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A small-sized protein binder specific for human PD-1 effectively suppresses the tumour growth in tumour mouse model

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ABSTRACT

Immune checkpoint inhibitors have drawn a consider attention as an effective cancer immunotherapy, and several monoclonal antibodies targeting the immune checkpoint receptors, such as human programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), are clinically used for treatment of various cancers. Here we present the development of a small-sized protein binder which specifically binds to hPD-1. The protein binder, which is composed of leucine-rich repeat (LRR) modules, was selected against hPD-1 through phage display, and its binding affinity was matured up to 17 nM by modular evolution approach. The protein binder was shown to be highly specific for hPD-1, effectively inhibiting the interaction between hPD-1 and its ligand, hPD-L1. The protein binder restored T-cell function in vitro, and exhibited a strong anti-tumour activity in tumour mouse model, indicating that it acts as an effective checkpoint blockade. Based on the results, the developed protein binder specific for hPD-1 is likely to find a potential use in cancer immunotherapy.

Introduction

Immunotherapy has drawn much attention as an effective way of treating various cancers by boosting patients’ own immune systems. Several types of immunotherapy have been used to treat cancers, and such treatments can help the body’s immune system fight cancer cells directly or stimulate the immune system in a more general way. Immune checkpoints, such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), have been considered promising targets for immunotherapy. PD-1 is an inhibitory receptor which is normally expressed on activated T-cells [1], and it has two ligands, namely programmed death 1-ligand 1 (PD-L1) (also known as B7-H1) and PD-L2 (also known as B7-DC). It has been known that binding of PD-1 to PD-L1 hampers T-cell proliferation and cytokine production through downregulation of ZAP70-mediated T-cell receptor (TCR) signalling pathways [2,3]. Importantly, cancer cells also express PD-L1 in tumour microenvironment to make T-cells “exhausted” as one of the mechanisms to avoid immune-surveillance in the body [4,5].

As an approach to stimulating the body’s immune system for cancer treatment by inhibition of the PD-1 and PD-L1 interaction, monoclonal antibodies targeting PD-1 have been developed, and nivolumab and pembrolizumab are clinically used [6–8]. Anti-PD-1 antibodies are known to have significant effects on patients with various cancers, such as metastatic melanoma, renal cell carcinoma (RCC) and non-small cell lung cancer (NSCLC) [9–11]. Recently, more attempts have been made to broaden their use in other cancer types such as glioblastoma and pancreatic cancer through combination therapy [12,13]. Despite widespread use of monoclonal antibodies as therapeutics, they have several drawbacks. Their large size (~150 kDa) and disulphide bonds are known to cause aggregation and low penetration into tumour site, especially in solid tumours, resulting in low efficacy [14–17]. Their long half-life mediated by FcRn recycling might trigger unexpected immune-related toxicities [18–21]. In an effort to overcome such shortcomings, peptides and peptidomimetics targeting immune checkpoints were developed [22–26]. Peptide-based inhibitors were shown to have an antitumor activity, but they have some limitations, including low binding affinity and susceptibility to hydrolysis. Variants of PD-1 with high binding affinity for its ligand were also attempted [27,28], but they were expressed as inclusion body in E.coli, requiring a refolding process for purification mainly due to their conserved immunoglobulin (Ig)-like domains. Thus, development of new immune checkpoint inhibitors is of great significance.

As alternatives to immunoglobulin antibodies, small-sized non-antibody protein scaffolds have attracted considerable attention [29,30]. We previously developed a small-sized (~30 kDa) non-antibody scaffold, termed ‘repebody’, which is composed of leucine-rich repeat (LRR) modules [31]. The potential of the repebody scaffold was demonstrated by showing its use as antitumor agents, targeted drug delivery vehicles, and diagnosis tools of disease biomarkers [32–35]. Here, we present the development of a repebody which is highly specific for human PD-1 (hPD-1) through phage display and modular evolution approach. The utility of the hPD-1-specific repebody as an immune checkpoint blockade was investigated in vitro and in vivo. Details are reported herein.
Materials and methods

