Cancer Immunotherapy

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Blocking of the PD-1/PD-L1 Interaction by a D-Peptide Antagonist for Cancer Immunotherapy

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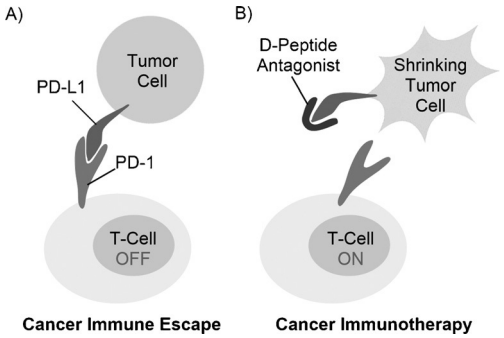
Abstract: *Blockade of the protein–protein interaction between the transmembrane protein programmed cell death protein 1 (PD-1) and its ligand PD-L1 has emerged as a promising immunotherapy for treating cancers. Using the technology of mirror-image phage display, we developed the first hydrolysis-resistant* D*-peptide antagonists to target the PD-1/PD-L1 pathway. The optimized compound* ^{D}*PPA-1 could bind PD-L1 at an affinity of 0.51* μM *in vitro. A blockade assay at the cellular level and tumor-bearing mice experiments indicated that* ^{D}*PPA-1 could also effectively disrupt the PD-1/PD-L1 interaction in vivo. Thus* D*-peptide antagonists may provide novel low-molecular-weight drug candidates for cancer immunotherapy.*

The amplitude and quality of the immune response of T cells is controlled by equilibrium between co-stimulatory and inhibitory signals, called immune checkpoints.[1] Tumor cells often overexpress immune checkpoint proteins to allow them to evade the host immune system by inhibiting T-cell attack.[2] The blockade of immune checkpoints by exogenously added antagonists may disrupt the immune-suppressing pathway and unleash and enhance pre-existing anti-cancer immune responses of T cells to destruct cancer cells. Preclinical and clinical data have shown that antibody blockade of immune checkpoints can indeed significantly enhance antitumor immunity.[3] This strategy is currently among the most promising approaches to activate therapeutic antitumor immunity for cancer immunotherapy.[4] However, antibody drugs have some problems associated with the production cost and immunogenicity. Therefore, alternative low-molecular-weight immune checkpoint antagonists also need to be investigated.

Compared to therapeutic antibodies, synthetic peptides have several advantages as drug candidates, including lower manufacturing costs, higher stability, reduced immunogenicity, and better organ or tumor penetration.[5] An array of therapeutic peptides have been successfully developed to treat diverse pathologies, but peptides targeting immune checkpoints remain to be investigated. Herein we wish to report our attempt to develop the first hydrolysis-resistant peptide inhibitor of immune checkpoint proteins (Scheme 1).



Scheme 1. A) The PD-1/PD-L1 interaction mediates cancer immune escape. B) D-Peptide antagonists targeting PD-L1 can inhibit the PD-1/PD-L1 interaction for cancer immunotherapy.

We selected programmed death ligand 1 (PD-L1) as our target for two reasons. First, PD-L1 is highly up-regulated on many types of tumor cells, such as melanoma and ovarian and lung cancers.[6] Second, multiple PD-L1 antibodies in different stages of clinical trials have been hypothesized to be accompanied by less immune-related toxicity, in part by regulating the immune response selectively in the tumor microenvironment.[4c,7,8]

Our initial plan to develop peptide inhibitors targeting PD-L1 was based on the technology of phage display.[9] The ectodomain of natural PD-L1 was expressed by *E. coli* and used as the bait for screening by a duodecimal peptide library displayed on M13 phage. After five rounds of biopanning, the highest frequency clone was obtained and characterized as ^{L}P1 (FPNWSLRPMNQM). However, when injected intraperitoneally in tumor-bearing mice models, ^{L}P1 failed to show any activity, possibly because of its rapid degradation by proteolytic enzymes in the blood plasma (Supporting Infor-

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mation). To test this hypothesis, a proteolytic stability experiment with ^{L}P1 in 10% human serum was conducted. Reverse-phase HPLC (RP-HPLC) was used to monitor time-dependent peptide hydrolysis. Our measurement showed that ^{L}P1 was completely digested within 8 h. The half-life of ^{L}P1 in 10% human serum was less than 90 min (Supporting Information).

