様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Targeting ALK1 using macrocyclic peptides: A novel approach for the regulation of angiogenesis in cancer

研究課題名（英文）Targeting ALK1 using macrocyclic peptides: A novel approach for the regulation of angiogenesis in cancer

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研究成果の概要（和文）：我々はBMP受容体ALK1に対してRaPID選択を行い、環状ペプチドALK1-3R6-5を単離した。さらに追加のインビトロ阻害アッセイ、バイオレイヤー干渉法、およびプルダウンアッセイを用いてALK1--3R6-5を分析した。最終的には、環状ペプチドは小さすぎ、ナノボディが示す結合よりは劣ると考えられた。これらの知見に基づいて、我々はモノボディーライブラリを作成した。これはフィブロネクチンをスキャフォールドとしている。我々はこのライブラリーを作製し、現在ALK1結合モノボディの選択を行っている。

研究成果の学術的意義や社会的意義
The significance of our findings brings us closer to inhibiting tumor growth in cancer. We learned that regulation of the ALK1 receptor and subsequent inhibition of angiogenesis requires a ligand larger than a macrocyclic peptide and we are now developing monobodies against ALK1.

研究成果の概要（英文）：We performed the RaPID selection against the BMP receptor ALK1 and isolated the macrocyclic peptide ALK1-3R6-5. We characterized ALK1-R6-5 using biolayer interferometry, in vitro inhibition assays, and pull-down assays. Our conclusion is that ALK1-3R6-5 is too small for our experiments. I have produced a monobody library to address this problem. This library is based on the scaffold of fibronectin, which has a larger and better-defined structure. We have validated production of the library and are currently in the process of performing selection for ALK1-binding monobodies.

研究分野：Medicine

キーワード：Macrocyclic peptides　In vitro selection　RaPID system　angiogenesis

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景

Introduction

Cancer is the dysregulated proliferation of cells. As a cancer tumor grows, cells in the center of the tumor mass lose the ability to efficiently intake nutrients and expel metabolic waste. The tumor cells avoid necrosis by signaling to nearby vasculature to sprout new blood vessels from existing vasculature, a process known as angiogenesis. The newly formed vasculature brings fresh nutrients and removes toxic metabolic waste. In addition, the increased vasculature provides a route for cancer cells to metastasize.

Strategies to inhibit angiogenesis and inhibit tumor growth have involved targeting vascular endothelial growth factor (VEGF) signaling, but development of resistance to anti-VEGF signaling therapeutics has forced researchers to explore alternative signaling pathways for therapeutic targeting. Activin receptor-like kinase (ALK) 1 is a cell surface receptor known to be critical for both developmental and pathological angiogenesis. ALK1 is highly expressed on proliferating vascular endothelial cells. ALK1 signaling is regulated primarily by the cytokine bone morphogenetic protein (BMP) 9. BMP9 binds to the extracellular domain of the receptor BMPR2, which promotes a conformational change allowing for the additional binding of ALK1 (Figure 1). The final heteromeric active complex consists of two ALK1 receptors, two BMPR2 receptors, and one BMP ligand. The intracellular kinase of constitutively active BMPR2 activates ALK1's intracellular kinase activity though phosphorylation. ALK1 in turn propagates the signal inside the cell via phosphorylation of SMAD proteins.

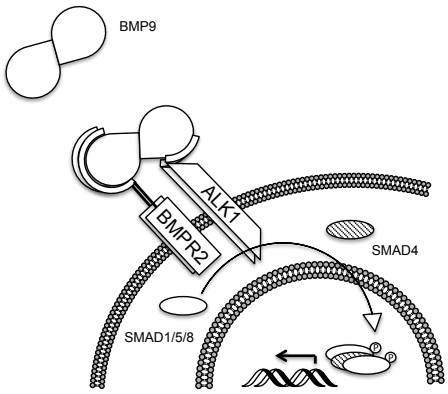


Figure 1. Extracellular and intracellular components of the BMP signaling pathway.

An anti-ALK1 antibody, PF-03446962, has been developed by Pfizer, but unfortunately was shown in Phase 2 clinical trials to lack efficacy and could cause adverse effects[1]. Despite this setback in targeting ALK1 signaling and pathological angiogenesis, the continued discovery of (non-antibody) ligands against ALK1 signaling is a valuable pursuit. In our current study, we characterize a previously identified macrocyclic-peptide ligand for binding and inhibitory activity against ALK1. After evaluating the macrocyclic peptide's inhibitory activity, we designed a monobody library, which we are now using for a subsequent selection.

