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Peptide-based and small synthetic molecule inhibitors on PD-1/PD-L1 pathway: a new choice for immunotherapy?

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Abbreviations

PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; hPD-1/hPD-L1, human-PD-1/human-PD-L1; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; mAbs, monoclonal antibodies; CD152, cluster of differentiation 152; Tregs, regulatory T cells; NKT, natural killer T cells; DCs, dendritic cells; TAMs, tumor-associated macrophages; ITIM, immune tyrosine-based inhibitory motif; ITSM, immune receptor inhibitory tyrosine-based switch motif; TME, tumor microenvironment; HIV, human immunodeficiency virus; MAITs, Mucosal-associated invariant T cells; FDA, Food and Drug Administration; irAEs, immune-related adverse events; EGFR, epidermal growth factor receptor; STAT3, signal transducer and activator of transcription 3; PI3K, phosphatidylinositol 3-kinase; HER2, human epidermal growth factor receptor-2; RCC, renal cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small-cell lung cancer; IFNγ, interferon gamma; IDO, indoleamine 2,3-dioxygenase; SHP-1, src homology region 1; SHP-2, src homology region 2; TCR, T-cell receptor; ERK, extracellular-signalregulated kinase; sPD-1, soluble PD-1; sPD-L1, soluble PD-L1; mPD-1, mouse PD-1; PPI, protein-protein interaction; LArg113, Arg113 in hPD-L1; SBDD, structure-based drug design; BMS, Bristol-Myers Squibb; ArgH99, Arg99 of the Pembrolizumab Fab heavy chain; ArgL96, Arg96 of the Pembrolizumab Fab light chain; RMSD, root-mean-square deviation; HTRF, homogenous time resolved fluorescence; PBMC, peripheral blood mononuclear cells; SAR, structure-activity relationships; sPD-L1, soluble PD-L1; ELISA, enzyme-linked immunosorbent assay; TR-FRET, time-resolved fluorescence resonance energy transfer; SPR, surface plasmon resonance; CQAs, caffeoylquinic acid compounds; ITC, isothermal titration calorimetry; CFSE, 5, 6-carboxyfluorescein diacetate, succinimudyl ester.

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Abstract: Blockade the interaction of the programmed cell death protein 1 (PD-1) and its ligand, programmed death-ligand 1 (PD-L1) can prevent immune evasion of tumor cells and significantly prolong the survival of cancer patients. Currently marketed drugs targeting PD-1/PD-L1 pathway are all monoclonal antibodies (mAbs) that have achieved great success in clinical trials. With the constantly emerging problems of antibody drugs, small molecule inhibitors have attracted the attention of pharmaceutical chemists due to their controllable pharmacological and pharmacokinetic properties, which make them potential alternatives or supplements to mAbs to regulate PD-1/PD-L1 pathway. However, the insufficient target structure information hinders the development of small molecule inhibitors. Since the publication of human-PD-1/human-PD-L1 (hPD-1/hPD-L1) crystal structure, more and more cocrystal structures of mAbs, cyclopeptides and small molecules with PD-1 and PD-L1 have been resolved. These complexes provide a valuable starting point for the rational design of peptide-based and small synthetic molecule inhibitors. Here we reviewed the non-antibody inhibitors that have been

published so far and analyzed their structure-activity relationships (SAR). We also summarized the cocrystal structures with hot spots identified, with the aim to provide reference for future drug discovery.

Key words: Peptide-based and small synthetic molecule inhibitors, PD-1/PD-L1 pathway, rational design, tumor immunotherapy, cocrystal structures, structure-activity relationship

1. Introduction

As a host defense system, the immune system protects body against various diseases such as cancer. However, cancer cells can escape from the immune system and continue to grow via a variety of immune evasion strategies, including down-regulation of cell-surface MHC class I molecules, secretion of immunosuppressive factors, and lack of T cell costimulation[1]. Therefore, restoring the anti-cancer effect of immune system is an important direction in anti-cancer research. Compared to the traditional cancer therapies such as chemotherapy and radiotherapy, the cancer immunotherapy marks a completely different way of treating cancer by targeting the immune system rather than the tumor itself[2]. In recent years, cancer immunotherapies including immune checkpoint inhibitors[3], chimeric antigen receptor T cell therapy[4] and adoptive cell therapy with tumor-infiltrating lymphocytes[5] have achieved great clinical success, which make the cancer immunotherapy a current topic of intensive research and bring new hope to cancer patients. Among these, the immune checkpoint inhibitor is the most

mature immunotherapy and has the highest share in market than the rest[6].

Immune checkpoints act as the regulators of the immune system and are almost B7/CD28 and TNF/TNFR superfamily members. They can be divided into stimulatory checkpoint molecules and inhibitory checkpoint molecules whose function is to maintain self-tolerance and prevent the immune system from invading cells indiscriminately[7] through the real-time coordination with lymphocyte activity. Until now, the best-studied immune checkpoints are cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), PD-1 and PD-L1. With the encouraging clinical results of Ipilimumab (targeting CTLA-4) and Pembrolizumab (targeting PD-1), immune checkpoint inhibitors have cemented its potential in patients and were selected as the most important scientific breakthrough of 2013 by the journal *Science*[8]. However, the anti-CTLA-4 therapy encountered several problems in cancer treatment and drug development[9-13]. In contrast to CTLA-4, the drug development of PD-1/PD-L1 pathway has made rapid progress in recent years.

PD-1, also known as CD279, is the second-generation of immune checkpoint protein isolated by Ishida Y in 1992[14]. PD-1 belongs to the CD28 family and expresses on T cells, regulatory T cells (Tregs), exhausted T cells, B cells, natural killer cells, natural killer T (NKT) cells, dendritic cells (DCs), and tumor-associated macrophages (TAMs)[15, 16]. As a 55-kDa monomeric type I surface transmembrane glycoprotein, PD-1 molecule consists of an extracellular IgV domain, a transmembrane domain and an intracellular cytoplasmic domain containing two structural motifs, immune

tyrosine-based inhibitory motif (ITIM) and immune receptor inhibitory tyrosine-based switch motif (ITSM)[17].The two ligands of PD-1 molecule, PD-L1 (CD274) and PD-L2 (CD273), belong to B7 family and share 37% sequence homology. PD-L1 has been detected on antigen presenting cells, non-lymphoid organs and non-haematopoietic cells[11, 18], whereas PD-L2 expression is more restricted and is mainly found in DCs and a few tumor lines[19]. The 40-kDa PD-L1 is a type I transmembrane protein comprising extracellular IgV and IgC domains, a transmembrane domain and intracellular domains. The PD-1/PD-L1 interaction releases a co-inhibitory signal to activated T cells, which induces T cell apoptosis, anergy, and functional exhaustion[20, 21]. As a result, T cells activities to antigenic stimuli including proliferation, cytokine secretion and cytolytic activity are reduced. Normally, PD-1 and widely expressed PD-L1 act as an intrinsic negative feedback system to down-regulate the immune system and promote self-tolerance by suppressing T cell inflammatory activity. However, the overexpressed PD-L1 on the surface of tumor cells can induce T cells dysfunction and suppress antigen-specific T cell responses[22]. And in contrast to CD80 and CD86, PD-L1 is overexpressed by various solid tumors, indicating PD-1/PD-L1 pathway is the dominant immune checkpoint pathway in the tumor microenvironment (TME). It develops an important mechanism for tumor immune evasion in adaptive immune resistance[23]. Therefore, PD-1/PD-L1 pathway has become an important target to restore tumor-specific T-cell function for eliminating tumor cells. In addition to cancer,

PD-1/PD-L1 pathway is closely related to autoimmunity. It has been proven that in PD-1 knockout mice the disruption of peripheral tolerance would lead to a negative regulation of lymphocyte activation, which ultimately resulted in the appearance of autoimmune features, such as glomerulonephritis and destructive arthritis[24, 25]. For humans, the PD-1/PD-L1 signaling pathway is associated with type 1 diabetes, systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis and so on. In rheumatoid arthritis, the expression of both soluble PD-1 in synovial fluid and plasma and PD-1 in synovial fluid-derived CD4+ T cells is increased[26, 27]. Moreover, PD-L1 expressed in synovial fluid myeloid DCs is also elevated. Viral infection can lead to chronic infection by affecting the PD-1/PD-L1 signaling pathway, avoiding the surveillance and killing effects of immune system. For example, PD-1 expression in human immunodeficiency virus (HIV) infection mediates the depletion of CD8 T cells, which causes reduced immune clearance in virally infected host cells[28]. Moreover, Tregs and Mucosal-associated invariant T cells (MAITs) in HIV patients also overexpress PD-1[29, 30]. The abnormal expression of PD-1 are positively related to impaired immune function, disease progression, and decreased CD4 T cell count. Inhibitory signaling through the PD-1/PD-L1 pathway is also associated with neurological diseases, which is supposed to regulate immune cells in the CNS as a mechanism to avoid inflammation in the brain. In patients with Alzheimer's disease and mild cognitive impairment, PD-1 expression on CD4 + T cells and PD-L1 expression on CD14 + macrophages/monocytes are

reduced[31].