To overcome the stability problem of the L-peptide inhibitors, we turned our attention to developing an enzymolysis-resistant D-peptide inhibitor of PD-L1. Previous studies have indicated that D-peptides or D-proteins composed of D-amino acids are potential therapeutic agents with long half-lives in vivo and even oral bioavailability.[10] To find D-peptides that can bind to a target protein of interest, the method of choice is mirror-image phage display.[10a,11] In this technology, the mirror image (a D-protein) of the natural L-target protein is made by chemical synthesis. This D-protein is used to screen for binding of L-peptides displayed on phages. D-Versions of the selected L-peptides are then synthesized with D-amino acids. By symmetry, these D-peptides should bind to the natural L-target.[12]

To implement mirror-image phage display, we analyzed the PD-L1 ectodomain which contains an immunoglobulin-like variable (IgV) domain and an immunoglobulin-like constant (IgC) domain, typical of the B7 family. The crystal structure of PD-L1 in complex with its receptor, programmed cell death protein 1 (PD-1), indicates that they interact through their IgV domains.[13] As a result we chose the D-version of the IgV domain of PD-L1 (designated as ^{D}IgV$^{PD\text{-}L1}$) as our synthetic target. ^{D}IgV$^{PD\text{-}L1}$ contains two antiparallel β sheets linked by an intersheet disulfide bond.

To synthesize ^{D}IgV$^{PD\text{-}L1}$ containing 124 D-amino acids, we divided it into four peptide segments with Ala^{35}, Ala^{68}, and Ala^{107} temporarily mutated to Cys (Figure 1A). Peptide segments **1**–**4** were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis. An azide group was used to protect the N-terminal Cys of segment **3** to avoid self-cyclization and oligomerization. After the ligation was finished, the azide was reduced to NH_2 by adding tris(2-carboxyethyl)phosphine (TCEP).[14] An Arg8 tag was added to the C terminal of segment **4** to improve the solubility of the final protein product. After three steps of native chemical ligations,[15,16] desulfurization[17] and deprotection of the acetamidomethyl (Acm) group[18] were conducted, furnishing the full-length peptide **8** in milligram scale with an overall yield of 13% for the isolated product (Figure 1C). Gradient dialysis against decreasing concentrations of urea was conducted to give the folded ^{D}IgV$^{PD\text{-}L1}$ **9**.

