 72 hours with different concentrations of oligopeptide (50 nM, 500 nM, 5 μM, none). All experiments were carried out in triplicate.

14. Ethics statement

All experiments were conducted according to the guidelines for the care and use of laboratory animal (National Research Council, USA), IACUC, and the 3R principles.

15. Statistics

All values are expressed as mean ± standard deviation and median, and the statistical analysis was performed using and GraphPad Prism 5 software. Mann-Whitney U test was used to analyze the statistical significance, and the differences were considered to be statistically significant when $p < 0.05$.

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III. RESULTS

1. Solution structures of the cyclized oligopeptide

A series of screening tests was done previously and the cyclized oligopeptide was selected for further investigation. This oligopeptide was predicted to have similar structure and effects as the DKK1 protein. To study the *in vitro* and *in vivo* effects of the oligopeptide, the cyclized oligopeptide was synthesized chemically by Peptron Inc. Korea, and complete proton resonance assignments were achieved using the standard assignment procedure. Once the individual spin systems had been classified, backbone sequential resonance assignment was easily completed by $d\alpha N$ (i, i+1) NOE connectivities in the 2D-NOESY spectra. The side chain assignment was performed with TOCSY connectivities (Figure 1A). All NOEs were observed at mixing times of 600 ms. A total of 100 starting structures were calculated in the initial simulated annealing stage. The 20 lowest-energy structures (<SA>$_{k}$) were selected out of the 100 final simulated-annealing structures for structural analysis. The average structure was generated from the geometrical average of 20 structure coordinates and was subjected to restrained energy minimization to correct bond length and angle distortions. The average NMR structure exhibited 0.68-Å root-mean-square deviation for backbone atoms with respect to 20 (<SA>$_{k}$) structures. A best-fit superposition of all final structures and the backbone conformation for the average restrained energy minimized structure (<SA>$_{kr}$) are displayed in figure 1B. The overall fold of the cyclized oligopeptide is presented by ribbon diagram in figure 1C. The cyclized oligopeptide shows a loop conformation with no regular secondary structure.

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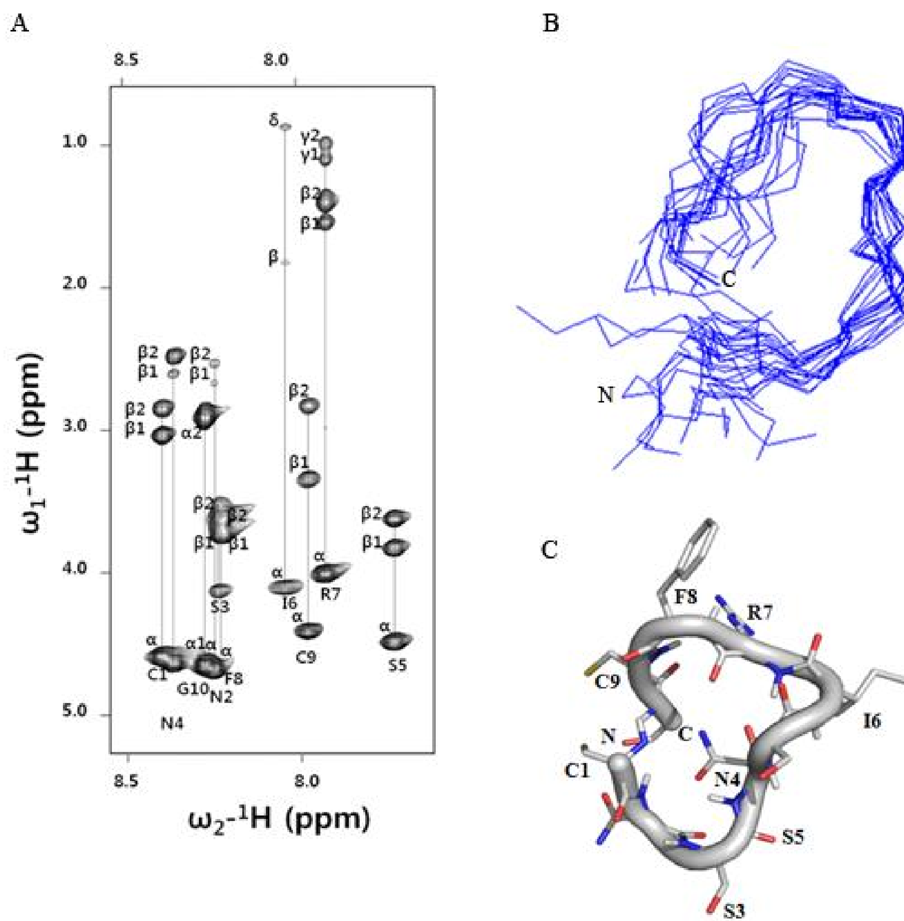