The anti-tumor mechanisms of immune checkpoint inhibitors comprise the blockade of inhibitory signals transmission and the direct stimulation to activate cytotoxic T lymphocytes. Currently, the marketed drugs targeting PD-1/PD-L1 pathway are all mAbs (Table 1). In 2014, Food and Drug Administration (FDA) approved the first two mAbs (Pembrolizumab and Nivolumab) targeting PD-1 molecule into market. Another three mAbs (Atezolizumab, Durvalumab and Avelumab) targeting PD-L1 molecule appeared on the market during the next few years. These antibody drugs have achieved tremendous clinical success and significantly improved the survival of patients with certain cancers. However, along with the impressive clinical effect, the problems of antibody drugs have also emerged over time[32, 33]. First, antibody drugs are not orally bioavailable and have a poor diffusion due to their high molecular weight. Second, mAbs always have a poor permeability. Third, high-affinity antibodies often bind tightly to the first encountered antigen on the periphery of the tumor, which makes them inaccessible to internal targets[34]. Moreover, the Fc domain of IgG antibody can interact with various receptors on different cells surface which in turn affect their retention in the circulation. The last but the most important is mAbs are immunogenic that can lead to severe immune-related adverse events (irAEs) with deadly outcomes in rare cases. The long half-lives and strong target occupancy of PD-1- and PD-L1-directed mAbs result in the sustained target inhibition, which also contributes to the intractability of irAEs. In terms of production,

the high production cost and poor stability of mAbs hinder the promotion and application of the immunotherapy. In order to address the limitations of the current antibody drugs, low-molecular-weight PD-1/PD-L1 inhibitors with better pharmacokinetic profiles are becoming the focus of research. However, the development of small molecule immune checkpoint inhibitors lags far behind mAbs. To the best of our knowledge, only one patented PD-1/PD-L1 inhibitor CA-170 has undergone clinical trials. Other few series of peptide-based and small molecule inhibitors have been reported primarily in patent applications, but no further information is disclosed so far. The lack of target structure information restricted the rational design of small molecule inhibitors targeting PD-1/PD-L1 pathway. However, since the publication of the hPD-1/hPD-L1 complex in 2015[35], more and more cocrystal structures of antibodies, cyclopeptides, and small molecules targeting PD-1 and PD-L1 have been published. In recent years, several reviews have summarized the disclosed peptide-based and small molecule inhibitors[6, 36-40] and the published cocrystal structures[41-43] in detail. In addition to summarize the recent progress in drug development, our review specifically describes the SAR of representative peptide-based and small molecule compounds and we focus on analyzing the hot spots identified in published cocrystal structures with the aim to provide reference for future drug discovery.

Table 1. FDA-approved PD-1/PD-L1-directed mAbs

Generic Name (Brand Name)	Target	Company	Indication	Affinity
Nivolumab (Opdivo)	PD-1	Bristol-myers squibb	melanoma, non-small-cell lung cancer, renal cell carcinoma (RCC), squamous cell carcinoma of the head and neck (SCCHN), Hodgkin lymploma, hepatocellular carcinoma, urothelial carcinoma, colorectal cancer, stomach cancer.	1.45 nM
Pembrolizumab (Keytruda)	PD-1	Merck	melanoma, head and neck squamous cell carcinoma (HNSCC), non-small-cell lung cancer (NSCLC), Hodgkin lymphoma, urothelial carcinoma, solid tumors, gastric or gastroesophageal junction adenocarcinoma, colorectal cancer, stomach metastatic cancer.	27 pM
Atezolizumab (Tecentriq)	PD-L1	Roche	urothelial carcinoma, NSCLC, bladder cancer	400 pM
Avelumab	PD-L1	Merck/Pfizer	metastatic Merkel cell carcinoma,	42 pM

(Bavencio)			urothelial carcinoma	
Durvalumab (Imfinzi)	PD-L1	AstraZenaca	urothelial carcinoma, NSCLC	667 pM

2. PD-1/PD-L1 pathway

There are two mechanismsfor the overexpression of PD-L1 on tumor cell surface (Figure 1). The inherent mechanism relies on the change of the internal signaling pathways, transcriptional factors and epigenetic factors in tumor cells. It is demonstrated that up-regulation of PD-L1 can be caused by activation of transcriptional factors epidermal growth factor receptor (EGFR)[44], signal transducer and activator of transcription 3 (STAT3)[45], hypoxia-inducible factor alpha (HIF-1α)[46] and NF-κB[47] as well as signaling pathways such as AKT–mTOR pathway[48], mitogen-activated protein kinase (MAPK) pathway[49], COX2/mPGES1/PGE2 pathway[50] and phosphatidylinositol 3-kinase (PI3K) signaling pathway[51]. And PD-L1 expression is negatively correlated with human epidermal growth factor receptor-2 (HER2)[44] and several microRNAs (miR-570, miR-513, miR-197, miR-34a and miR-200)[52]. External mechanismalso induces tumor cells to overexpress PD-L1. Activated T cells, natural killer cells and other cells involved in tumor clearance could secrete a dimerized soluble cytokine, interferon gamma (IFNγ), to kill tumor cells. In order to resist the clearance effect of IFNγ, tumor cells develop an adaptability mechanism in which PD-L1 and indoleamine 2,3-dioxygenase (IDO) are overexpressed to take immunosuppressive effects

in TME[7]. When engaged with a ligand, the intracellular ITIM and ITSM motifs of PD-1 become phosphorylated. In turn phosphorylated ITIM and ITSM recruit the phosphatases src homology region 1 (SHP-1) and src homology region 2 (SHP-2) to the PD-1 intracellular domain[53]. SHP-1 and SHP-2 can suppress the downstream signaling pathways of the T-cell receptor (TCR) including PI3K/Akt, RAS, extracellular-signal regulated kinase (ERK), c-Myc pathways and so on[54-56](Figure 1). The consequences are decreased T cell activation, proliferation and survival as well as the reduction of cytokine production.These consequences are formed by three mechanisms. . First, overexpressed PD-L1 on tumor cells delivers apoptotic signal thus promoting the apoptosis of activated tumor antigen-specific T cells, in which cytokines IL-10 and Fasl are involved[57]. Second, PD-1/PD-L1 interaction controls the initiation of T-cell anergy andexhaustion in peripheral and lymphoid organs[58, 59] and hinders $CD8^+$ T cells differentiation into functional cytolytic T lymphocytes[60]. Last, PD-1/PD-L1 pathway promotes the development and function of Treg cells to maintain immune homeostasis, suppress Teff cells and keep the threshold of T cells activation high enough to prevent autoimmuity[61]. Recent studies revealed that PD-1/PD-L1 interaction is not limited in TME and PD-L1 expressed on immune cells also influences antitumor $CD8^+$ T cell responses[60]. It can be concluded that PD-1/PD-L1 checkpoint blockade therapies may enhance the priming but limit the survival of antitumor $CD8^+$ T cells by interfering with the intrinsic pro-survival/antiapoptotic role of $CD8^+$ T cells for PD-L1 signaling. Other

studies also revealed that membrane-bound PD-1 and PD-L1 have soluble forms[62]. It is hypothesized that the binding between soluble PD-1 (sPD-1) and membrane-bound PD-L1 or PD-L2 may prevent the PD-1/PD-L1 and PD-1/PD-L2 interactions from different cells, thus improving the antitumor immunity of T cells[63]. Frigola *et al*. discovered soluble PD-L1 (sPD-L1) can induce the apoptosis of $CD4^+$ and $CD8^+$ T lymphocytes[64] and several other studies confirmed that sPD-L1 is a negative therapeutic and prognostic biomarker in several malignant tumors[65, 66]. But whether soluble PD-L1 (sPD-L1) can bind to membrane-bound PD-1 to deliver an inhibitory signal remains unresolved. For these reasons, direct blocking of membrane-bound PD-1/PD-L1 interaction is the main strategy for current inhibitor development. The mechanism of action of the currently reported inhibitors targeting PD-1/PD-L1 is to inhibit the binding of PD-1 and PD-L1 by occupying their extracellular interaction sites. Although the binding sites and direction of different inhibitors is diverse, according to the crystal structure published, inhibitors mainly bind to the flat β-sheets of both PD-1 and PD-L1, which is dominant in PD-1/PD-L1 interaction. Macromolecular inhibitors include mAbs and cyclic peptides form 1:1 complexes with their targets, while small molecule inhibitors have been reported to inhibit PD-1/PD-L1 signaling by binding to the dimeric targets. The binding modes of these inhibitors will be described in detail in the following sections.

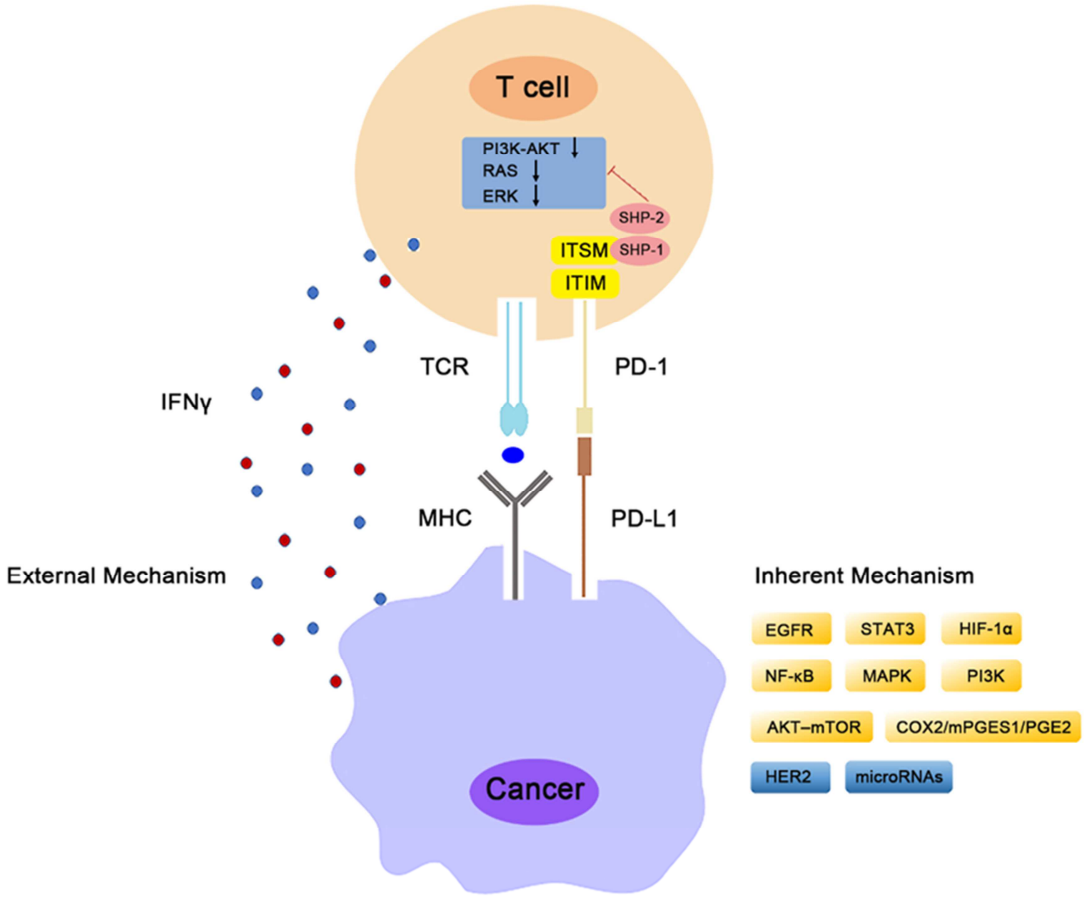


Figure 1. The internal mechanism and external mechanism of increased PD-L1 expression and the effect of PD-1/PD-L1 pathway on T cells.