Development of a repebody specific for hPD-1

A repebody library was constructed by mutagenic PCR using NNK degenerate codon as described elsewhere [31]. For selection of initial binders, three amino acids on LRRV2 (I91, T93, and G94) and three amino acids on LRRV3 (V115, V117, and E118) were randomised. Another four amino acids on LRRV4 (Y137, N139, A141, and H142) were randomly mutated to construct a second library for affinity maturation. The constructed library was inserted into a phagemid pBEL118M vector, and transformed into E. coli TG-1 strain by electroporation for a phage display. Repedebodies specific for human PD-1 were selected by phage display and soluble biopanning against extracellular domain (ECD) of hPD-1 using Dynabeads® M-280 Streptavidin magnetic beads (Invitrogen, Carlsbad, CA). ECD of biotinylated hPD-1 protein (Acro biosystems, Newark, DE) was immobilised onto Dynabeads and blocked with a blocking buffer (1X PBS, 0.05% Tween-20, and 1% BSA). To exclude phages which non-specifically bind to the surface of magnetic beads, 1 ml of phage solution containing 1.0 × 10^{12} cfu/ml was added to beads without hPD-1. After 1 h incubation at room temperature, phages were moved to hPD-1-immobilised beads for positive selection. Following incubation for 1 h at room temperature, unbound phages were washed out, and bound phages were eluted using 0.2 M Glycine-HCl (pH 2.2) and neutralised with 1 M Tris-HCl (pH 9.0). Eluted phages were infected to TG-1 (OD_{600} = 0.5) for generation of phage solution for the next round of biopanning. This process was repeated five times for selection of initial binders and three times for affinity maturation, respectively. After enrichment of phages, repedebodies specifically binding to hPD-1 were identified through phage ELISA.

Protein expression, purification and removal of LPS

Six histidine residues were fused to C-terminus of a repebody, cloned into pET21a vector, and transformed to E. coli strain Origami B. The cells were cultured in LB medium followed by addition of 0.5 mM IPTG when OD_{600} reached 0.5. A repebody was purified by affinity chromatography using Ni-NTA resin (Qiagen, Germany). Lipopolysaccharide (LPS) was removed using Triton X-114 (Sigma-Aldrich, St. Louis, MO), as described elsewhere [36,37]. Briefly, protein solution was treated with 1–2% Triton X-114 followed by incubation for 30 min at 4 °C with gentle shaking, and the mixture was further incubated at 37 °C for 10 min. The aqueous phase was separated by centrifugation at 13,000rpm. After repeating this step four times, residual Triton X-114 in protein solution was removed by Bio-Beads SM-2 (Bio-Rad, Hercules, CA).

Inhibition assay of the PD-1 and PD-L1 interaction

Inhibition of the interaction between PD-1 and PD-L1 by a repebody was assayed using competitive ELISA. Each well of a 96-well plate was coated with 1 μg/ml of extracellular domain (ECD) of hPD-1 (Sino Biological, China) by overnight incubation at 4 °C. After blocking with 250 μl of a blocking buffer (1X PBS, 0.05% Tween 20, 1% BSA), 100 μl of a working solution (0.6 μg/ml of ECD of hPD-L1 (Mouse Fc-tagged, Sino Biological) with different repebody concentrations) was added. Following incubation for 1 h at room temperature, each well was washed five times with PBST buffer (1X PBS, 0.05% Tween 20), HRP-conjugated goat-anti-mIgG antibodies (Bio-Rad) were added and incubated for 1 h at room temperature to detect remaining hPD-L1, followed by addition of 100 μl of TMB to each well. The reaction was stopped with 100 μl of 1 N H_{2}SO_{4}, and absorbance at 450 nm was measured. Competitive ELISA between PD-L1 and a repebody or nivolumab was carried out using the same procedure as described above. 1 μg/ml of hPD-1 was coated on 96-well plate. 100 μl of working solution containing 0.6 μg/ml of ECD of hPD-L1 (Biotinylated (Acrobiosystems)) and 200 nM of repebody or nivolumab (Anti-hPD1-Ni-hlgG4 (S228P), InvivoGen, San Diego, CA) was treated. Signals from hPD-L1 was detected by HRP-conjugated streptavidin (Biolegend, San Diego, CA). For competitive ELISA between a repebody and nivolumab, a 96-well plate coated with 2 μg/ml of hPD-1 was blocked by a blocking buffer followed by addition of 60 nM of a myc-tagged repebody with nivolumab at different molar ratios. Remaining repebody was detected by anti-c-myc-HRP antibody (Santa Cruz Biotechnology, Dallas, TX).