Figure 1. NMR structure of the cyclized oligopeptide.

(A) 2D TOCSY spectrum of DKK1 (1 mM) in 100% DMSO at pH 7.0, 283 K, recorded with a spin-lock mixing time of 70 ms. The spectrum shows the identification of amino acid spin systems based on scalar correlation of the backbone amide protons of (ω_2), with the respective side-chain protons (ω_1) of each spin system. (B) A backbone superposition of the energy-minimized average structure (<SA>$_{kr}$) over the family of 20 final simulated annealing structures (<SA>$_{kr}$). (C) A ribbon diagram of DKK1 with side-chain orientations is displayed as a stick model.

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2. Cyclized oligopeptide binds to the LRP5 E1 site and targets Wnt signaling

Then, further evaluation of whether the cyclized oligopeptide targets the Wnt signaling pathway. To study the binding activity of oligopeptide to the LRP5 E1 domain, SPR36 analysis was performed. It showed direct binding of the oligopeptide to the LRP5 E1 domain. The binding activity increased as the flowing oligopeptide concentration increased from 1 μM to 100 μM (Figure 2C). After it was proven that the oligopeptide can directly bind to LRP5, luciferase reporter activity assay was performed to study whether the oligopeptide affects the Wnt signaling pathway. MC3T3-E1-S-Top cells were treated with Wnts, Wnt inhibitors and oligopeptide. When the cells were treated with Wnt3a and DKK1, the activity increased as the concentration of oligopeptide increased; however, when treated with sclerostin, the activity could not overcome the inhibition effect (Figure 2A). Similar to this result when cells were treated with Wnt7a and DKK1, the luciferase reporter activity decreased and was overcome with oligopeptide treatment. However, when cells were treated with sclerostin, the activity showed no significant responses (Figure 2B). These results suggest that the oligopeptide is relatively DKK1 specific and that the oligopeptide targets the Wnt signaling pathway, abrogating the inhibition effects of DKK1.

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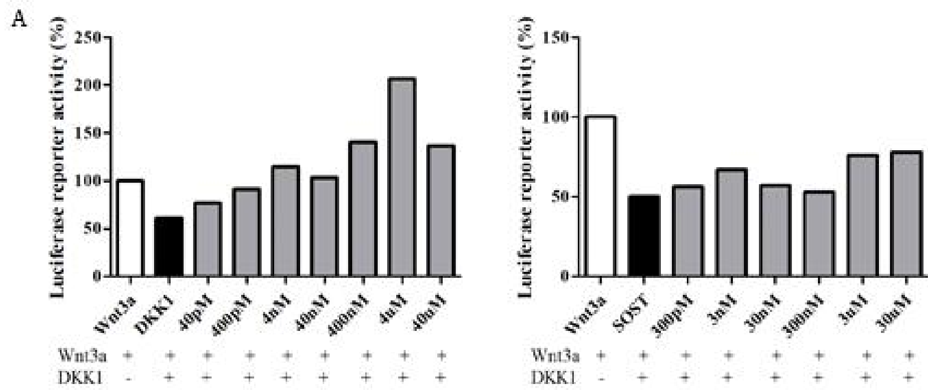