3. Structures of PD-1/PD-L1 Complexes and Cocrystal Structures of Monoclonal Antibodies

The first high-resolution crystal structure establishing the structural foundations of mouse PD-1 (mPD-1) and human PD-L1 (PDB ID: 3BIK) was published in 2008[67]. Despite the obtained crystals provide important information of the mPD-1/hPD-L1 interaction, they have differences in the details of the binding modes with the naturally occurring hPD-1/hPD-L1 complex because the sequence identity between human and

mouse PD-1 is only 64%. Within the crystal, mPD-1 and hPD-L1 form a 2:1 complex which is inconsistent with the 1:1 binding stoichiometry determined by biochemical assays. One of the mPD-1 molecules was noted as a crystallization artifact by authors afterwards since its limited interface is unable to support a strong protein-protein interaction (PPI)[68, 69]. Moreover, the mPD-1/hPD-L1 complex is not allowed to assess the extent of plasticity when starting from apo-proteins. Later the overall structures of apo-hPD-1 (PDB: 3RRQ) and apo-hPD-L1 (PDB: 3BIS) were published[68]. But these apo-proteins cannot account for the significant plasticity of hPD-1 upon complex formation.

The crystal structure of hPD-1/hPD-L1 complex was disclosed by Zak *et al* in 2015 (PDB ID: 4ZQK)[35]. The hPD-1 and hPD-L1 molecules are almost orthogonal to each other such that their interaction is completely mediated by the β-strands on the large and hydrophobic surfaces of their respective IgV domains (excluding IgV loops). The asymmetric unit within the complex contains one hPD-1 molecule and one hPD-L1 molecule, confirming the 1:1 stoichiometry. Within the complex, hPD-1 assumes a β-sandwich Ig V-type topology with a characteristic disulfide bridge formed by Cys54 and Cys123 but lacks the second disulfide bridge as other family members (CD28, CTLA-4, and ICOS). Similar to hPD-1, the N-terminal domain of hPD-L1 also assumes the Ig V-type topology. The hPD-1/hPD-L1 interaction is mainly constituted by the central hydrophobic core on the front CC'FG β-sheets of both partners. A buried region

of mixed polar/nonpolar interactions adjacent the hydrophobic core also plays an important role. The periphery of both regions is composed of networks of polar residues which form hydrogen bonds to provide additional interaction. Comparative analysis of the hPD-1 extracted from the hPD-1/hPD-L1 complex and the apo-hPD-1 demonstrated that the complex formation does not require large backbone reorganization of hPD-1 but causes subtle rearrangements of the CC' loop. The CC' loop in apo-hPD-1 assumes an "open" conformation where all amino acid side chains in the loop point towards the solvent. Complex formation changes the CC' loop from "open" conformation to ''closed'' conformation with a 90° twist and approximately 5 Å displacement of C_α carbons. The CC' loop rearrangement causes big movements of some residues, which results in the formation of four hydrogen bonds to stabilize the initial transient interaction of hPD-1 and hPD-L1 (Figure 2). It is worth noting that the CC' loop in apo-mPD-1 displays a "closed" conformation and has a slight displacement upon ligand binding, indicating the CC' loop rearrangement is unique to hPD-1. Similarly, the plasticity of hPD-L1 induced by complex formation does not entail large backbone reorganization but makes subtle changes to the arrangement of the side chains contributing to the interface. The majority of observed displacements of the side-chain atoms does not exceed 3 Å and can be considered as small adjustment of local space constraints on the interaction surface except Arg113 in hPD-L1 ($_L$Arg113). The distal side-chain atoms of $_L$Arg113 has a ~5 Å displacement to form an intermolecular salt bridge with Glu136 and a hydrogen bond

with $_{L}$Glu58. But the orientation of $_{L}$Arg113 in apo-hPD-L1 is possibly affected by crystal packaging, thus it is unreasonable to speculate the significance of $_{L}$Arg113 in complex formation. Other pronounced movement involves the side chains of $_{L}$Glu58, $_{L}$Met115, $_{L}$Tyr123, $_{L}$Ile54 and $_{L}$Ala121.

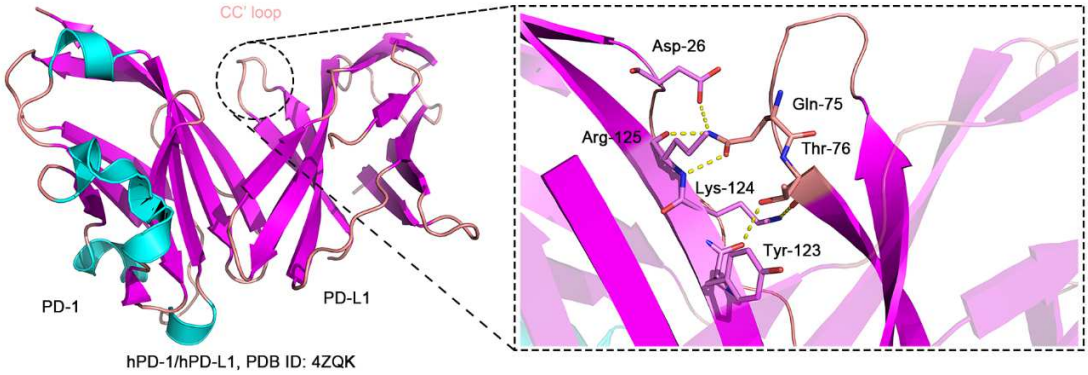


Figure 2. The overall structure of hPD-1/hPD-L1 and the hydrogen bonds formed between the CC' loop and PD-L1. The residues of CC' loop are colored by wheat and the residues of PD-L1 are colored by purple.

It appears that the 2.45 Å resolution hPD-1/hPD-L1 complex provides a perfect starting point for the rational structure-based drug design (SBDD) of small molecules against PD-1/PD-L1 interaction. Zak *et al* have put forward several hot spots (Figure 3) on the surface of hPD-1 and hPD-L1. They found the hot spot residues of hPD-1 in the hPD-1/hPD-L1 interaction are almost hydrophobic residues located on the front CC'FG β-strands comprising Asn66, Tyr68, Gly124, Ile126, Leu128, Ile134, and Glu136. Among these, Gly124 and surrounding residues form a hydrophobic cleft to accommodate

$_{L}$Tyr123 (Figure 3). Upon ligand binding, $_{L}$Tyr123 plays an important role in deepening the cleft and enforcing the conformation of CC' loop from "open" to "closed". $_{L}$Tyr123 also makes important interactions with the surrounding residues comprising π-π stacking interaction with Tyr68, alkyl-π interaction with Ile134 as well as hydrogen bond interaction with Glu136 and Thr76. Thereby the Gly124 cleft is a perfect anchor site to insert an aromatic ring. However, it is challenging to target the shallow pocket in apo-PD-1 which assumes a "closed" conformation. A hypothesis that the intrinsic flexibility of CC' loop could convert PD-1 between the "closed" and "open" conformation has been put forward. Nevertheless a fragment-based drug design using a limited number of tyrosine derivatives to occupy the Gly124 cleft did not succeed. It may due to the transient state of the Gly124 cleft is hard to target with fragments. Another reason may be the fragments are unable to provide sufficient binding energy to maintain the "open" conformation of hPD-1. Two promising hot spots have been defined on the surface of hPD-L1 (Figure 3). The first hot spot is a classical hydrophobic pocket comprising the side chains of $_{L}$Tyr56, $_{L}$Glu58, $_{L}$Arg113, $_{L}$Met115, and $_{L}$Tyr123. The pocket accommodates Ile134 in hPD-1 and a six-membered aromatic ring is supposed to have a suitable size to the pocket. The second hot spot next to the first one accommodates Ile126 in PD-1. It is composed of $_{L}$Met115, $_{L}$Ala121, and $_{L}$Tyr123 and can be anchored with a branched aliphatic moiety with a hydrogen bond donor to form interaction with $_{L}$Ala121. However, no fragment-based drug design has been carried out.