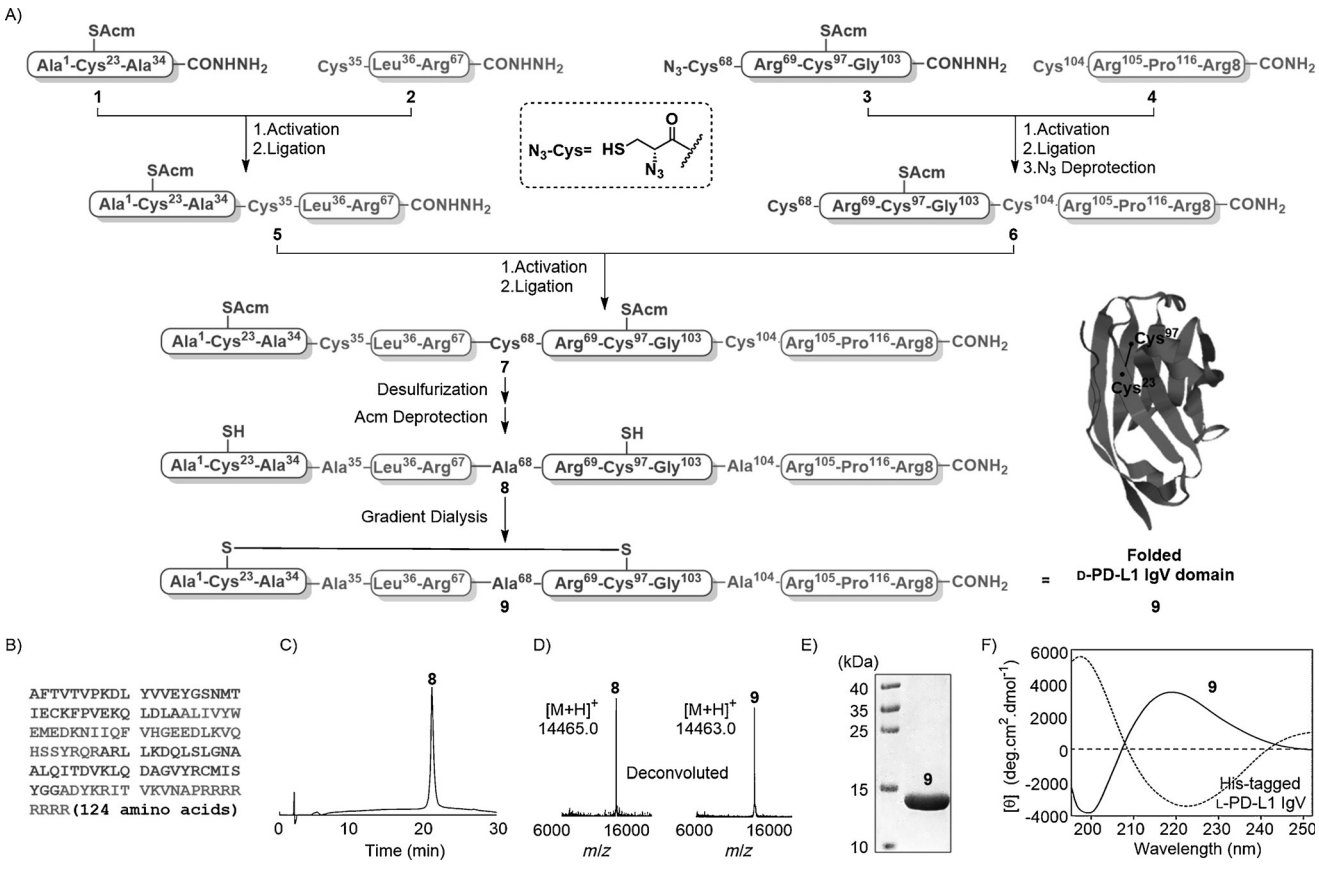

Figure 1. Chemical synthesis of ^{D}IgV$^{PD\text{-}L1}$ (**9**). A) Synthetic route. The structure of **9** was drawn as a mirror image of the literature structure (PDB code: 3BIS). B) The amino acid sequence of the human PD-L1 IgV domain with a C-terminal Arg8 tag. C) Analytical HPLC chromatogram ($\lambda = 214$ nm) of **8**. D) Deconvoluted electrospray ionization mass spectra of **8** and **9**. For **8**, detected mass = 14464.0 Da (calculated mass = 14463.8 Da, average isotopes). For **9**, detected mass = 14462.0 Da (calculated mass = 14461.8 Da, average isotopes). E) SDS-PAGE of **9**. F) Circular dichroism spectra of **9** and *E.coli*-expressed His-tagged L-PD-L1 IgV.

Electrospray ionization (Figure 1D) and MALDI-TOF (Supporting Information) analysis of **9** showed an exact 2 Da decrease from **8**, consistent with the formation of a disulfide bond between the two β sheets of the IgV domain. SDS-PAGE analysis of **9** also confirmed its purity and molecular weight (Figure 1E). The circular dichroism (CD) spectrum of **9** gave a positive band at $\lambda = 218$ nm and a negative band at 198 nm (Figure 1F). This CD spectrum was typical for anti-parallel D-configured β sheets and was of opposite sign to that measured for the His-tagged L-configured PD-L1 IgV domain expressed in *E. coli* (Figure 1F).[19]

Using **9** as bait, we screened a duodecimal peptide library displayed on M13 phage. Table 1 shows the amino acid sequences from seventeen binding clones obtained after five rounds of selection. D-peptide versions (^{D}PPA-1 and ^{D}PPA-2) of the two sequences with the highest frequency were synthesized for further experiments. Unlike ^{L}P1 which was quickly digested in 10% human serum, ^{D}PPA-1 and ^{D}PPA-2 were highly resistant to proteolysis. No degradation of these two D-peptides was observed after 24 h in 10% human serum (Supporting Information).