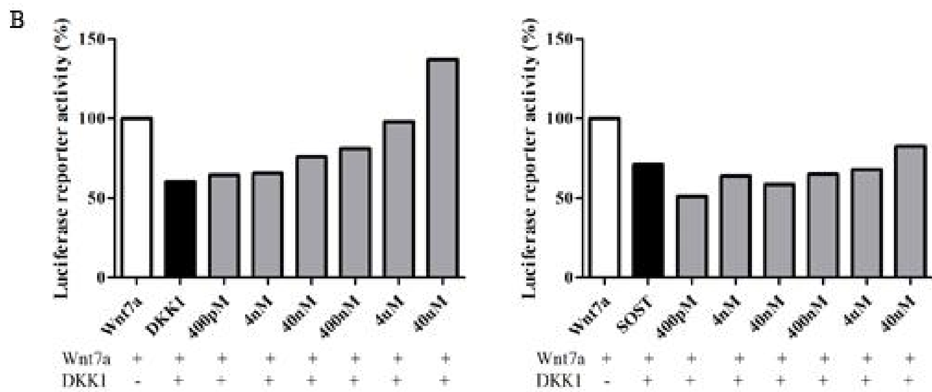

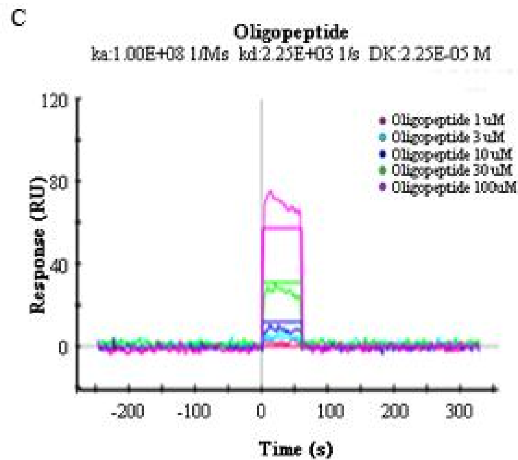

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Figure 2. Luciferase reporter activity of cells treated with cyclized oligopeptide and ProteOn XPR36 analysis. MC3T3-E1-S-Top cells were treated with Wnt3a, DKK1, sclerostin, and oligopeptide. (A) Graph on the left shows the dose increasing with luciferase reporter activity as the concentration of the oligopeptide increased. However, in the graph on the right, when treated with sclerostin, the same trend is not seen. (B) The same cells were treated with Wnt7a, DKK1, sclerostin and oligopeptide. (C) This graph shows the oligopeptide bound directly to the LRP5 E1 domain and the activity response increased dose dependently.

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3. The cyclized oligopeptide reverses the inhibitory effect of DKK1 on osteoblasts in vitro

It is known that Wnt signaling plays important roles in osteoblastic cells.[10] Here, the effects of the cyclized oligopeptide on the Wnt signaling pathway in the ST2 cell line were examined. ST2 cells were treated with Wnt7a, DKK1, and oligopeptide. Wnt7a treatment increased ALP and OPG mRNA expression levels, and the addition of DKK1 decreased mRNA expression levels. However, when cells were treated with oligopeptide, DKK1 and Wnt7a all together, the mRNA expressions of ALP and OPG increased again (Figure 3A). This result indicates that the oligopeptide activates the canonical Wnt signaling that is inhibited by DKK1. To further confirm this result, ST2 cells were treated with Wnt7a, DKK1, and oligopeptide for the ALP staining assay. The results of the ALP staining assay were similar to those seen in the RT-PCR. Cells treated with only Wnt7a expressed a stronger color, and the treatment of DKK1 decreased the staining. Cells treated with Wnt7a, DKK1, and oligopeptide could overcome the inhibition effect of DKK1 dose dependently (50 nM, 500 nM, 5 μM, 50 μM), shown by the increase in staining intensity (Figure 4). These results together suggest that this oligopeptide has bone-anabolic effects *in vitro*.