Flow cytometry

CHO-K1 cells expressing hPD-1 were constructed by transient transfection. Briefly, 3 μg of hPD-1/pCMV3 (Sino Biological) was introduced into 1 × 10^6 CHO-K1 cells using lipofectamine transfection reagent (Invitrogen). After incubation for 48 h post transfection, CHO-K1 cells were harvested and resuspended in 1 ml 1% BSA, DPBS. 200 μl of cell suspension (~1 × 10^6 cells) were treated with 10 μg/ml of a myc-tagged repebody for 30 min on ice, washed three times and stained with goat-anti-myc-FITC antibody (Abcam, UK). After incubation for 30 min on ice, cells were washed and analysed by LSR Fortessa (BD Biosciences, San Diego, CA) and FACSDiva software (BD Biosciences).

In vitro bioassay for blockade of PD-1 and PD-L1 interaction

Blockade of the interaction between PD-1 and PL-L1 in vitro was assayed using the commercial kit (PD-1/PD-L1 Blockade Bioassay kit, Promega, Fitchburg, WI). PD-1 effector cells (hPD-1-expressing jurkat T-cells) were manipulated to express luciferase by an NFAT response element (NFAT-RE) through TCR activation. PD-1 effector cells and PD-L1-expressing aAPC/CHO-K1 cells were co-cultured in the presence of a repebody or nivolumab. After incubation, luminescence was measured using a luminescence plate reader. EC_{50} values were determined using GraphPad Prism software.

Analysis of cytokine production

Human PBMCs were isolated by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare, Chicago, IL). CD4^+ T-cells were isolated from PBMCs using Dynabeads CD4 positive isolation kit (Invitrogen). Monocytes were isolated from PBMCs using Monocyte isolation kit II (Miltenyi Biotec, Germany), and used for generating dendritic cells (DCs). Briefly, isolated monocytes were cultured in RPMI-1640 medium containing 10% FBS, 5 ng/ml GM-CSF (R&D Systems, Minneapolis, MN), and 20 ng/ml IL-4 (R&D Systems), and the medium was refreshed every 2 days. At day 6, the medium was refreshed with RPMI-1640 containing 10% FBS, 5 ng/ml GM-CSF, 20 ng/ml IL-4, and 10 ng/ml TNF-α (R&D Systems), followed by incubation for 24 h and harvest. For assay of a mixed lymphocyte reaction (MLR), CD4^+ T-cells (1 × 10^5) were co-cultured with allogeneic DCs (1 × 10^5) in a 96-well cell culture plate in the presence of off-target repebody, anti-PD-1 repebody, or nivolumab. After 5 days of incubation, the supernatant was collected to determine the levels of IL-2 and...
IFN-γ by ELISA kit (BD OptEIA™ Set for human IL-2 and human IFN-γ, BD Biosciences) according to the manufacturer’s protocols.

In vivo experiments

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC, Korea) and carried out according to the guidelines described by the committee.

The pharmacokinetic profile of the repebody was analysed using male balb/c mice with 4–5 weeks of age (Joong Ah Bio, Korea). Mice were intravenously administered with the repebody (r_G9) (10 mg/kg, 100 μL), and blood samples were obtained at time intervals after single dosing. Serum concentrations of the repebody were determined by sandwich ELISA. Briefly, 10 μg/mL of polyclonal anti-repebody antibody were coated on a 96-well ELISA plate by overnight incubation at 4 °C. Each well was blocked by SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific, MA). The body weight and tumour volumes were monitored every 3 or 4 days from a week post implantation.

Tumour volumes were measured using calliper and calculated with 100 μL of anti-PD-1 repebody or off-target repebody was treated to detect the levels of the repebody in serum. Signals from HRP were developed by TMB, and the reaction was stopped by 1% SDS. Absorbance measured at 450 nm was converted to percent for relative comparison of serum concentration of the repebody. The initial and terminal half-lives of the repebody were determined using GraphPad Prism software.