Table 1: Amino acid sequences from 17 PD-L1-binding phage clones and the corresponding K_D values.[a]

Name	Sequence	Frequency	K_D [μM]
^{D}PPA-1	NYSKPTDRQYHF	7	0.51
^{D}PPA-2	KHAHHTHNLRLP	7	1.13
^{D}PPA-3	AAKMDGHLHGGQ	1	NB
^{D}PPA-4	MRNRERYPKPYY	1	22.0
^{D}PPA-5	TLYQRPSTNLER	1	NB
^{D}PPA-1*	RHTNDYSQFYPK	–	NB

[a] Sequences obtained after five rounds of biopanning selection. K_D values measured by SPR. NB = No binding. ^{D}PPA = D PD-1/PD-L1 interaction antagonist. ^{D}PPA-1* was a scrambled peptide as negative control.

To test the activity of all the selected peptides, surface plasmon resonance (SPR) spectroscopy (Biacore T200, at 25 °C) was used to measure their binding affinities to the extracellular domain of human PD-L1 (hPD-L1). Recombinant hPD-L1 ectodomain was immobilized onto the gold surface of a CM5 sensor chip by direct amine coupling. To confirm that the immobilized hPD-L1 ectodomain was functional, we first checked its affinity to the recombinant human PD-1 (hPD-1) ectodomain. Our measurement showed that the binding of hPD-L1 to hPD-1 had a K_D value of 0.18 μM and 0.16 μM when PD-1 ectodomain was immobilized onto a CM5 chip, comparable to previous experimental data ranging from 0.11 μM to circa 8 μM.[13,20] Next, the K_D values of ^{D}PPA-1 and ^{D}PPA-2 for the binding to hPD-L1 were measured to be 0.51 and 1.13 μM. The low frequency peptides showed much worse binding affinity or even no binding at all. A scrambled peptide ^{D}PPA-1* was also synthesized as a negative control and it showed no binding. Not surprisingly, our measurements showed that neither ^{D}PPA-1 nor ^{D}PPA-2 bound to immobilized hPD-1 (Supporting Information).

To test that the D-peptides could block the PD-1/PD-L1 interaction, we conducted competition SPR experiments. Taking ^{D}PPA-2 as an example, 50 nM of the hPD-L1 ectodomain was incubated with increasing concentrations of ^{D}PPA-2 before SPR analysis on a hPD-1 immobilized CM5 chip. As shown by the response unit (RU) values, an increasing concentration of ^{D}PPA-2 led to a decreasing SPR signal (Supporting Information). This observation indicated that ^{D}PPA-2 effectively inhibited the binding of hPD-L1 to immobilized hPD-1. Furthermore, microscale thermophoresis (MST) was also used to measure the binding affinity of PD-1 (0.054 μM), ^{D}PPA-1 (0.99 μM), and ^{D}PPA-2 (21 μM) to hPD-L1 (Supporting Information).[21] These data are close to the values measured by the SPR method.

To test whether ^{D}PPA-1 and ^{D}PPA-2 could block PD-1/PD-L1 interaction at the cellular level, we conducted flow cytometry experiments (Figure 2).[22] hPD-1-EGFP plasmid

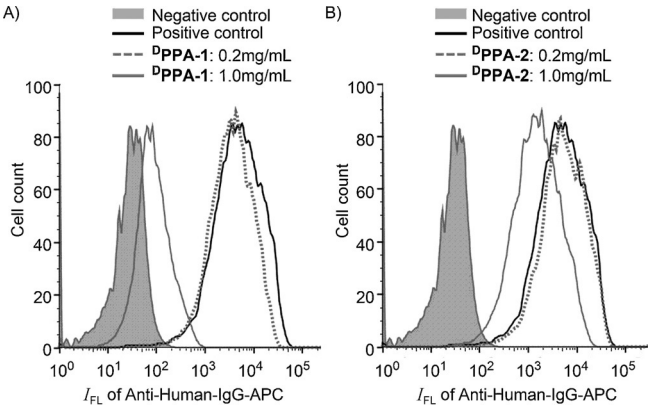

Figure 2. Flow cytometry analysis to investigate the PD-1/PD-L1 interaction using different concentrations of A) ^{D}PPA-1 or B) ^{D}PPA-2. I_{fl} = fluorescence intensity.

