Graduate School of Advanced Science and Engineering
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博　士　論　文　概　要
Doctoral Dissertation Synopsis

論　文　題　目
Dissertation Title

Development of immune checkpoint inhibitors made of PD-1 variants or small molecule derivatives

PD-1変異体あるいは小分子誘導体からなる免疫チェックポイント阻害剤の開発

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Cancer is a leading cause of death worldwide. Immune checkpoint inhibitors (ICIs) block the interaction between programmed cell death-1 (PD-1)/ programmed cell death-ligand 1 (PD-L1) to reactivate the tumor-suppressed T cell, opening a new era of cancer treatment. Compared to the approved antibody ICIs, recently reported non-antibody ICIs have advantages such as smaller molecular weight, shorter serum half-life, and lower manufacturing costs. However, the non-antibodies ICIs have a limited therapeutic effect due to their immunogenicity or low bioactivities. To obtain the ICIs with low manufacturing cost, small molecular size, and higher therapeutic effect, this thesis aimed to develop three improved non-antibody ICIs: a low-immunogenicity protein ICI, a high-bioactivity small molecular ICI-incorporated peptide (SMI-peptide) ICI, and a high-bioactivity SMI conjugated polymer ICI. All the developed ICIs are promising to expand the current repertories of ICIs and their development method are general that can be used for developing other inhibitors against other targets.

In Chapter 1, the background and motivation of my research were described. Although ICIs have gained great success, so far, only antibody is approved as ICI drug; however, its high molecular weight limits its distribution amount into the tumor sites which may be responsible for its low clinical response rate. Besides, its long serum half-life extends the inevitable immune-related adverse events, and its expensive manufacturing cost ends up limiting the number of affordable patients. Non-antibody ICIs could overcome the above shortcomings of antibody ICI but each category of non-antibody ICIs has its shortcomings that limit their therapeutic effect. Specifically, non-antibody protein ICIs are either made based on non-human proteins or contain many mutations, which may generate high immunogenicity and thus decrease their therapeutic effect. Small molecular ICIs lack enough PD-1/PD-L1 contact surface area to provide enough specificity which presumably led to their low bioactivity and high cytotoxicity. Peptide ICIs lack the key molecules to provide the target-binding interaction for providing enough antibody-like bioactivity. For obtaining non-antibody ICIs with high therapeutic effects, three improved non-antibody ICIs were developed to overcome their shortcomings in the next three chapters.

In Chapter 2, the development of protein inhibitors of a PD-1 variant was described. As the native PD-L1 binder, soluble PD-1 has lower immunogenicity than non-human protein scaffold. To increase the affinity of PD-1 without introducing many mutations, I performed accurate mutagenesis by combining the *in silico* mutagenesis and in-cell verification.

Based on the crystal structure of human PD-1/PD-L1, *in silico* mutagenesis was performed by calculating the difference of free binding energy between the mutated PD-1/PD-L1 complex and the wild-type PD-1/PD-L1 complex. Point mutations that showed lower binding energy was selected as candidates since their resulting complex are more thermodynamically stable. To verify the *in silico* selected candidates, a split luciferase reporter assay was performed by co-expressing two fusion proteins within the cell. One fusion protein was composed of a PD-1 variant and a subunit of luciferase, while the other fusion protein was composed of PD-

L1 and a complementary subunit of luciferase. The binding between PD-1 variants and PD-L1 could reconstitute the subunits of luciferase and recover the luciferase activity. Since the luminescence intensity was correlated with the binding affinity between PD-1 variants and PD-L1, the candidates could be accurately and rapidly evaluated in the intracellular environment through luminescence. From the assay, PD-1(A132V) and PD-1(T76Y) were selected, and their double mutant called 2-PD-1 was found to have a synergic effect. Kinetics analysis revealed that 2-PD-1 had a 100-times lower dissociation constant (K_d) than wild-type PD-1. Besides, *in vitro* PD-1/PD-L1 inhibition assay confirmed that 2-PD-1 had a 30-times higher inhibitory effect than wild-type PD-1. Furthermore, 2-PD-1 had a 100-times higher bioactivity in the reactivation of the tumor-suppressed T cell. Although the activity of 2-PD-1 was about 10 to 50 times weaker than the divalent antibodies, the possible low immunogenicity from the only two mutations still indicated that the 2-PD-1 would be a more potent non-antibody protein ICI.

In Chapter 3, the development of the SMI-peptide ICI was described. The current SMIs lack enough PD-L1 contact surface area while the current peptide ICIs lack the key molecules for enhanced PD-L1 interaction, which limits their bioactivity. Therefore, I considered to incorporate SMI into the random peptide library (SMI-peptide library) for screening out a potent SMI-peptide ICI.