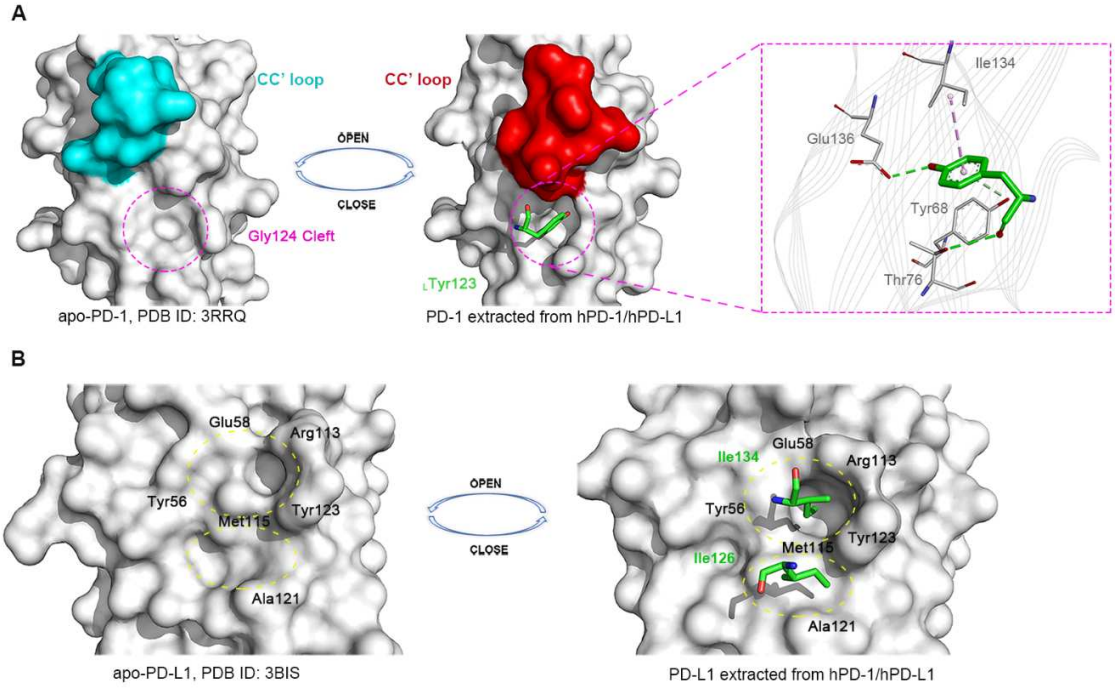


Figure 3. Hot spots of PD-1 and PD-L1. (A) The Gly124 cleft in the "open" and "closed" conformations of PD-1 and the detailed interaction between $_{L}$Tyr123 and PD-1. The green dash line indicates a hydrogen bond and the pink dash line indicates an alkyl-π interaction. (B) The two proposed small molecule binding pockets in the "open" and "closed" conformations of PD-L1.

So far, only two crystal structures of mAbs bound to hPD-1 have been reported, the Nivolumab/hPD-1 complex (PDB ID: 5GGR, 5WT9)[42, 70] and the Pembrolizumab/hPD-1 complex (PDB ID: 5JXE, 5B8C, 5GGS)[42, 71, 72]. Pembrolizumab and Nivolumab developed by Merck & Co. and Bristol-Myers Squibb (BMS) were proved by FDA in the same year for the treatment of patients with NSCLC, melanoma and HNSCC. Nivolumab and Pembrolizumab are both IgG4 antibodies and

form a 1:1 complex with hPD-1. They both utilize heavy chains and light chains to interact with PD-1 with 1932.5 $Å^2$ and 1774 $Å^2$ buried surfaces respectively. However, a comparative analysis of Pembrolizumab/hPD-1 complex and Nivolumab/hPD-1 complex revealed the distinct binding modes of these two antibodies. In the Nivolumab/hPD-1 complex, the interaction surface of hPD-1 involves the N-terminal loop as well as the FG and BC loops in the IgV domain. Though it is the first time that the N-terminal loop (L25-P34) outside the IgV domain has been observed, it dominates the Nivolumab/hPD-1 interaction. Structural analysis revealed the N-terminal loop contributes 10 out of 16 hydrogen bonds in the Nivolumab/hPD-1 interaction. Specifically, the residues Leu25, Ser27, Pro28, Asp29 and Arg30 in N-terminal loop forms 10 hydrogen bonds with Ser30, Asn31 and Gly33 in HCDR1 as well as Trp52, Tyr53 and Lys57 in HCDR2 (Figure 4). Superposition of the Nivolumab/hPD-1 complex and the hPD-1/hPD-L1 complex reveals Nivolumab outcompetes hPD-L1 for binding to hPD-1. By contrast, Pembrolizumab assumes a different orientation to interact with hPD-1 and has a steric clash with Nivolumab. As exhibited in Figure 4, their binding surfaces are close to each other but not overlap. It is worth noting that the C'D loop within the IgV domain of hPD-1 contributes predominantly polar, charged and hydrophobic interactions to the Pembrolizumab/hPD-1 complex. Specifically, Asp85 of hPD-1 establishes a salt bridge with Arg^{H99} (Arg99 of the Pembrolizumab Fab heavy chain). Arg86 involves in a T-shaped stacking interaction with Arg^{L96} (Arg96 of the Pembrolizumab Fab light chain).

The residues Glu84, Ser87, Gln88 and Gly90 in C'D loop make several hydrogen bonds in the complex formation. The structural superposition shows that Pembrolizumab has an overlapping interface with PD-L1 and PD-L2. Although the loops of hPD-1 are disordered in the hPD-1/hPD-L1 complex, these two crystal structures demonstrate that the loops can also provide important binding energy for the compound binding. Thus, in comparison to the flat β-sheets lacking small molecule binding sites, the loop domain may become potential target for future structure-based small immunomodulators design.

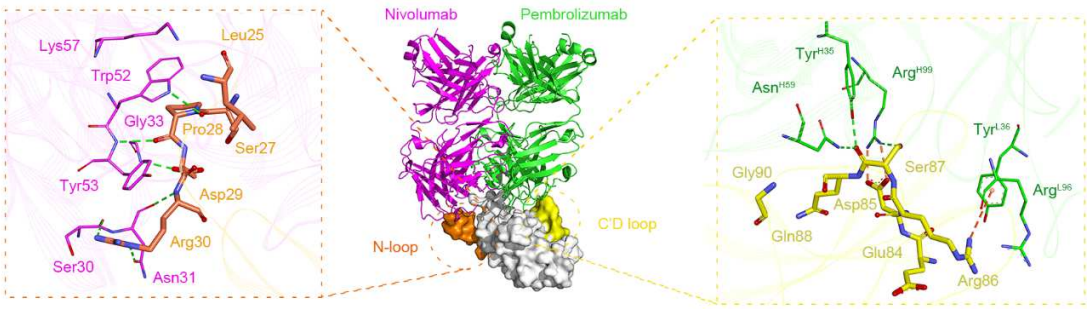


Figure 4. The superposition structure of Pembrolizumab/PD-1 complex and Nivolumab/PD-1 complex and the detailed interaction of the N-loop with Nivolumab as well as the C'D loop with Pembrolizumab. The green dash line indicates a hydrogen bond and the orange dash line indicate a π-π stacking interaction.

Recently, the cocrystal structures of hPD-L1 in complex with Avelumab (PDB ID: 5GRJ)[73], BMS-936559 (PDB ID: 5GGT)[42], Atezolizumab (PDB ID:5XXY)[74] and Durvalumab (PDB ID: 5X8M)[75] have been published, revealing the precise epitopes and binding modes involved in the antigen-antibody interactions (Figure 5). A comprehensive comparison study of these hPD-L1-directed antibodies helps us better

understand the mechanism of hPD-L1 inhibition and provides new insights into the rational design of small molecule modulators. As the first hPD-L1-directed mAb approved by FDA in 2016, Atezolizumab developed by Tecentriq is a IgG1 antibody. Avelumab and Durvalumab are another two IgG1 antibodies marketed in 2017, which are developed by Bavencio and Imfnzi respectively. BMS-963559 is a fully human IgG4 antibody under clinical trials. Comparative analysis revealed the binding epitopes of these antibodies and hPD-1 are overlapped, resulting in the blockade of paired molecules to bind each other. Although the binding directions and binding sites of these antibodies are different, they all form key interactions with the central CC′FG β-sheets and share five identical hot spot residues (Tyr56, Glu58, Arg113, Met115 and Tyr123). However, the flat CC′FG β-sheets is hard for SBDD to discoverlead compounds. In addition to the front β-sheets, the loop domains of hPD-L1 provide additional interactions to stabilize the complexes. Specifically, Atezolizumab makes extensive interactions with the BC, CC′, C′C″, and FG loops of hPD-L1. Durvalumab also interact with the CC′ loop and the N-terminal region of hPD-L1 through a salt bridge and van der Waals contacts. The interactions between the BC, C′C″, and FG loops and BMS-963559 provide additional affinity. As for Avelumab, it forms multiple hydrogen bonds with Asp61 in CC’ loop. More importantly, these loops in the cocrystal structures adopt similar conformations as in apo-PD-L1, indicating these loops are in the productive binding conformation before ligand binding. In the current situation thatthe CC’FG β-sheets of hPD-L1 lacking

suitable hot spots, these loops have the potential to be valuable targets for future small molecule inhibitors design. However, small molecule design targeting loop domains still have some problems. For example, the loop domains are not recommended to target in medicinal chemistry because they are labile and hard to present its actual conformation in crystal complexes. Moreover, there are rare successful examples to borrow.

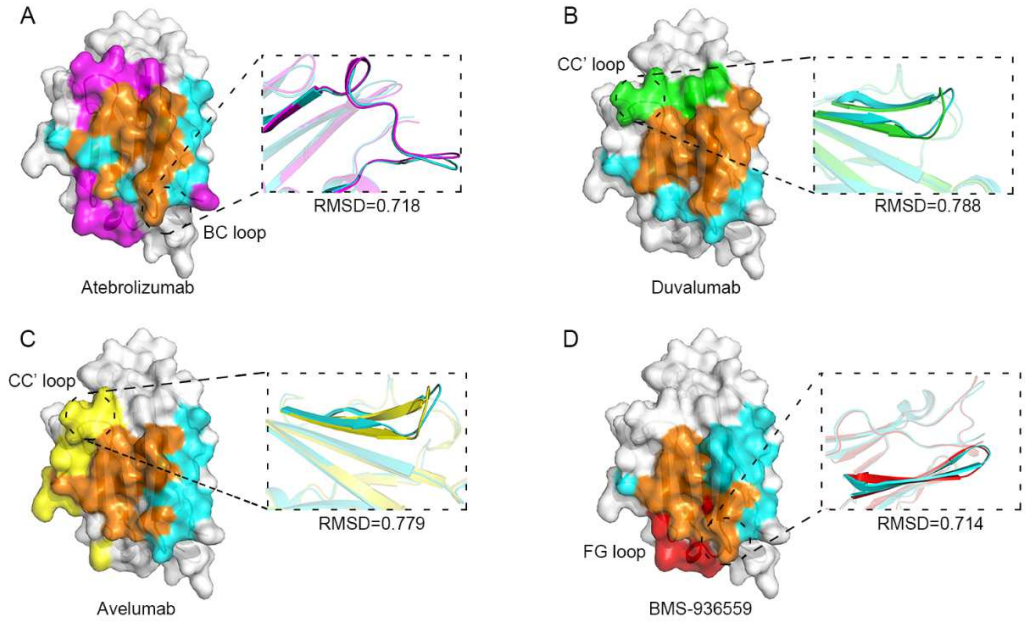


Figure 5. The superposition of the PD-1 binding site and anti-PD-L1 mAbs binding sites on PD-L1. The binding site of PD-1 is colored by cyan and the binding sites of Atebrolizumab (A), Duvalumab (B), Avelumab (C) and BMS-936559 (D) are colored by purple, green, yellow and red, respectively. The overlapped binding site is colored by orange. The dashed boxes show the superposition of the same PD-L1 loops in mAb/PD-L1 complex (colored as mAb binding site) and apo-PD-L1 protein (colored by cyan). The structural similarity is evaluated by the root-mean-square deviation (RMSD)

values.