was transfected and expressed in the Plat-E cell line by X-treme GENE HP reagent. A commercial Fc-labeled human PD-L1 recombinant protein (where Fc is the tail fragment of the antibody) was incubated with these cells, with or without the D-peptides at different concentrations (0.2 or 1.0 mg mL^{-1}). After the cells were washed twice by the phosphate buffer containing 10% fetal bovine serum, APC-labeled anti-Fc antibody (APC = allophycocyanin) was added to stain the remaining PD-L1 bound to PD-1 on the cell surfaces. As shown in Figure 2A, in the negative control no hPD-L1-Fc was added into suspended cells expressing hPD-1 while the positive control had. After stained by APC-anti-Fc antibody, the average fluorescence intensity of the positive control showed a signal three orders of magnitude stronger than the negative one, indicating that the staining was specific, as no staining was observed without PD-L1-Fc (Figure 2). When added at a low concentration (0.2 mg mL^{-1}), ^{D}PPA-1 showed a weak inhibitory effect (Figure 2A) whereas ^{D}PPA-2 showed none (Figure 2B). When the concentration was increased to 1.0 mg mL^{-1}, significant inhibition of the PD-L1/PD-1 interaction by ^{D}PPA-1 was detected (Figure 2A) whereas ^{D}PPA-2 showed a weaker inhibition (Figure 2B), in agreement with the SPR and MST data that ^{D}PPA-1 was more active than ^{D}PPA-2. Furthermore, during the flow cytometric

analysis, toxicity was not observed on the tested cells at inhibitory concentrations up to 1 mg mL^{-1}, indicating a high therapeutic index of the D-peptides.

To examine the therapeutic efficacy of the D-peptides in vivo, ^{D}PPA-1 was used to conduct the tumor growth and survival experiments in mice. 36 Balb/c mice were injected with 5×10^5 CT26 cells subcutaneously in the right flank. After nine days, when the tumor volume reached 50 to 100 mm^3, the mice were divided randomly into six groups each containing six mice. ^{D}PPA-1 (2 mg kg^{-1}) was injected in two different manners, subcutaneously around the tumor once every day (Figure 3A) or intraperitoneally once every day for seven days (Figure 3B). Normal saline and 5-fluorouracil (5-Fu) at 10 mg kg^{-1} were injected separately as negative and positive controls. The tumor diameter was measured every day with a caliper, and the volume was calculated by using the formula, $\pi/6\times a\times b^2$. As shown in Figure 3, 5-Fu injection was effective as a positive control, but it caused significant bodyweight loss because of its toxicity (Supporting Information). On the other hand, in both peritumoral and intraperitoneal injection models, ^{D}PPA-1 significantly inhibited the growth of implanted CT26 in Balb/c mice (with $P<0.05$ and $P<0.01$ compared to the negative control, respectively), and no sign of bodyweight loss was detected.

In the survival experiments, three groups of CT26-bearing mice were injected intraperitoneally with saline, ^{D}PPA-1 at 2 mg kg^{-1}, or 5-Fu at 10 mg kg^{-1} once every day for twelve days (Figure 3C). From the thirteenth day, no further injection of saline, ^{D}PPA-1, or 5-Fu was given because mice injected with 5-Fu became very sick because of drug toxicity. The survival of mice in each group was checked every day until all the mice were dead. The results indicated that ^{D}PPA-1 treatment could prolong the survival time of CT26 tumor bearing mice to nearly 50% longer. The average survival time of saline-injected mice was 27 days (17 to 36 days), and that of the mice treated with ^{D}PPA-1 was prolonged to 44 days (36 to 52 days), similar to 5-Fu treated mice. These results indicated that ^{D}PPA-1 could be a promising drug candidate for cancer immunotherapy.