Ribosome display is a powerful peptide screening technique that uses a designed DNA library to first *in vitro* biosynthesize billions of unique peptide/ribosome/mRNA complexes (PRMs) and then obtain the sequences of the target-binding peptide by reading their DNA coding sequence. However, the SMI cannot be directly biosynthesized into the peptides. To circumvent this problem, a noncanonical amino acid of (exoBCN)-L-lysine was first biosynthesized into the peptide (BCN-peptide).BCN refers to bicyclo[6.1.0]non-4-yne, which could occur the high speed and specific copper-free click-reaction with tetrazine. Therefore, SMI was modified with tetrazine (SMI-tetrazine) and SMI-peptide could be synthesize from the reaction between BCN-peptide and SMI-tetrazine. To demonstrate this SMI-peptide biosynthesis method could be applied for the synthesis of SMI-PRM for ribosome display, a demo selection was designed by mixing the BCN-PRM encoding sequence with a normal PRM encoding sequence and using a tetrazine modified fluorescein of FAM (FAM-tetrazine) as “SMI-tetrazine” to generate FAM-PRM. By observing the FAM-PRM coding sequence enriched in the target of an anti-FAM antibody, I confirmed that the click chemistry is compatible with ribosome display. Next, one of the PD-1/PD-L1 SMIs (BMS-1166) was chemically modified with the tetrazine group to give the BMS-1166-tetrazine and together with the random BCN-PRMs encoding DNA library to perform the real selection for the target of PD-L1. Low affinity wild type PD-1 and medium affinity 2-PD-1 were used to competitively pre-elute the low affinity and medium affinity PD-L1 binding BMS-1166-PRMs. DNA recovered from the high-affinity PD-1 eluted BMS-1166-PRMs was used for the next round of selection. After six recursive rounds of selection, two aliquots of BMS-1166-PRMs library were selected against either the true target of PD-L1 or a fake target of PD-1. Three peptides which were statistically significantly highly enriched in the true target of PD-L1 were selected as candidates. High-accuracy protein

modeling software of AlphaFold2 confirmed that all three peptides could form the complex with PD-L1 while a negative control peptide (44.4 % sequence similarity) could not. Besides, the calculated binding free energy of the predicted candidates/PD-L1 complex is lower than the known peptide ICI/PD-L1 complex. These *in silico* results together indicated the SMI-peptides will be the more promising non-antibody peptide ICIs.

In Chapter 4, the development of an SMI-conjugated polymer ICI was described. The SMI could bind to PD-L1 but has low bioactivity as well as cytotoxicity which is not suitable as a single inhibitor. As known as the multivalency effect, a compound with multiple target-binding units would have an enhanced binding ability than a compound that has a single target-binding unit. Therefore, a polymer with multiple surface SMI conjugation would become a more potent ICI with higher inhibitory ability.

To obtain a high surface SMI conjugation number, generation four of poly(amidoamine) dendrimer (PAMAM-G4) with sixty-four conjugatable surface amines was employed as the polymer for the conjugation with the carboxyl acid group-containing SMI, BMS-1166. After synthesizing and removing the free BMS-1166 via the desalting column, the successful synthesis of PAMAMG4-BMS1166 was confirmed by ^{1}H-NMR and UV-vis spectrophotometry. Mass spectrometry indicated that each PAMAMG4-BMS1166 conjugated an average number of fifteen BMS-1166 molecules. Owing to the small size of BMS-1166, the molecular weight of PAMAMG4-BMS1166 is still 7-times smaller than the antibody. PAMAM-BMS-1166 showed the inhibition effect with half maximal inhibitory concentration (IC_{50}) of around 2 nM, which is a comparable inhibitory effect as current antibodies. Although the inhibitory effect is similar to the free BMS-1166, PAMAMG4-BMS1166 has an advantage as a nanocarrier, which could encapsulate chemotherapy drugs for achieving better anti-tumor effects.

In Chapter 5, to sum up, this thesis used novel methods to develop the improved protein ICI, the SMI-peptide ICI, and the SMI-conjugated polymer ICI for achieving a better therapeutic effect. As the final results, the protein ICI of 2-PD-1 has only two mutations from the native PD-1 scaffold, which is likely to have lower immunogenicity for a better therapeutic effect than other protein ICI. SMI-peptide candidates were target-specifically selected and *in silico* modeling indicated they have a higher binding affinity than known peptide ICI. SMI-conjugated polymer ICI of PAMAMG4-BMS1166 showed an equivalent inhibitory effect as the antibody ICI or SMI while its therapeutic effect could be further enhanced by encapsulating the chemotherapy drug or increasing the conjugation number. Meanwhile, the novel development methods are general and could be used for developing inhibitors for other targets. For further prospects, the inhibitory effect of 2-PD-1 could be further enhanced by dimerization to become a bivalent inhibitor like the antibody. Besides, biosynthesis of SMI-peptide candidates will be performed for the experimental analysis of their activity and a cell experiment would be conducted for the evaluation of the bioactivity of PAMAMG4-BMS1166. Moreover, a flexible spacer could be added to PAMAMG4-BMS1166 to decrease the steric hindrance for a higher inhibitory effect.

List of research achievements for application of Doctor of Life Science, Waseda University

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academic paper	Eun-Hye Kim, Boyang Ning, Masuki Kawamoto, Hideyuki Miyatake, Eiry Kobatake, Yoshihiro Ito, and Jun Akimoto, "Conjugation of biphenyl groups with poly(ethylene glycol) to enhance inhibitory effects on the PD-1/PD-L1 immune checkpoint interaction", J. Mater. Chem. B, 8, 10162-10171 (2020)