In addition to the crystal structures mentioned above, other compounds that have crystal structures reported include nanobody KN035[76] and two macrocyclic-peptide inhibitors[77]. As a smaller PD-L1-directed antibody, KN035 not only obtains high affinity, good pharmacokinetic profiles, satisfactory permeability and stability but also it can be administered as a subcutaneous injection. Macrocyclic-peptide inhibitors also showed excellent inhibitory activities (IC_{50}<10 nM) towards hPD-L1 in the homogenous time resolved fluorescence (HTRF) assay. The binding modes of these compounds are similar with the mAbs like Avelumab and BMS-936559, forming major interactions with the flat surface of the IgV domain of PD-L1.

4. Inhibitors targeting the PD-1/PD-L1 pathway

4.1 Peptides and peptidomimetics as inhibitors of the PD-1/PD-L1 pathway

Among the reported peptides and peptidomimetics, inhibitors developed by Aurigene have excellent activities toward hPD-1 and have a clear optimization process as shown in Figure 6A. In 2014, Aurigene Discovery Technologies and Laboratoires Pierre Fabre have announced a collaboration license on the peptide AUNP-12 (Figure 6B) for the treatment of cancer. AUNP-12 is the first peptide inhibitor of PD-1/PD-L1 pathway and has a shorter half-life to overcome the pharmacokinetic limitations of current antibody modulators and better control irAEs. Sequences of the hPD-1 extracellular

domains which are critical for ligand-receptor interaction were considered as starting points for the design of 7- to 30-mer peptide derivatives with a non-linear fashion. As one of the most active peptides, AUNP-12 (a 29-mer)[78] exhibited an EC_{50} value of 0.72 nM on HEK293 cells expressing hPD-L2 and 0.41 nM on MDA-MB-231 cells expressing hPD-L1 in a rat peripheral blood mononuclear cells (PBMC) proliferation assay. *In vivo* studies showed AUNP-12 was potent in IFNγ production assay and efficiently restored the proliferation and effector functions of splenocytes and PBMCs. In preclinical models, AUNP-12 inhibited the growth of B16F10 mouse melanoma cells by 44% (5 mg/kg, subcutaneously once daily, 14 days). It also inhibited the growth of 4T1 cells by 44% in the mouse breast cancer animal model (3 mg/kg, subcutaneously, once daily, 40 days) in which 10% of the animals showed complete remission and other 10% reached partial remission. AUNP-12 also reduced lung metastasis of B16F10 cells in mice (5 mg/kg, subcutaneously, once daily, 11 days) and reduced tumor burden in kidney injected with renal carcinoma cells (5mg/kg, once daily, 21 days). Further *in vivo* studies revealed AUNP-12 had a sustained PD (> 24h) and was well tolerated with no significant toxicity at any of the tested doses. Because AUNP-12 was the first peptide inhibitor of PD-1/PD-L1 pathway and showed superior efficacy compared to therapeutic agents used in the clinic, the activity of AUNP-12 in mouse splenocyte proliferation CFSE (5, 6-carboxyfluorescein diacetate, succinimudyl ester) based assay at 100 nM was identified as the standard for Aurigene compounds[79, 80]. The SAR of AUNP-12 shows

shortening C-terminal chain length of branched peptides would result in loss of activity. And the acylation on C-terminal lysine side chain by various long-chain aliphatic acids is well tolerated, whereas the acylation on N-terminal serine side chain results in loss of activity. Moreover, unbranched peptides, particularly the N-terminal heptapeptide show excellent activities.

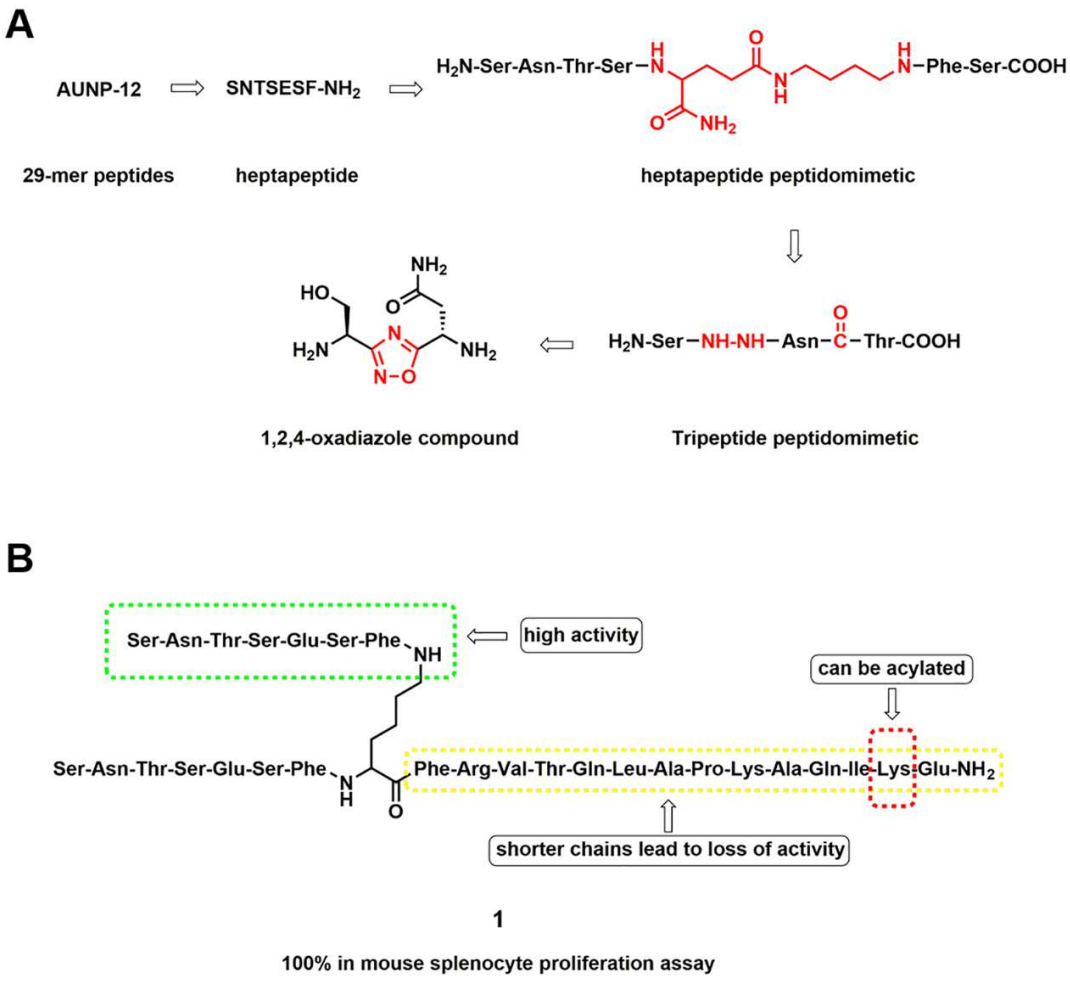


Figure 6. (A) The optimization procedure of Aurigene's peptides and peptidomimetics. (B) The structure and SAR of AUNP-12.

In the AUNP-12 discovery process, Aurigene scientists found compound **2** (Figure

7), a shorter heptapeptide, is equipotent to AUNP-12 in the mouse splenocyte proliferation rescue assay [81]. Compound **2** comprising the modified BC loop (amino acid 24-30) of hPD-1 extracellular domain led to the further development of several series of linear hepta- and octapeptides derived from the BC loop with a central linker of various nature and length between amino acids 28 and 29. Compound **2** exhibited a 4-fold induction in the mouse splenocyte proliferation assay inhibited by human breast cancer MDA-MB-231 cells overexpressing PD-L1. Further *in vivo* studies demonstrated a reduced lung metastasis by 64% in C57BL/6J mice bearing melanoma B16F10 cells (5 mg/kg, subcutaneously, once daily, 14 days). SAR of this series of linear peptides tolerates small substituents on the phenyl ring, acetylation on the N-terminal and D-amino acids in certain positions including an all-D analog. In addition to the linear peptides, 7- to 9-mer cyclic peptides derived from the same BC loop were rigidified by amide bonds between the side chains of lysine and glutamate or between the N-terminal serine and C-terminal arginine to give lactams. Compound **3** (Figure7) was shown to be active in rescuing splenocyte proliferation up to 78% at 100 nM concentration. Further *in vivo* studies demonstrated compound **3** reduced the rate of tumor metastases by 54% in a lung metastasis model of B16F10 melanoma in mice (5 mg/kg, subcutaneous, once daily, 14 days), compared to a 48% reduction for Taxol at the same dose. SAR reveals that replacement of the N-terminal serine or other residues in the central positions of the peptide by various amino acids does not lead to decreased activity[82].