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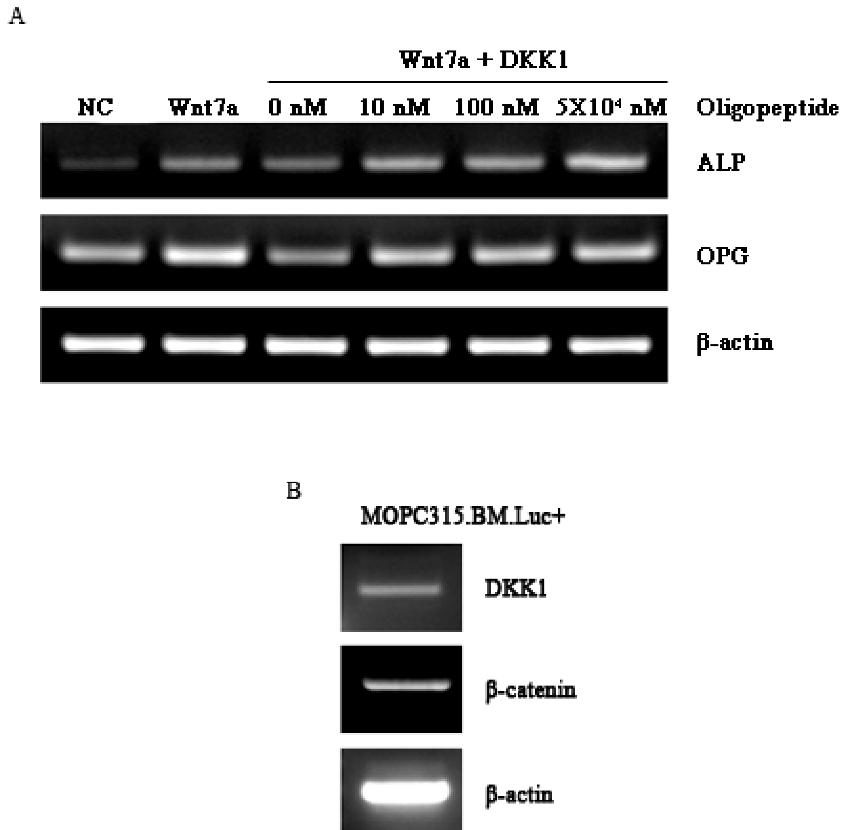

Figure 3. Reverse transcription-PCR of ST2 and MOPC315.BM.Luc cells. (A) ST2 cells were treated with Wnt7a, DKK1, and oligopeptide. The mRNA expression of both ALP and OPG were increased using Wnt7a and suppressed with DKK1 treatment. However, when oligopeptide was used, the mRNA expression levels of ALP and OPG increased again, overcoming the DKK1 inhibition effect. (B) The expressions of DKK1 and β-catenin were checked in the MOPC.315.BM.Luc cells prior to the experiment.

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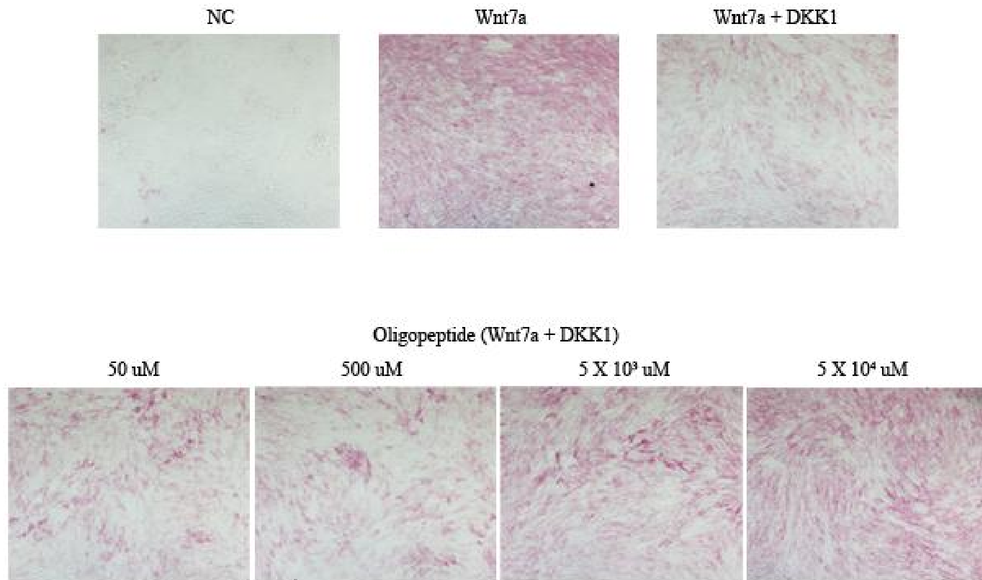

Figure 4. **Alkaline phosphatase staining assay of ST2 cells.** Cells were treated with Wnt7a only, Wnt7a and DKK1, or Wnt7a and DKK1 with oligopeptide. Similar to the RT-PCR results, when Wnt7a alone was used, the staining was stronger than when Wnt7a and DKK1 were used together. The staining intensity also increased dose dependently when oligopeptide was used in four increasing concentrations from 50 nM to 50 μM. Original magnification × 40.