２．研究の目的

Purpose

The purpose of this research is to discover non-antibody ligands that can inhibit angiogenesis by blocking ALK1's interaction with either the signaling ligand BMP9 or with the receptor BMPR2. We hypothesize that blocking either of these interactions with a novel ligand will inhibit ALK1 activation and pathological angiogenesis.

３．研究の方法

Research method

We have previously used the Random non-standard Peptide Integrated Discovery (RaPID) system[2] to isolate a macrocyclic peptide, ALK1-R6-5, that appeared to be a potential ALK1-binding ligand (Figure. 2). We chemically synthesized this lariat-shaped macrocyclic peptide as well as fluorescein-labeled and biotin-labeled derivatives. In addition, we synthesized just the minicycle without the linear tail to assess the minimal scaffold for inhibitory activity.

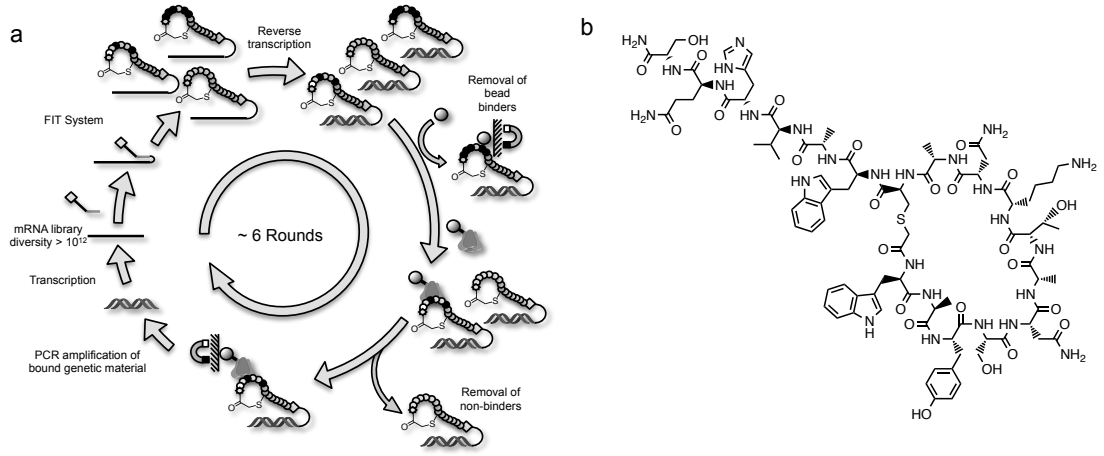


Figure 2. *In vitro* selection of macrocyclic peptide ALK1-R6-5 using the RaPID system. a) Schematic representation of a selection cycle protocol of the RaPID system. b) Chemical structure of ALK-R6-5.

We attempted to biophysically measure the macrocyclic peptide's affinity for ALK1 using biolayer interferometry (BLI). Next, we examined ALK1's ability to affect signaling using a BMP Response Element (BRE)-luciferase assay. Afterward, we performed pull-down of ALK1-HA using biotinylated ALK1-R6-5. After testing macrocyclic peptide ALK1-R6-5 led us to the conclusion that it could not effectively interfere with ALK1 signaling, I produced a new ligand library based on monobodies and am currently using this library for further studies.

4．研究成果

Results

Biolayer Interferometry

Our first test with the chemically synthesized peptides was our attempted measurement of the binding affinity of the macrocyclic peptide ALK1-R6-5 to ALK1. We used a Pall Bioforte BLItz machine to perform biolayer interferometry (BLI). For this experiment, we immobilized ALK1-R6-5-biotin to a streptavidin biosensor from time 40 seconds to 150 seconds (Figure. 3). After a 30-second wash, ALK1 protein was associated from time 180 seconds to 300 seconds. The associated protein was allowed to dissociate from 300 seconds to 420 seconds. A negative control was performed, where buffer was used in place of the

Figure 3. Binding affinity measurement using Biolayer Interferometry. a) Run 1 was performed with ALK1-R6-5-biotin immobilized on the biosensor (40s-150s), followed by wash (150s-180s), association with ALK1 (180s-300s), and finally, dissociation of ALK1 (300s-420s). Negative control Run 3 was performed in the same manner as Run 1 except buffer was used in place of ALK1-R6-5-biotin solution. b) Graphs baseline corrected at 180 seconds.

ALK1-R6-5-biotin solution. Unfortunately, there was no noticeable difference in binding between the two runs during the association steps (Figure 3b). This suggests that the ALK1-R6-5 peptide does not bind strongly to ALK1 in this experiment.