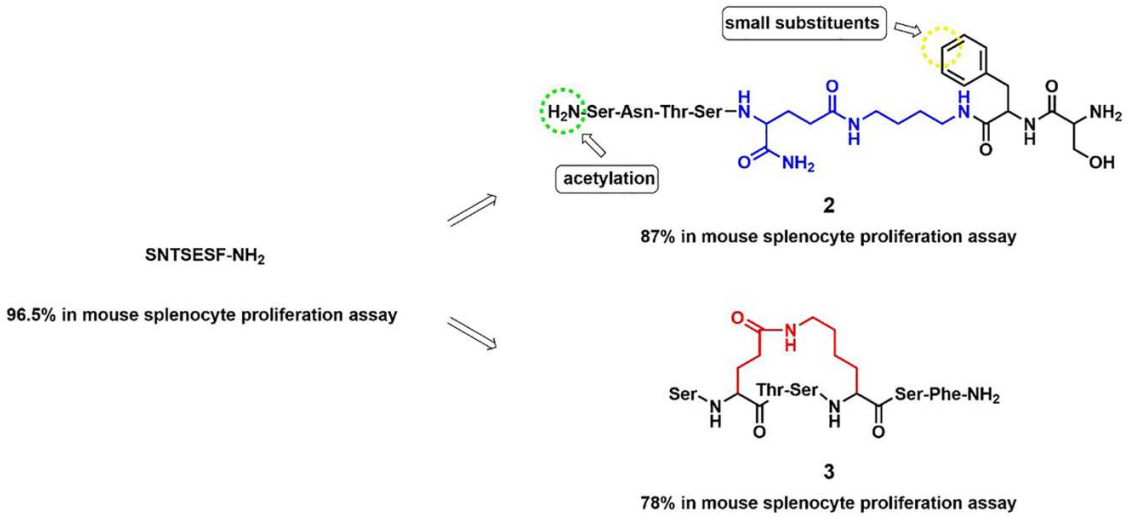


Figure 7. The structures and SAR of compound **2** and **3**.

A peptidomimetic is a small-protein like chain designed to mimic a peptide. By applying this concept in drug development, Aurigene researchers found tripeptide peptidomimetics with diacylhydrazine and urea groups potently inhibiting both PD-1/PD-L1 and PD-1/PD-L2 signaling pathway in the splenocyte effector function assay by monitoring IFNγ release. Peptidomimetic **4** (Figure 8) is one of the most active compounds with high activities in rescuing splenocyte proliferation up to 68% at 100 nM[83]. Peptidomimetic **4** also showed EC_{50} values in the nanomolar range to both PD-L1 (EC_{50}=30 nM) and PD-L2 (EC_{50}=40 nM). *In vivo* studies showed that peptidomimetic **4** reduced tumor growth by 46% in a CT-26 colon cancer mouse model (3mg/kg, 25 days) and was active against *Pseudomonas aeruginosa* in a lung infection model in mice (10 mg/kg, three times daily, 11 days). SAR shows that the methylation of the N-terminal hydroxy and amino groups as well as the amidation of the C-terminal

carboxyl would result in decreased activity. However, the methyl esterification of the C-terminal threonine carboxyl group was well tolerated. Further modification of this scaffold using various amino acid side chains led to the identification of new series of tripeptide peptidomimetics with improved activities. As a selected compound, peptidomimetic **5** (Figure 8) with a lysine side chain gave splenocyte proliferation rates of 87% in mouse splenocyte proliferation rescue assays [84]. SAR demonstrates that connecting the C-terminal carboxyl group with an asparagine or glycine increases activity, whereas connecting the N-terminal amino group to a serine and displacement of the diacylhydrazine group would impair activity. In addition to linear tripeptide peptidomimetics, cyclic tripeptide peptidomimetics also have been discovered as immunomodulators by Aurigene researchers. Compound **6** (Figure 8) with a glycol chain gave splenocyte proliferation rates of 92% in mouse splenocyte proliferation rescue assays[85]. Further SAR studies show that compounds with a glycol chain is more potent than that with an aliphatic chain.

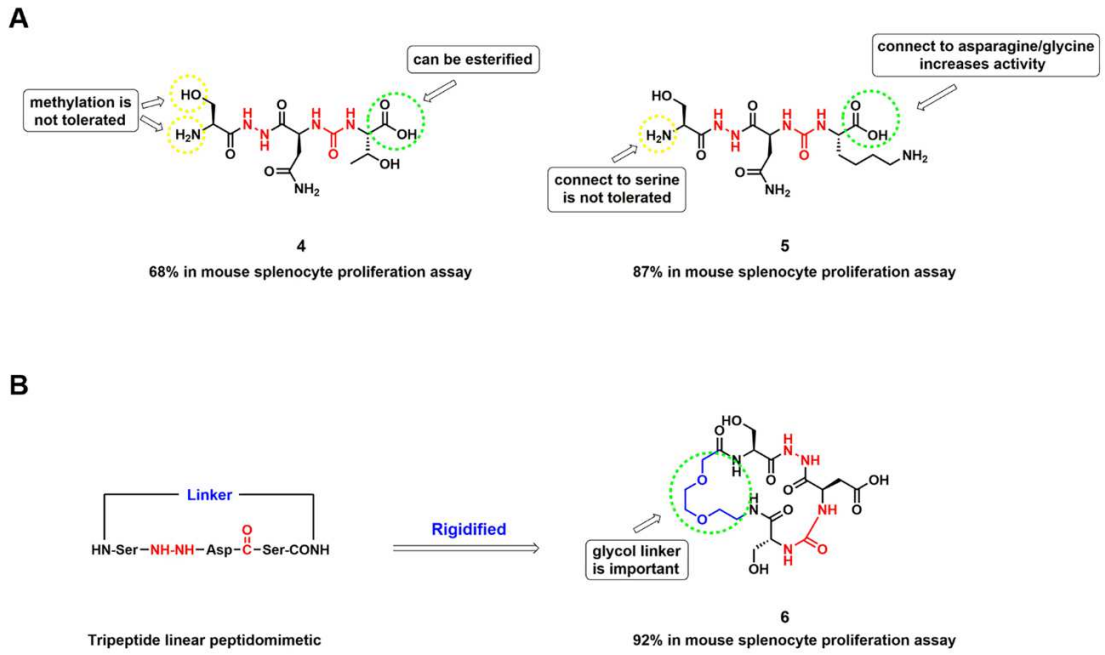


Figure 8. (A) The structures and SAR of compound **4** and **5**. (B) The schematic diagram of structural transformation as well as the structure and SAR of compound **6**.

In a further peptidomimetic study, Aurigene researchers designed and synthesized a novel series of PD-1/PD-L1 antagonists in which the diformylhydrazine moiety is rigidified by an oxadiazole or thiadiazole ring. In contrast to the above bulky peptides, some peptidomimetics with excellent biological activities in this series have similar volume and molecular weight as small molecules. For instance, compound **7** (Figure 9) with a molecular weight of 215 rescued splenocyte proliferation by 93% at 100 nM in CFSE proliferation assays[86]. Further modifications of compound **7** include linking the serine or threonine amino acid to the 1,2,4-oxadiazole structures through a urea group and replacing the original serine and asparagine side chains with structurally similar side chains, like threonine and glutamine. The derivative compound **8** (Figure 9) gave

splenocyte proliferation rates of 91% while no further activity data have been provided[87]. SAR demonstrates that the amidation of the C-terminal carboxyl group and the methylation of the N-terminal hydroxy group would reduce the activity. In addition to the 1,2,4-oxadiazole scaffold, similar modifications were implemented in 1,3,4-oxadiazole (thiadiazole) scaffold. In a pair of structurally similar compounds, the compound with an 1,3,4-oxadiazole ring is more potent than that with a 1,3,4- thiadiazole ring. Other modifications focus on altering the asparagine side chain into aspartic acid, glutamic acid and glutamine side chains. However, the effect on the activity of both 1,2,4-oxadiazole and 1,3,4-oxadiazole compounds is uncertain. As a positive example, compound **9** (Figure 9) gave splenocyte proliferation rates of 92% in mouse splenocyte proliferation rescue assays[88].

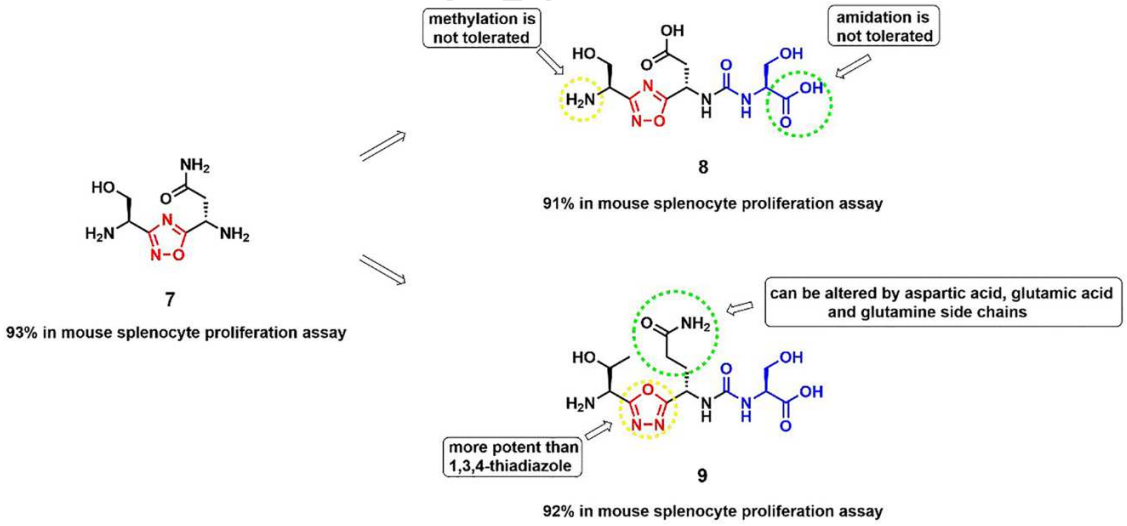


Figure 9. The structures and SAR of compound **7**, **8** and **9**.

Further researches led to the identification of 3-substituted oxadiazole structures

reported in patent WO2016142886 and WO2016142894. The substituent groups include aliphatic, aromatic and heterocyclic rings with different sizes. SAR of 3-substituted 1,2,4-oxadiazole structure appeares to tolerate pyrazine, piperidine and morpholine rings. For example, compound **10** (Figure 10) with a pyrazine ring showed the highest 92% rescue in the above mouse splenocyte assays[89]. Coincidentally, 1,3,4-oxadiazole compound **11** (Figure 10) with a piperazine substituent is also highly active compared to other 3-substituted 1,3,4-oxadiazole compounds [90].