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4. The cyclized oligopeptide inhibits tumor growth in a multiple myeloma mouse model

To determine the effect of oligopeptide on MM cell growth *in vivo*, DKK1 expression was confirmed. The mRNA expression of both DKK1 and β-catenin were detected (Figure 3B), indicating that the canonical Wnt signaling pathway may have certain effects on MM progression and bone lesions. After the expression of DKK1 was proven, MOPC315.BM.Luc cells (2×10^5 cells) were injected into the tail vein of 20 BALB/c mice (day -5). Five days after the injection of cells, mice were treated with either the oligopeptide or vehicle alone, injected subcutaneously six days a week, for a total of four weeks (Figure 5A). Tumor burden was measured via BLI on day 0 and day 28. One representative mouse from each group is shown (Figure 5B). On day 0 (5 days after MOPC315.BM.Luc injection), no significant radiance (p/sec/cm^2/sr) was detected in any of the three groups of mice. In contrast, on day 28, all mice injected with MM cells invoked a BLI signal. Importantly, larger radiances were detected in the vehicle group than in the oligopeptide group. No significant radiances were detected in the control group, as no cells were injected. The signals of oligopeptide-treated mice were predominantly detected in the spleen, while the signals of the vehicle-treated mice were more diffused all over the body. The median fold change of the vehicle group had the highest value, and the median of the oligopeptide group was about three times lower ($p = 0.046$) than the median of the vehicle group (Figure 5C). This data was divided into three ranges, 10 to less than 50, 50 to 500, and more than 500. In the lower range of fold change, there were more mice from the oligopeptide group (7) than the vehicle group (3). However, in the fold change of 50 and higher, the result was opposite; the number of the vehicle group mice was much larger (7) than the number of mice in the oligopeptide group (3; Figure 5D). All mice from the control group had fold changes of less than two. In accordance with the BLI results, the ELISA assessed M315

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protein level of the peptide group showed lowering trend compared to the vehicle group, but with no significance (Figure 6). This may be resulted because of the small number of mice in each group and their large variations of the M315 protein level. This observation shows that oligopeptide affects the growth of MM cells *in vivo*, although the detailed mechanism remains unknown.