For generation of the xenograft mouse model, immune deficient NOD/ShiLtJ-Rag2<sup><sppem1AmC</sup> I2g<sup><sppem1AmC</sup> (NRGA) mice (6–8 weeks of age, female) were purchased from Joong Ah Bio. NCI-H292 tumour cells (2 × 10<sup>6</sup> in 25 μL) and freshly isolated human PBMC (5 × 10<sup>5</sup> in 25 μL) were mixed with 50 μL of BD Matrigel Matrix (BD Biosciences), and subcutaneously implanted into the flanks of NRGA mice (8 mice per each group). After inoculation of tumour cells, 100 μL of anti-PD-1 repebody or off-target repebody was intraperitoneally (IP) administered at day 1, 3, 7, and 10. DPBS was also used as a negative control. Dose of a repebody was 10 mg/kg per each injection. The body weight and tumour volumes were monitored every 3 or 4 days from a week post implantation. Tumour volumes were measured using calliper and calculated according to the formula: V = (W<sup>2</sup> × L)/2 (mm<sup>3</sup>) (V is tumour volume, W is tumour width, and L is tumour length). Mice were monitored for a month and sacrificed at day 31 for extirpation of tumours. Statistical analysis was performed by one-way ANOVA using GraphPad Prism software.

Results

Development of a repebody specific for hPD-1

To develop a repebody that specifically binds to hPD-1, we constructed a repebody library by randomising six variable sites on two nearby modules (LRRV2 and LRRV3 modules) using NNK degenerate codon followed by phage display selection against ECD of hPD-1 (Figure 1(A)). After five rounds of soluble biopanning, a repebody clone (r_A1) with the highest binding affinity was selected. The selected repebody was shown to have three mutations on LRRV2 (I91N, T93W, and G94E) and additional three mutations on LRRV3 (V115T, V117F, and E118L) (Figure 1(B)). The binding affinity (K<sub>D</sub>) of r_A1 for hPD-1 was estimated to be 617 nM through isothermal titration calorimetry (ITC) (Supplementary Figure S1A). Although hPD-1 is known to have a low binding affinity for hPD-L1 (K<sub>D</sub> = 8.2 μM) [38], a repebody with higher affinity for hPD-1 is expected to be more effective for inhibiting the interaction between PD-1 and its ligand, PD-L1. To increase the binding affinity of r_A1, random mutations were further introduced into additional four variable sites on LRRV4 module of r_A1 (Figure 1(A)), followed by phage display selection and three rounds of soluble biopanning. As a result, r_G9 with two additional mutations (A141K and H142F) was finally selected (Figure 1(B)). The binding affinity of r_G9 for hPD-1 was determined to be about 17.6 nM, showing a 35-fold increase compared to the initially selected repebody (Supplementary Figure S1B). It is known that hPD-1 has four N-glycosylation sites on ECD (49 N, 58 N, 74 N, and 116 N). We thus checked whether glycosylation of hPD-1 affects its binding to r_G9. To this end, we measured a binding affinity of r_G9 for ECD of hPD-1 expressed by E. coli. The K<sub>D</sub> value of r_G9 for non-glycosylated ECD of hPD-1 was estimated to be 28.9 nM (Supplementary Figure S1C), and this implies a negligible effect of glycosylation on its binding to r_G9.

We next investigated a binding specificity of r_G9, and observed that it had high specificity for hPD-1 (Figure 1(C)). Even though hPD-1 has the sequence identity as high as 64% with mouse PD-1 (mPD-1), r_G9 showed a negligible cross-reactivity against mPD-1 as well as human and mouse PD-L1. We checked if the repebody binds to a serum albumin, because its binding to a serum albumin could affect its half-life and distribution [39]. The repebody was shown to have a negligible binding activity for serum albumins from mouse, rat, rabbit, and human (Supplementary Figure S2), verifying high specificity for hPD-1.

Inhibitory effect on the PD-1 and PD-L1 interaction

To check a potential use of r_G9 as an immune checkpoint inhibitor, we tested its inhibitory effect on the interaction between hPD1 and hPD-L1 through competitive ELISA. As a result, r_G9 was shown to effectively block the interaction between hPD-1 and hPD-L1 (Figure 2(A)). In contrast, inhibition by initially selected repebody (r_A1) was much lower than r_G9, and this seems to be due its lower binding affinity for hPD-1. Off-target repebody (r_Off) had a negligible inhibitory effect on the interaction between hPD-1 and hPD-L1 (Figure 2(A)). We tested the effect of the r_G9 concentration, and observed a dose-dependent inhibition on the binding of hPD-1 to hPD-L1, whereas off-target repebody had a marginal effect (Figure 2(B)). Interestingly, r_G9 was shown to exhibit a comparable inhibitory effect to anti-PD-1 antibody, nivolumab (Figure 2(C)). These results indicate that r_G9 specific for hPD-1 has a potential as an immune checkpoint inhibitor. We further attempted to get insight into a binding region of r_G9 by comparing with nivolumab of which crystal structure in complex with hPD-1 is available [40]. When nivolumab was added to a mixture of hPD-1 and r_G9 at the increasing molar ratio, the binding of r_G9 to hPD-1 decreased, but became saturated even though the molar ratio was above 1 (Figure 2(D)). This seems to be due to the fact that binding affinity of nivolumab (~3 nM) for hPD-1 is comparable to r_G9 [41]. Based on the result, it is likely that r_G9 shares the epitope with nivolumab.