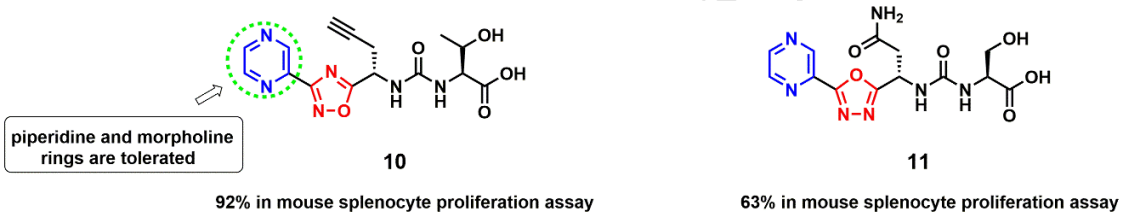


Figure 10. The structures and SAR of compound **10** and **11**.

In addition, 124 compounds with the 1,2,4-oxadiazole scaffold and 55 compounds with the 1,3,4-oxadiazole scaffold were synthesized with various amino acid side chains. Surprisingly, the activity of the 1,2,4-oxadiazole compound **12** (Figure 11) with an alkylated glutamine side chain exceeds the activity of AUNP-12 with a 119% rescue of splenocyte proliferation at 100 nM[87]. Activity data of 1,2,4-oxadiazole scaffold also shows that attaching another amino acid and esterification at the C-terminal carboxyl would lead to a decline in activity. Moreover, substitution of the urea group with a sulfonyl group resulted in decreased activity while sulfonylurea groups could restore partial activity. In contrast, the overall activity of 1,3,4-oxadiazole compounds is lower.

The most potent compound **13** (Figure 11) gave 79% in mouse splenocyte proliferation CFSE based assay which is weaker than the original compound with serine, asparagine and threonine side chains (80.3% in mouse splenocyte proliferation assay)[91].

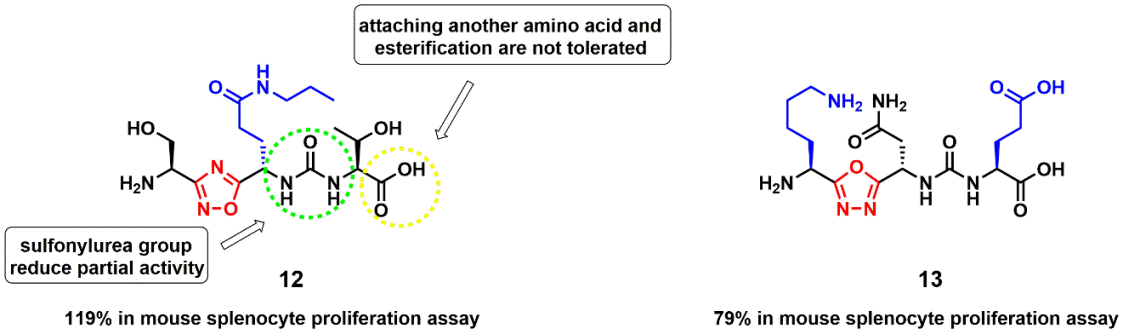


Figure 11. The structures and SAR of compound **12** and **13**.

4.2 Small molecules as inhibitors of the PD-1/PD-L1 pathway

Sharpe *et al*. from Harvard University first screened small molecules containing sulfamonomethoxine and sulfamethizole scaffold (Figure 12) can inhibit PD-1/PD-L1 pathway in 2011 (Figure 12). The *in vitro* efficacy of these compounds was tested by using wild type PD-1^{c} T cells and PD-$1^{-/-}$ T cells. The results showed the sulphonamide derivatives have inhibitory activity at a concentration of 0-10 mmol/L. And these inhibitors rescued PD-1 mediated inhibition in PD-1 Tg cells only when PD-L2 was present. Sulphonamide derivatives also rescued PD-1 mediated inhibition of IFNγ secretion in the micromolar range in an IFNγ-release assay using transgenic mouse T-cells expressing PD-1. In terms of toxicity, the cytotoxicity of such compounds is weak because toxic compounds are eliminated in the initial screen using PD-1^{c} T cells.The

substitution patterns of sulfamonomethoxine (compound **13**) focus on the methoxypyrimidine part. The pyrimidine ring can be replaced by pyridine and substituted by methyl and phenyl groups. For sulfamethizole, the thiadiazole derivatives of compound **14** is active and the amino group can be replaced by carbamate (compound **16**) and ureido (compound **17**). Furthermore, compound **18** with a pyridine ring also showed good results in cell assays. As the first discovered small molecule inhibitors, sulphonamide derivatives are promising as lead compounds for further modification[92].

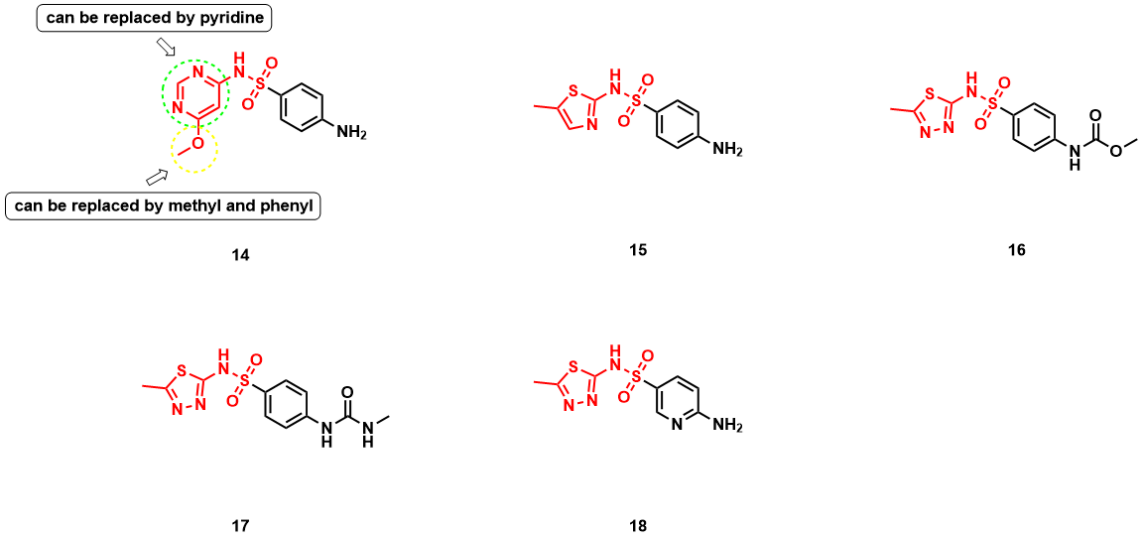


Figure 12. The structures of sulphonamide derivatives **14**, **15**, **16**, **17** and **18**.

Scientists from BMS have discovered a novel series of small molecule inhibitors targeting PD-L1[93]. These small molecule inhibitors contain a mono-ortho substituted biphenyl moiety which links to another phenyl ring via a methylene amine group. A novel HTRF binding assay using europium cryptate-labeled anti-Ig was established to detect the activity of these compounds. As a result, most of the synthesized compounds showed

PD-L1 affinity in the nanomolar range. As selected compounds, compound **19** and **20** (Figure 13) showed IC_{50} values of 146 nM and 18 nM respectively. There are no further *in vitro* and *in vivo* studies to support the biological activity of this series of compounds. Recently, the cocrystal structures of compound **19** and **20** (PDB IDs: 5J8O and 5J89) as PD-L1 antagonists were resolved by Holak group[94, 95] with the resolution of 2.3 Å and 2.2 Å respectively. In contrast to the antibodies with a 1:1 binding stoichiometry, the asymmetric unit in the crystal contains four protein molecules which could be organized into two dimers with one small molecule inhibitor locating at the center of each dimer. As shown in the Figure 13, the small molecule inserts into a deep, hydrophobic channel with a roughly cylindrical shape in the cross section which is formed by the dimerization of PD-L1. The channel is open to the solvent on the one side and is closed on the other side since BMS compounds provoke a rearrangement of the side chain of $_{A}$Tyr56 thereby closing the other side of the cleft (the protein molecules are annotated by subscripts A and B). Unlike antibody drugs directly occupying the PD-1 binding epitope on PD-L1 surface, further biological assays demonstrated that compounds **19** and **20** induce the dimerization of targets and play an important role in the homodimer stabilization by forming hydrophobic and electrostatic interactions with both PD-L1 proteins. Docking studies also showed dimerization was sterically feasible at the cell surface. Taking the BMS-202/PD-L1 complex as an example, the 2-methylbiphenyl group anchors at the bottom of the pocket. The distal phenyl ring within the biphenyl group forms a T-stacking

interaction with the sidechain of $_{A}$Tyr56 and is further stabilized via π-alkyl interactions with the sidechains of $_{A}$Met115 and $_{B}$Ala121. The methyl phenyl ring rotates about 45° with respect to the distal phenyl ring and is located in the center of the cleft. It forms hydrophobic interactions with $_{B}$Met115 and $_{A}$Ala121 and the its methyl group inserts into a pocket formed by $_{A}$Met115, $_{A}$Ala121 and $_{A}$Tyr123. The methyl group also serves to turn the phenyl groups out of coplanarity. The interactions of methoxy-pyridine moiety involve the π-π stacking interaction with $_{B}$Tyr56, the carbonyl-π interaction with the backbone of $_{A}$Ala121, the anion-π interaction with $_{A}$Asp122, the water-mediated lone-pair-π interaction with $_{A}$Phe19 and the water-mediated interactions of the methoxyl group with the sidechains of $_{A}$Asp122 and $_{A}$Lys124 and the backbone carbonyl of $_{A}$Tyr123. The extended N-(2-aminoethyl) acetamide moiety forms additional electrostatic interactions with the A monomer including a water-mediated interaction with the backbone carbonyl of $_{A}$Phe19. But due to the flexibility of the acetamide group and solvent-exposed environment, further interaction of the extended moiety differs between the monomers. The binding surface of BMS-8 and BMS-202 explicitly occlude the PD-1 binding site in both PD-L1 proteins, which provide a rational for their PD-1/PD-L1 blocking function. In addition, the internal interaction between the monomers also occupies the PD-1 binding epitope. After illuminating the binding mode of compound **19** and **20**, the previously proposed hot spots based on the hPD-1/hPD-L1 complex was redefined by authors. A single continuous area consisting of $_{L}$Tyr56, $_{L}$Met115, $_{L}$Ile116,

$_{L}$Ala121 and $_{L}$Tyr123 is treated as new hot spot. In conclusion, the substituted biaryl derivatives are the first series of true small-molecule inhibitors of PD-1/PD-L1 interaction and they clearly demonstrated that PD-1/PD-L1 interaction could be inhibited by non-peptide-derived small molecules with high potency. Although these molecules lack suitable drug-like properties to become lead compounds, their scaffold and binding mode provide a blueprint for the future small molecule modulators design.