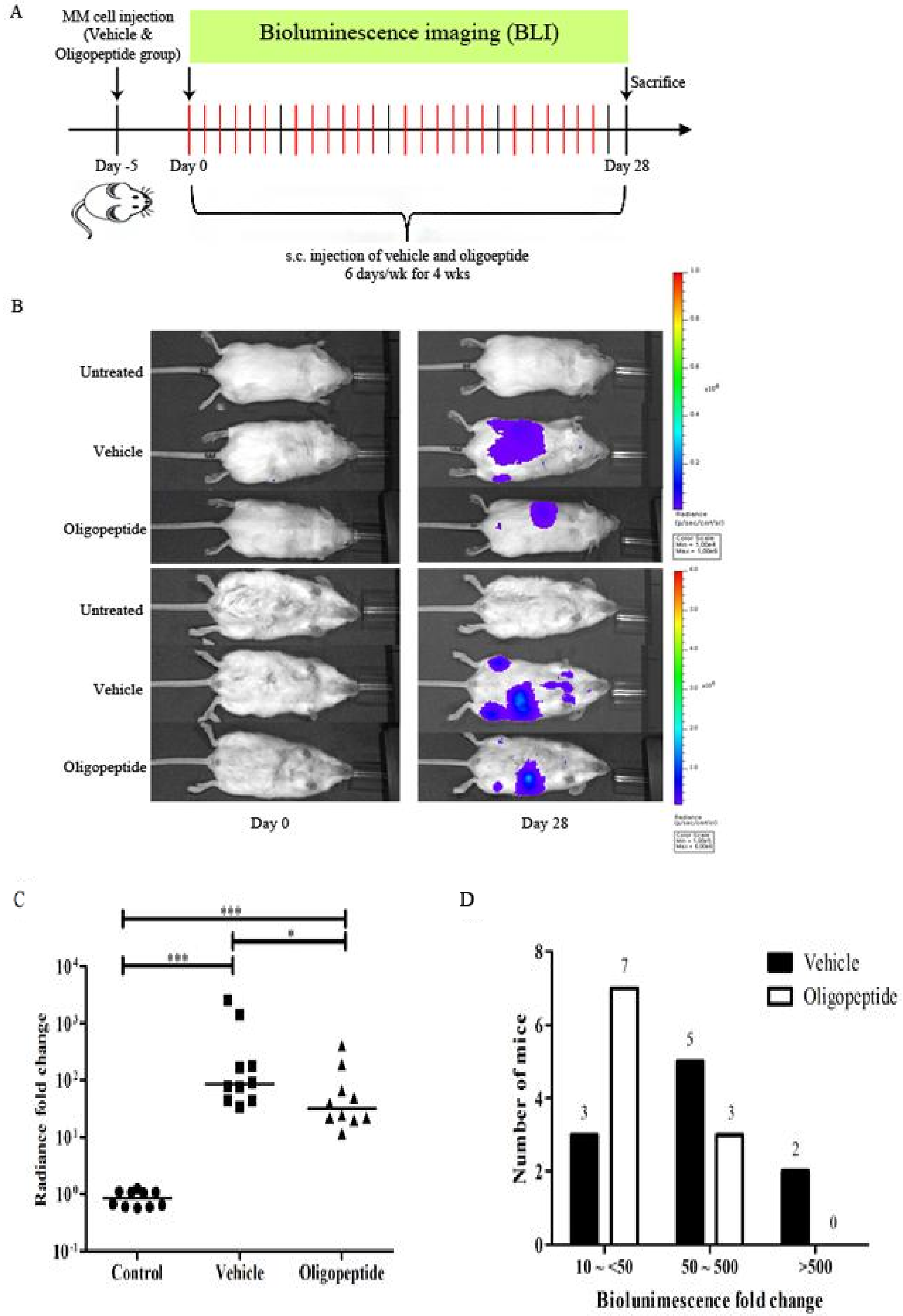
A
MM cell injection
(Vehicle &
Oligopeptide group)
Bioluminescence imaging (BLI)
Sacrifice
Day -5
Day 0
Day 28
s.c. injection of vehicle and oligoeptide
6 days/wk for 4 wks
B
Untreated
Vehicle
Oligopeptide
Untreated
Vehicle
Oligopeptide
Day 0
Day 28
C
***
*
***
Radiance fold change
Control
Vehicle
Oligopeptide
D
Vehicle
Oligopeptide
Number of mice
10 ~ <50
50 ~ 500
>500
Biolunimescence fold change

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Figure 5. Tumor growth was reduced in the MOPC315.BM.Luc cell induced multiple myeloma mouse model. (A) A brief timeline of the animal study is shown. (B) One representative mouse from each group was chosen based on the closest radiance activity to the median value. Images taken on day 0 and day 28 are shown and compared. On day 0, BLI was taken before the oligopeptide treatment. No radiance was detected in the untreated mouse. (C) Bioluminescence radiance fold changes of each group from day 0 to day 28 are shown. Each group was comprised of 10 mice. * indicates $p < 0.05$ and *** indicates $p < 0.0001$ by Mann-Whitney U test. (D) Mice numbers according to the fold changes of bioluminescence radiance are shown. Fold changes were calculated simply by dividing the final value by the initial value.

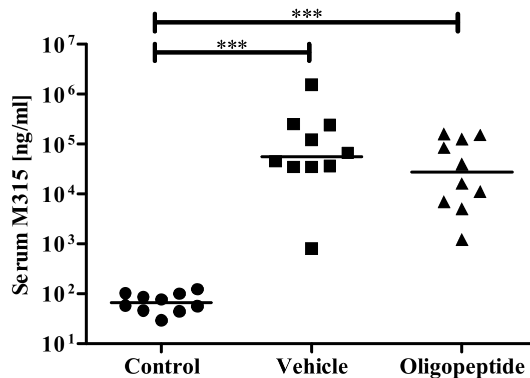

Figure 6. Serum M315 ELISA assay of multiple myeloma mouse model. Serum M315 ELISA assay showed the decreasing trend of M315 protein in mice after the 4 week s.c. treatment of oligopeptide. *** indicates significance by Mann-Whitney U test.