To evaluate the tumor-targeting and delivery efficiency of ^{D}PPA-1, we conjugated fluorescent cyanine 5.5 to the N terminal of ^{D}PPA-1.[12f,23] To avoid space overlap of the liver and kidneys, CT26 tumor cells were injected in the back. When the tumor volume reached about 200 mm^3, CT26 bearing Balb/c mice were injected with 200 µL (40 µg/mouse) of Cy5.5-^{D}PPA-1 or Cy5.5-^{D}PPA-1* into the tail vein. After 24 hours, in vivo near-infrared fluorescence imaging was used to detect Cy5.5 distribution. The results showed that except for the liver, kidney, stomach, and lung, ^{D}PPA-1 was accumulated at the site of tumor tissue whereas this was not the case for the negative control (Figure 3D), indicating that ^{D}PPA-1 had the ability to target tumor tissue. We also tested the cytotoxicity of ^{D}PPA-1 for CT26 tumor cells. After ^{D}PPA-1 addition, CT26 cells were still able to grow normally (Figure 3E), which suggested that ^{D}PPA-1 would not kill tumor cells directly. According to these results, we propose that the activity of ^{D}PPA-1 might come from the activation of the antitumor immune system, rather than by directly killing CT26 tumor cells.[4]

To summarize, we have developed the first proteolysis-resistant peptide antagonists of the immune checkpoint protein hPD-L1 by combining the techniques of chemical protein synthesis and mirror-image phage display. These

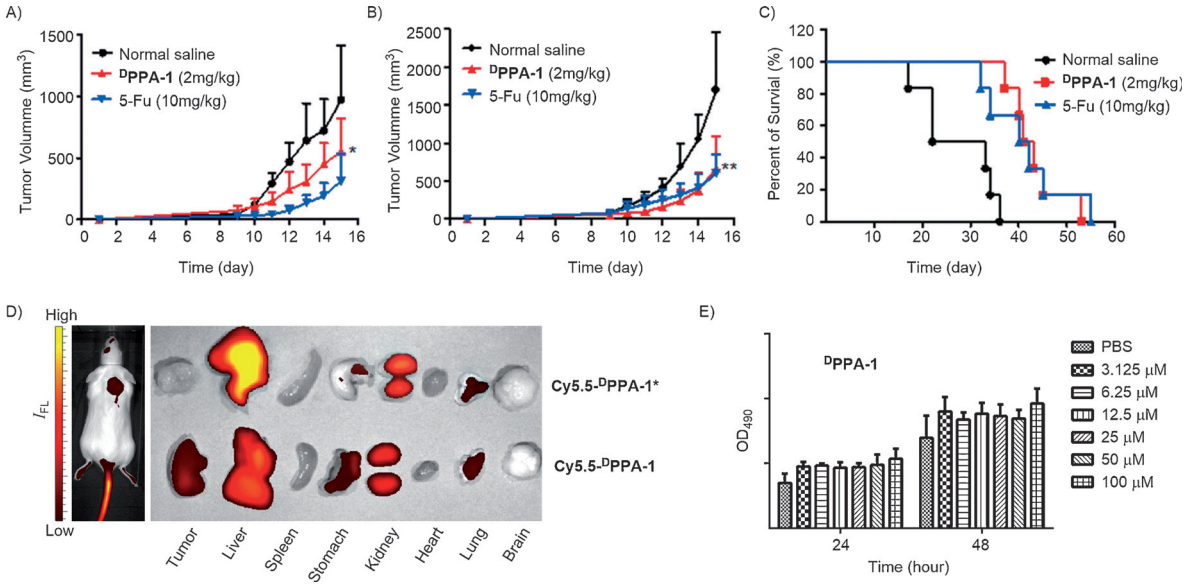

Figure 3. Tumor growth inhibition in CT26-tumor-bearing Balb/c mice injected with ^{D}PPA-1 and 5-Fu either A) subcutaneously around the tumor or B) intraperitoneally. (Significant differences of the tumor volume between the ^{D}PPA-1 group and the negative control was determined by student's *t*-test, $*P<0.05$, $**P<0.01$). C) Survival prolonging curves for CT26-tumor-bearing Balb/c mice treated with ^{D}PPA-1 and 5-Fu. D) In vivo near-infrared fluorescence imaging of CT26-tumor-bearing Balb/c mice, 24 h after injection with Cy5.5-^{D}PPA-1* or Cy5.5-^{D}PPA-1 into the tail vain (Cy5.5 = 2-((1*E*,3*E*,5*E*)-5-(3-(5-carboxypentyl)-1,1-dimethyl-1*H*-benzo[e]indol-2(3*H*)-ylidene)penta-1,3-dienyl)-3-ethyl-1,1-dimethyl-1*H*-benzo). I_{fl} = fluorescence intensity. E) OD_{490} of CT26 cells after incubation with different concentrations of ^{D}PPA-1 for 24 and 48 hours (OD = optical density).