BRE-luciferase reporter assay

Despite being unable to detect binding using BLI, we used full-length ALK1-R6-5 (FULL) and a truncated version lacking the linear portion (MIN) in a coupled BMP response element (BRE)-luciferase assay. In this assay, overexpression of luciferase is under the control of BRE, which is stimulated by BMP9-mediated signaling. If the macrocyclic peptides are able to disrupt BMP9-mediated signaling by blocking ALK1, a reduction in luciferase activity should occur. The tail-truncated ALK1-R6-5 showed no inhibitory activity up to 1 μM, whereas the addition of full-length ALK1-R6-5 caused a very slight drop concentration-dependent drop in signal (Figure. 4). After observing this slight inhibitory activity, we next examined if the cause of the inhibition is due to specific ALK1-R6-5 binding to ALK1.

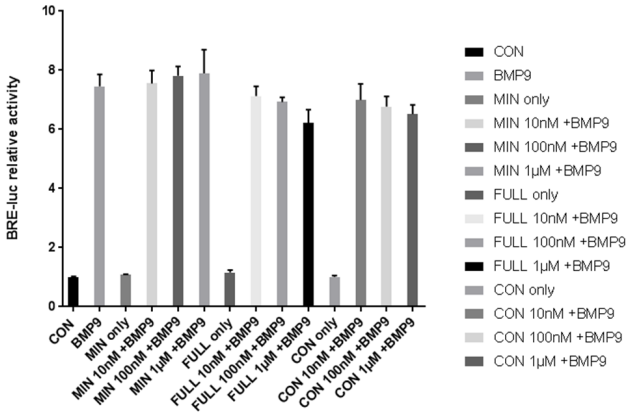


Figure 4. Inhibition of BMP signaling using a coupled BRE-luciferase assay. A non-related macrocyclic peptide was used as a negative control (CON).

ALK1-R6-5-mediated pull-down of ALK1

We tested the ability of ALK1-R6-5-biotin (FULL-B) to pull-down soluble ALK1-HA fusion protein. In this experiment, an anti-ALK1 nanobody (AE8) that was selected for in a different *in vitro* selection and an anti-HA antibody (HA) were used as positive controls. ALK1-R6-5-biotin, anti-ALK1 nanobody-Fc (AE8), or and anti-HA antibody were incubated with ALK1-HA fusion protein-overexpressing cells for 2 hours at 4°C. Lysis buffer and either neutravidin beads for ALK1-R6-5-biotin or protein G beads for AE8 and anti-HA antibody were added. The mixtures were incubated for 1 hour at 4°C. The beads were then washed, treated with mouse anti-HA antibody, washed again, and finally, treated with anti-mouse Fc antibody for detection using a secondary antibody.

The initial exposure time could not be used to detect ALK1-HA in the pull-down experiment using ALK1-R6-5-biotin (Figure 5). Only after lengthening exposure time, was the captured ALK1-HA detectable. Using up to 1 μM of ALK1-R6-5-biotin did not significantly capture more ALK1-HA over the beads-only negative control. This observation once again verifies that the macrocyclic peptide binds weakly, if at all, to ALK1 when compared to AE8 and HA.

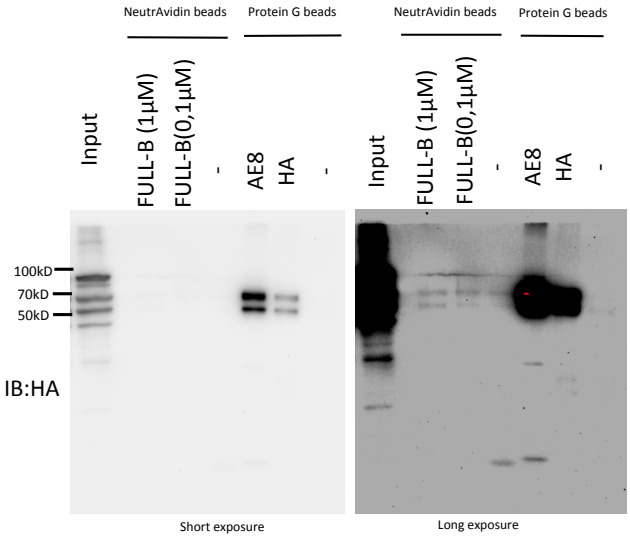


Figure 5. Pulldown of ALK1 using immobilized Ligand. Exposure time was increased to facilitate detection of ALK1 captured by ALK1-R6-5-biotin (right)

Despite the weak binding, the observation of slight inhibitory activity in the BRE-luciferase reporter assay warranted one more test to detect binding *in vitro*. This time, ALK1-Fc was used as the capture target and the pull-down process was repeated. The captured ALK1 was visualized using a fluorescent secondary anti-mouse antibody and fluorescence microscopy (Figure 6). A very slight increase in fluorescent is detected when using 6000 µg of ALK1-R6-5-biotin, again suggesting weak binding.