Restoration of T-cell function

We tested if the hPD-1-specific repebody can restore T-cell function. For this, we first examined if r_G9 binds to native hPD-1 on the cell surface using hPD-1-expressing CHO-K1 cells (Supplementary Figure S3). FACS analysis revealed a shifted peak only when r_G9 was treated with hPD-1-expressing CHO-K1 cells (Figure 3(A)). It is thus likely that r_G9 specifically binds to native
hPD-1 expressed on the cell surface as well as ECD domain of hPD-1 in solution. Inhibition assay for the interaction between PD-1 and PD-L1 was carried out to assess the biological activity of r_G9 in vitro. Human PD-1-expressing jurkat T-cells were co-cultured with aAPC/CHO-K1 cells expressing PD-L1 followed by addition of r_G9, and expression of luciferase which is driven by activation of TCR signalling pathway was measured. As a result, TCR-mediated luminescence signal was shown to increase with

Figure 1. Construction of a library and selection of anti-PD-1 repebodies through phage display. (A) Randomised sites for a library construction are shown. Six variable sites on LRRV2 and LRRV3 modules were mutated for screening of initial binders (yellow color). Additional four variable sites on LRRV4 module (colored in purple) were mutated for affinity maturation. (B) Amino acid sequences of selected repebodies from the first round of biopanning (r_A1) and the second round of affinity maturation (r_G9). (C) Binding specificity of r_G9 was tested by ELISA. Each ECD of hPD-1, hPD-L1, mPD-1, and mPD-L1 was coated on a 96-well plate. Binding of myc-tagged r_G9 was detected using anti-c-myc-HRP antibody. BSA and Off-target repebody (r_Off) were used as negative control. Data represent the mean ± standard deviation (n = 3).
the increasing concentration of \( r_{G9} \), and EC\(_{50} \) of \( r_{G9} \) was estimated to be 0.84 \( \mu \)g/mL, which is comparable to nivolumab (EC\(_{50} \approx 0.32 \mu \)g/mL) (Figure 3(B)). This result indicates that \( r_{G9} \) effectively inhibited the interaction between PD-1 and PD-L1 in vitro, leading to activation of TCR signalling pathway.

We next performed a mixed lymphocyte reaction (MLR) to check if \( r_{G9} \) could restore T-cell function. PD-1 blockades have been known to result in an increase in cytokine secretion through T-cell activation [42]. T-cells isolated from human PBMCs were co-cultured with allogeneic DCs, and a repebody was treated for 5 days, and the levels of IL-2 and IFN-\( \gamma \) secreted from T-cells were measured. As expected, \( r_{G9} \) was observed to effectively enhance the secretion of IL-2 and IFN-\( \gamma \) in a dose-dependent manner, whereas off-target repebody had a negligible effect (Figure 4(A,B)). Interestingly, \( r_{G9} \) exhibited a comparable effect on the secretion of cytokines by T-cells to nivolumab (Figure 4(C,D)). As for the secretion levels of IL-2 and IFN-\( \gamma \), no significant difference was observed between \( r_{G9} \) and nivolumab (\( p \) values of 0.88 and 0.48, respectively). The results indicate that \( r_{G9} \) binds to hPD-1 and inhibits PD-1 signalling pathway, resulting in activation of TCR signalling.

**Pharmacokinetics and in vivo anti-tumour activity**

We analysed the pharmacokinetic profile of the repebody in mice to assess its half-life. To this end, \( r_{G9} \) (10mg/kg) was intravenously administered into 4–5-week old male balb/c mice. Blood samples were collected over time, and the levels of the blood-circulating repebody were determined (Supplementary Figure S4). The initial and terminal serum half-lives were estimated to be 0.2 and 4.5 h, respectively. The repebody with a small size (~30 kDa) seems to have a short half-life due to fast renal clearance compared to immunoglobulin antibodies [43].