peptide inhibitors could bind to PD-L1 with low micromolar affinity. Their ability to inhibit PD-1/PD-L1 protein–protein interaction was also demonstrated at the cellular level. Experiments with tumor-bearing mice models showed that the peptides could inhibit tumor growth and prolong animal survival. Collectively our results suggested the potential of developing proteolysis-resistant peptides as low-molecular-weight drug candidates for cancer immunotherapy.

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[1] L.-P. Chen, D. B. Flies, *Nat. Rev. Immunol.* **2013**, *13*, 227–242.
[2] a) I. Mellman, G. Coukos, G. Dranoff, *Nature* **2011**, *480*, 480–489; b) S. L. Topalian, G. J. Weiner, D. M. Pardoll, *J. Clin. Oncol.* **2011**, *29*, 4828–4836.
[3] a) A. J. Korman, K. S. Peggs, J. P. Allison, *Adv. Immunol.* **2006**, *90*, 297–339; b) D. M. Pardoll, *Nat. Rev. Cancer* **2012**, *12*, 252–264.
[4] a) F. S. Hodi, S. J. O'Day, D. F. McDermott, et al., *N. Engl. J. Med.* **2010**, *363*, 711–723; b) S. L. Topalian, F. S. Hodi, J. R. Brahmer, et al., *N. Engl. J. Med.* **2012**, *366*, 2443–2454; c) J. R. Brahmer, S. S. Tykodi, L. Q. M. Chow, et al., *N. Engl. J. Med.* **2012**, *366*, 2455–2465; d) J. R. Brahmer, *J. Clin. Oncol.* **2013**, *31*, 1021–1028; e) J. Couzin-Frankel, *Science* **2013**, *342*, 1432–1433; f) L.-F. Deng, H. Liang, B. Burnette, M. Beckett, T. Darga, R. R. Weichselbaum, Y.-X. Fu, *J. Clin. Invest.* **2014**, *124*, 687–695.
[5] P. Vlieghe, V. Lisowski, J. Martinez, *Drug Discovery Today* **2010**, *15*, 40–56.
[6] a) H.-D. Dong, G. Zhu, K. Tamada, L.-P. Chen, *Nat. Med.* **1999**, *24*, 1365–1369; b) H.-D. Dong, S. E. Strome, D. R. Salomao, et al., *Nat. Med.* **2002**, *8*, 793–800; c) Y. Iwai, M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, N. Minato, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12293–12297; d) W. Zou, L.-P. Chen, *Nat. Rev. Immunol.* **2008**, *7*, 467–477; e) A. Dömling, T. A. Holak, *Angew. Chem. Int. Ed.* **2014**, *53*, 2286–2288; *Angew. Chem.* **2014**, *126*, 2318–2320.
[7] R. S. Herbst, J.-C. Soria, M. Kowanetz, et al., *Nature* **2014**, *515*, 563–567.
[8] G. K. Philips, M. Atkins, *Int. Immunol.* **2014**, *27*, 39–46.
[9] P. Molek, B. Strukelj, T. Bratkovic, *Molecules* **2011**, *16*, 857–887.
[10] a) M. T. Weinstock, M. T. Jacobsen, M. S. Kay, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 11679–11684; b) Y.-L. He, S. Murby, L. Gifford, A. Collett, G. Warhurst, K. T. Douglas, M. Rowland, J. Ayrton, *Pharm. Res.* **1996**, *13*, 1673–1677.
[11] T. N. M. Schumacher, L. M. Mayr, D. L. Minor, Jr., M. A. Mihollen, M. W. Burgess, P. S. Kim, *Science* **1996**, *271*, 1854–1857.