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5. The cyclized oligopeptide has no direct effect on MOPC315.BM.Luc cell proliferation in vitro

One concern of using this oligopeptide is that the oligopeptide may affect the Wnt signaling pathway and stimulate cancer cell growth. In our *in vivo* experiment, however, it was shown that the tumor burdens were reduced with the treatment of the oligopeptide. To better define the effect of the oligopeptide on MM cell growth, the oligopeptide was tested *in vitro*, examining cell viability via WST assay. MM cells were treated with 50 nM, 500 nM, and 5μM of the oligopeptide or none for 24 h and 48 h. The MM cell population did not vary significantly in the differently treated groups (data not shown). This result demonstrated that oligopeptide does not affect MM cell growth directly, despite the MM cells exhibiting a Wnt/β-catenin signaling pathway.

These overall findings indicate that the oligopeptide affects MM cells and osteoblastic cells; it remains uncertain as to how and through what kind of mechanism this oligopeptide affects osteoblasts and MM cells. The anti-MM effect may be mediated by the indirect effects on the bone microenvironment by the cyclized oligopeptide. Variable factors in the microenvironment may contribute strongly to the MM tumor burden; however, further studies are needed.

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IV. DISCUSSION

This present study showed that a cyclized oligopeptide treatment reduced tumor burden in the MOPC315.BM.Luc MM mouse model. To study the effects of this oligopeptide targeting the DKK1-LRP5/6 binding pocket and containing the NXI motif in MM bone disease, the DKK1 NXI motif–containing cyclized oligopeptide was developed, and a series of *in vitro* and *in vivo* experiments was performed.

Wnt signaling is well known for the osteoblast lineage specification from mesenchymal cells, further differentiation of osteoblast precursor cells, and skeletal development.[33] DKK1, an endogenous Wnt inhibitor, binds to LRP5/6 with Kremen to inhibit the canonical Wnt signaling pathway. Most MM cells express DKK1;[16] furthermore, the secreted DKK1 disrupts the balance of osteoblastogenesis and osteoclastogenesis. As a result, the RANKL/OPG expression ratio is shifted in preosteoblasts, which enhances osteoclast-mediated osteolysis and simultaneous osteoblast inhibition.[19] As DKK1 has been a potential therapeutic target for the treatment of MM, some effective therapies targeting DKK1 have been developed, including DKK1-neutralizing antibodies, proteasome inhibitors, DKK1 vaccines, and tumor-produced endothelin-1.[34] A study using the SCID-rab mouse model of myeloma showed that anti-DKK1 antibody treatment increased bone formation and reduced tumor burden in a rabbit bone implanted with MM.[25] Human anti-DKK1 monoclonal antibody (BHQ880) was also found to increase osteoblasts and blocked MM cell proliferation when MM cells were co-cultured with bone marrow stromal cells.[26]

The cyclized oligopeptide against the DKK1-LRP5/6 binding pocket abrogated the Wnt–β-catenin signaling inhibited by DKK1 (not by sclerostin) dose dependently ($IC_{50} \approx 5 \times 10^{-8}$ M). The NMR structure was obtained by complete proton resonance assignment following the standard procedure. Isoleucine residue of the cyclized peptide should target the second pocket of LRP5/6. The ribbon diagram (Figure 1C) showed that DKK1 is a linear peptide chain with a loop conformation.