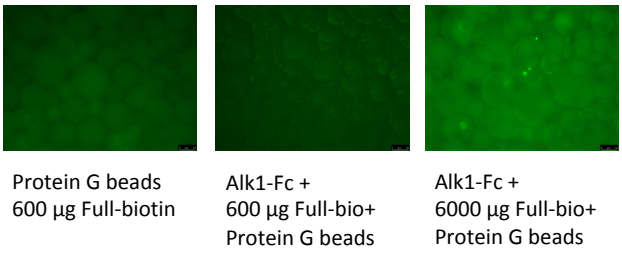
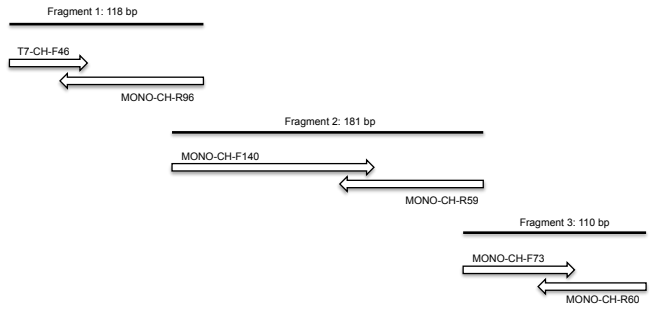


Figure 6. Staining of ALK1-Fc captured on beads using a biotin-labeled ALK1-R6-5 macrocyclic peptide.

Production of a monobody library for in vitro selection against ALK1

From our experiments, we concluded that the small size of our macrocyclic peptide and any peptide originating from our initial macrocyclic peptide library would be too small to effectively bind and inhibit the signaling function of ALK1. We therefore produced a new monobody library[3], which is based on a fibronectin scaffold (Figure. 7). This library is currently being used in a new *in vitro* selection against ALK1. Our current experimental plan is to complete the *in vitro* selection and repeat the assays using newly identified ligands.

a



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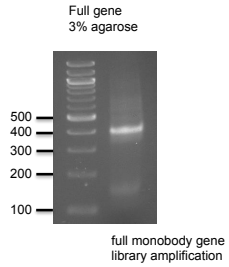

c

Monobody Library

MSVSDVPRDLEVVAATPTSLLISW**XXXXXXXXX**Y**X**ITYGE**XXXXXXX**Q**X**F**X**VP**XXXXX**ATISGLKPGVDYTITVYAVT**XXXXXXXXX**
XPISINYRTGSGSGS

Clone 1

MSVSDVPRDLEVVAATPTSLLISWAIRDHLAWGYWITYGEDALRRRWQEFIVPVRSRGATISGLKPGVDYTITVYAVTGCVYLCMNV
VPISINYRTGSGSES

Clone 2

MSVSDVPRDLEVVAATPTSLLISWRSRLSWQLAYKITYGEGTPHRQGQFFFVPSHCLSATISGLKPGVDYTITVYAVTLLPLVGCTG
GPISINYRTGSGSGS

Figure 7. Production of a monobody library. a) Schematic depicting the gene fragment assembly using primer extension of chemically-synthesized oligonucleotides. b) Agarose gel showing the fully assembled monobody library gene. c) The peptide sequences of the monobody library design, where **X** indicates a randomized amino acid, and two isolated clones from an early round in a new selection against ALK1.

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3. Koide, A.; Koide, S. *Methods Mol Biol.* **2007**;*352*:95-109.

５．主な発表論文等

〔雑誌論文〕（計　1　件）

Kusakizako, T.; Tanaka, Y.; **Hipolito, C.J.**; Suga, H.; Nureki, O. Crystallographic Analysis of MATE-Type Multidrug Exporter with Its Inhibitors. *Methods Mol Biol.* **2018**, *1700*, 37-57. doi: 10.1007/978-1-4939-7454-2_3. (Peer-reviewed)

〔学会発表〕（計　4　件）

〔図書〕（計　0　件）

〔産業財産権〕
○出願状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
出願年：
国内外の別：

○取得状況（計　0　件）

名称：
発明者：
権利者：
種類：
番号：
取得年：
国内外の別：

〔その他〕
ホームページ等
http://www.md.tsukuba.ac.jp/tmrc/foundation_core/peptide/peptide_en.html

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