[12] a) D. M. Eckert, V. N. Malashkevich, L.-H. Hong, P. A. Carr, P. S. Kim, *Cell* **1999**, *99*, 103–115; b) K. Wiesehan, K. Bruder, R. P. Linke, S. Patt, M. Stoldt, E. Unger, B. Schmitt, E. Bucci, D. Willbold, *ChemBioChem* **2003**, *4*, 748–753; c) B. D. Welch, A. P. VanDemark, A. Heroux, C. P. Hill, M. S. Kay, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16828–16833; d) T. van Groen, K. Wiesehan, S. A. Funke, I. Kadish, L. Nagel-Steger, D. Willbold, *ChemMedChem* **2008**, *3*, 1848–1852; e) M. Liu, M. Pazgier, C.-Q. Li, W.-R. Yuan, C. Li, W.-Y. Lu, *Angew. Chem. Int. Ed.* **2010**, *49*, 3649–3652; *Angew. Chem.* **2010**, *122*, 3731–3734; f) M. Liu, C. Li, M. Pazgier, C.-Q. Li, Y. Mao, Y.-F. Lv, B. Gu, G. Wei, W.-R. Yuan, C.-Y. Zhan, W.-Y. Lu, W.-Y. Lu, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 14321–14326; g) K. Mandal, M. Uppalapati, D. Ault-Riché, J. Kenney, J. Lowitz, S. S. Sidhu, S. B. H. Kent, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 14779–14784.
[13] D.-Y. Lin, Y. Tanaka, M. Iwasaki, A. G. Gittis, H.-P. Su, B. Mikami, T. Okazaki, T. Honjo, N. Minato, D. N. Garboczi, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 3011–3016.
[14] M. Pan, Y. He, M. Wen, F.-M. Wu, D.-M. Sun, S.-J. Li, L.-H. Zhang, Y.-M. Li, C.-L. Tian, *Chem. Commun.* **2014**, *50*, 5837–5839.
[15] a) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776–779; b) V. R. Pattabiraman, J. W. Bode, *Nature* **2011**, *480*, 471–479.
[16] G.-M. Fang, J.-X. Wang, L. Liu, *Angew. Chem. Int. Ed.* **2012**, *51*, 10347–10350; *Angew. Chem.* **2012**, *124*, 10493–10496.
[17] Q. Wan, S. J. Danishefsky, *Angew. Chem. Int. Ed.* **2007**, *46*, 9248–9252; *Angew. Chem.* **2007**, *119*, 9408–9412.
[18] B. L. Pentelute, S. B. H. Kent, *Org. Lett.* **2007**, *9*, 687–690.
[19] N. J. Greenfield, *Nat. Protoc.* **2006**, *1*, 2876–2890.
[20] a) P. Youngnak, Y. Kozono, H. Kozono, H. Iwai, N. Otsuki, H. Jin, K. Omura, H. Yagita, D. M. Pardoll, L.-P. Chen, M. Azuma, *Biochem. Biophys. Res. Commun.* **2003**, *307*, 672–677; b) M. J. Butte, M. E. Keir, T. B. Phamduy, A. H. Sharpe, G. J. Freeman, *Immunity* **2007**, *27*, 111–122; c) X.-W. Zhang, J.-C. D. Schwartz, X.-L. Guo, S. Bhatia, E. Gao, L.-P. Chen, Z.-Y. Zhang, M. A. Edidin, S. G. Nathenson, S. C. Alomo, *Immunity* **2004**, *20*, 337–347.
[21] C. J. Wienken, P. Baaske, U. Rothbauer, D. Braun, S. Duhr, *Nat. Commun.* **2010**, *1*, 100.
[22] K. Weiskopf, A. M. Ring, C. C. M. Ho, J.-P. Volkmer, A. M. Levin, A. K. Volkmer, E. Özkan, N. B. Fernhoff, M. van de Rijn, I. L. Weissman, K. C. Garcia, *Science* **2013**, *341*, 88–91.
[23] a) V. Ntziachristos, E. A. Schellenberger, J. Ripoll, D. Yessayan, E. Graves, A. Bogdanov, Jr., L. Josephson, R. Weissleder, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 12294–12299; b) M. Veiseh, P. Gabikian, S.-B. Bahrami, et al., *Cancer Res.* **2007**, *67*, 6882–6888.

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