We next evaluated anti-tumour activity of the repebody in tumour mouse model. NCI-H292 tumour cells were co-implanted with human PBMCs into the right flank of immune deficient NRGA mice, and tumour growth was traced. Compared to the controls using DPBS and off-target repebody, \( r_{G9} \) was shown to significantly suppress the tumour growth (\( p < 0.001 \)) (Figure 5(A)). When \( r_{G9} \) was administered four times over two weeks after tumour implantation, tumour progression was significantly blocked until 31 days. Average tumour volumes of mice treated with \( r_{G9} \) were maintained within the range of 97 mm\(^3\) to 106 mm\(^3\), whereas tumours were grown to 501 mm\(^3\) and 582 mm\(^3\) for DPBS- and off-target repebody-treated mice, respectively. Representative images of tumours from sacrificed mice on day 31 also confirmed a strong anti-tumour activity of \( r_{G9} \) (Figure 5(B)). This result indicates that \( r_{G9} \) blocked effectively the interaction between PD-1 and PD-L1, thus significantly suppressing the tumour growth. Based on the results, it is likely that \( r_{G9} \) can act as an efficacious immune checkpoint inhibitor.

**Discussion**

We demonstrated an effective anti-tumour activity of a small-sized repebody specific for hPD-1 as an immune checkpoint blockade.
The repebody, r\textsubscript{G9}, which has a binding affinity of 17.6 nM for hPD-1, showed high specificity for hPD-1. The repebody was shown to bind to hPD-1 about 470-times stronger than hPD-L1 (KD of hPD-L1 = 8.2 \mu M) [38], effectively inhibiting the interaction between hPD-1 and its ligand PD-L1. The repebody was predicted to share the epitope with clinically used monoclonal antibody, nivolumab. As an effective antagonist of PD-L1, the repebody restored the TCR signalling pathway, stimulating cytokine secretion.

In vivo study using tumour mouse model showed that the repebody has a strong anti-tumour activity, significantly suppressing the tumour growth. These results strongly support that the repebody specific for hPD-1 can have a potential use for cancer immunotherapy.

Cancer immunotherapy with immune checkpoint inhibitors has shown notable successes, and several monoclonal antibodies, including ipilimumab (anti-CTLA-4 antibody), nivolumab and pembrolizumab (anti-PD-1 antibody), have been approved by FDA, and are clinically used. However, monoclonal antibodies have several disadvantages, including aggregation and poor tumour penetration due to a large size, difficulty with engineering, high production cost and immune-related adverse effect [18,44,45]. As an alternative to immunoglobulin antibodies, the repebody scaffold was shown to offer some favourable properties: overproduction by bacterial expression system, easy and simple affinity maturation, easy engineering and design, high tissue penetration [31,46,47]. In this study, the hPD-1-specific repebody (r\textsubscript{G9}) with high binding affinity was successfully developed through phage display and modular evolution approach. Its high binding affinity can be a distinct advantage over other small molecules or peptides showing weak binding affinity for hPD-1. High specificity of the repebody seems to stem from intrinsic property of the LRR proteins with \( \beta \)-strands at the concave region. Considering the mode of action of immune checkpoint inhibitors, high tissue penetration of the repebody due to small size (\( \sim 30 \) kDa) would be another distinct property in their applications to solid tumours compared to immunoglobulin antibodies [48,49]. Although engineered high affinity PD-1 or PD-L1 variants with a similar molecular size to the repebody were attempted, they were expressed as inclusion body in \( E. coli \) or obtained from insect cell expression system [27,28].

In recent, combination therapy has attracting much attention because of an increased therapeutic efficacy. Anti-PD-1 repebody might be used with conventional immune checkpoint antibodies (e.g. anti-CTLA-4 antibody, ipilimumab) since dual blockade of CTLA-4 and PD-1 is known to be most effective combination in terms of therapeutic efficacy [50]. It is also worth a combination with other repebodies targeting various pathways related to cancers. As an example, VEGF-targeting repebody [51], which was
previously developed, might be a candidate for combination therapy because combinatorial inhibition of the VEGF and PD-1 pathways exhibited a synergistic effect in advanced RCC [52,53]. At the same time, anti-PD-1 repebody can be employed for in vivo imaging as companion diagnostics to check the expression of PD-1 on the cells [35,54]. In conclusion, anti-PD-1 repebody is likely to find a potential use as an immune checkpoint inhibitor in cancer immunotherapy.

Disclosure statement
No potential conflict of interest was reported by the authors.

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