Administration of the oligopeptide abrogated the suppression of canonical Wnt

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signaling by DKK1 and inhibited tumor burden significantly. As matrix protein contains many growth factors and cytokines stimulating proliferation of tumor cells, inhibition of bone resorption by decreasing the RANKL/OPG ratio via the oligopeptide could reduce the release of these growth factors and cytokines from matrix proteins and inhibit the overgrowth of MOPC315.BM.Luc cells. Similar to what was achieved in this study, the DKK1 neutralizing antibody BHQ880 has been shown to not only improve bone formation but also reduce tumor burden.[26] Additionally, findings reported that DKK1 suppressed β-catenin in myeloid-derived suppressor cells (a heterogeneous population of myeloid lineage immune cells in the stromal compartment) and thus inhibited tumor growth in mice.[35] To explore the mechanism for the reduction of tumor burden more, the effects of oligopeptide on proliferation of tumor cells were studied. However, no direct effects of oligopeptide on the proliferation of tumor cells were found, despite the fact that DKK1 is expressed in MOPC315.BM.Luc cells (data not shown). This result was supported by a previous study showing that while BHQ-880 did not have a direct anti-tumor effect on myeloma cells, it inhibited myeloma growth in the presence of bone marrow stromal cells (BMSC).[26] The MM niches contain bone marrow, fat cells, and immune cells, and they interact with each other. Several other cell types within the bone microenvironment produce significant amounts of DKK1 including megakaryocytes, endothelial cells, and osteoblasts and may therefore contribute to the regulation of tumor growth.[36,37] TGF-β, VEGF and FGF, as well as the increase of bone resorption by osteoclasts seem to support MM cell growth.[38,39] In the previous study, DKK1 indirectly contributed to myeloma growth by regulating IL-6 in the bone microenvironment. BHQ-880 significantly reduced the production of IL-6 levels in BMSC supernatants, and the addition of IL-6 reversed the majority of growth inhibitory effects.[26] Therefore, the overall results obtained in this study may have derived from DKK1 acting on these cells differently.

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V. CONCLUSION

In conclusion, the cyclized oligopeptide based on the DKK1-LRP5/6 interaction abrogates the suppression of canonical Wnt signaling by DKK1. Treatment of the oligopeptide reduced tumor burden significantly in the MOPC315.BM.Luc MM mouse model. The indirect effect of the oligopeptide to the bone microenvironment likely have resulted the reduction of tumor burden. This cyclized oligopeptide may be an option for proper management of tumor burden in MM; however, further research is required to determine the details of the mechanisms involved.

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ABSTRACT (IN KOREAN)

LRP5/6-DKK1 상호작용을 타깃 하는 고리화 올리고펩타이드가 다발성 골수종 마우스 모델에서 종양에 미치는 영향

<지도교수 임 승 길>

연세대학교 대학원 의과학과

박 보 미

DKK1은 골 용해성 병변이 특징인 다발성 골수종의 쥐 모델에서 많이 연구되어왔다. 골수종 환자의 골수 혈장과 혈청에서 상승된 DKK1은 환자의 뼈 병변과 연관이 있고, 혈청에 함유된 높은 양의 DKK1이 조골 세포 전구체의 분화를 억제한다. 현재, 골 전이 치료에 승인받은 약제는 골 흡수 억제제로, bisphosphonates와 denosumab 등이 있다. 본 연구에서 본인은 DKK1과 LRP5/6의 상호 작용을 방해하는 고리화 올리고펩타이드를 개발하였고, 올리고펩타이드가 종양 크기에 미치는 영향을 조사하였다. DKK1-LRP5/6의 상호 작용에 기초하여 고리화 올리고펩타이드를 화학적으로 합성하였고, NXI motif를 함유하는 이 올리고펩타이드는 SPR분석에서 효과적으로 LRP5/6의 E1 도메인에 결합하는 것을 확인하였다. 올리고펩타이드의 NMR 구조도 분석하였다. 이 올리고펩타이드가 DKK1에 의해 억제된 Wnt/β-catenin 신호를 회복하지만, slerostin에 의한 억제된 Wnt/β-catenin 신호는 회복시키지 못 했다. MOPC315.BM.Luc 세포는 쥐의

꼬리 정맥에 주사하였고, 주사 후 5일 뒤에 올리고펩타이드를 4주 동안 일주일에 6회 피하에 주사하였다. Bioluminescence 이미징을 통해 올리고펩타이드를 주사한 그룹에서 종양의 성장을 억제하는 효과를 확인하였다. RT-PCR 과 ALP staining 을 통해서 조골세포의 마커들이 올리고펩타이드를 처리한 그룹에서 더 증가하는 것을 확인하였다. 결과적으로, 이 고리화 올리고펩타이드는 다발성 골수종의 종양을 치료할 수 있는 약제 후보물질이다.

핵심되는 말: DKK1, 다발성 골수종, 올리고펩타이드, 종양, Wnt시그널