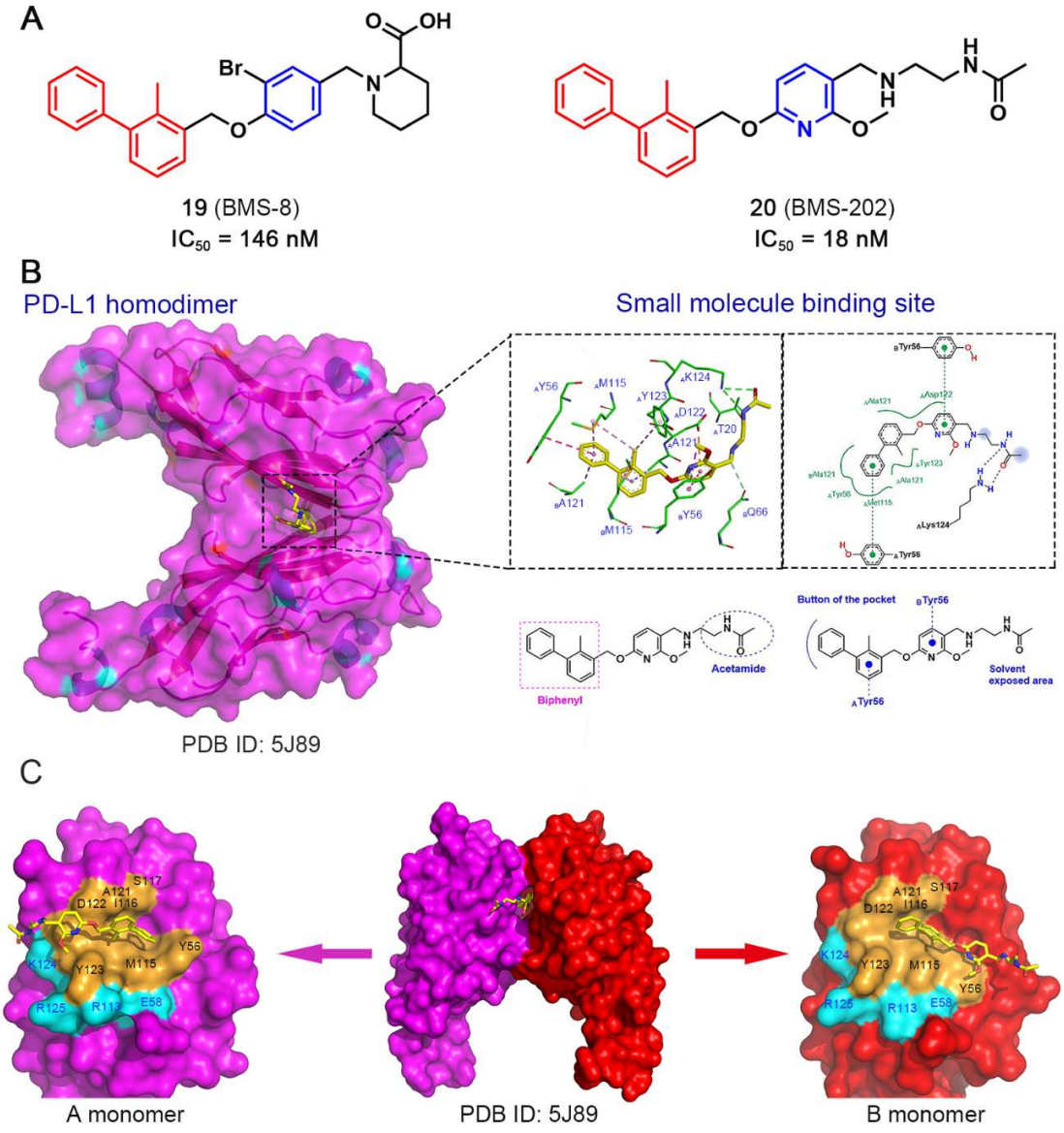


Figure 13. Detailed analysis of the crystal structures of PD-L1 and compound **19** and **20**. (A) The structures of compound **19** and **20**. (B) Detailed intermolecular interactions between compound **20** and the PD-L1 homodimer. The surface of PD-L1 molecules are expressed in purple while the small molecule inhibitors are colored by yellow. The green dash line indicates a π-π stacked interaction and the black dash line indicates a

conventional hydrogen bond in the 2D diagram. (C) "Hot spots" on the surface of each PD-L1 monomer. The BMS-202 interaction surface is colored by orange with corresponding residues labeled black while the previously described additional sites are depicted in cyan with corresponding residues labeled with blue.

Later Holak group published two novel cocrystal structures of BMS-37 (compound **21**) and BMS-200 (compound **22**) to a resolution of 2.35 Å and 1.7 Å respectively (PDB IDs: 5N2D, 5N2F)[94]. The IC_{50} values of both compound **21** and **22** (Figure 14) are less than 100 nM. Cocrystal structures showed compound **21** shares the similar binding mode with compound **19** and **20** since they all contain the (2-methyl-3-biphenylyl) methanol scaffold. However, compound **22** with a [3-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-methylphenyl] methanol scaffold surprisingly induces the significant conformation change of $_{A}$Tyr56 (Figure13). It is discovered that the 2,3-dihydro-1,4-benzdioxine moiety of BMS-200 provokes the rotation of $_{A}$Tyr56 thereby making the hydrophobic cleft accessible to solvent on both sides. It not only eliminates the steric hindrance but also provides additional interaction between $_{A}$Tyr56 and the 2,3-dihydro-1,4-benzodioxane moiety. The distal phenyl ring of BMS-200 interacts with $_{A}$Tyr56 through a π-σ interaction and forms a hydrogen bond with $_{A}$Ala121. The central methyl phenyl ring is also rotated roughly around 45° to make hydrophobic contacts with $_{A}$Met115, $_{B}$Met115 and $_{A}$Ala121 and alkyl interactions with $_{A}$Tyr123. The substituted 2,5-difluorophenyl ring is stabilized by π-π stacking interaction with $_{B}$Tyr56

and the halogen bonding between fluorine atom and $_{A}$Asp122. Furthermore, the extended (S)-4-amino-3-hydroxybutyric acid moiety make two hydrogen bonds with $_{A}$Thr20 and $_{B}$Gln66. In conclusion, the rotation of $_{A}$Tyr56 demonstrates that the binding site within the homodimer is flexible and provides further guidance for the design of small molecule inhibitors to PD-L1.

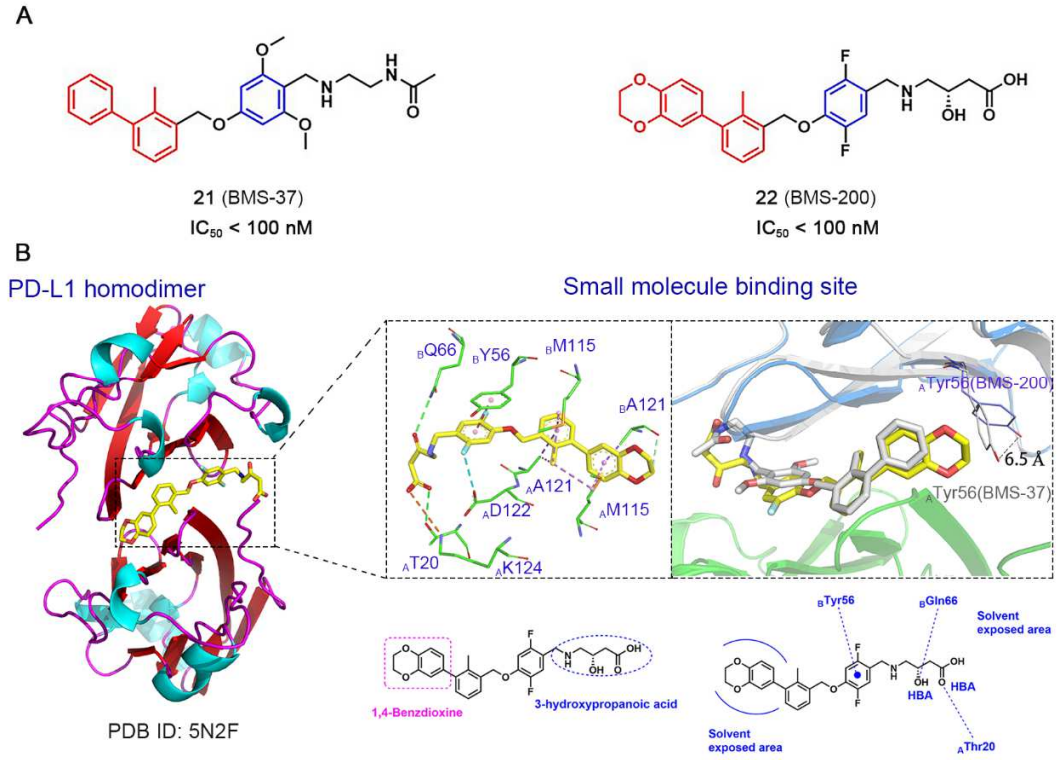


Figure 14. Detailed analysis of the crystal structures of PD-L1 and compound **21** and **22**. (A) The structures of compound **21** and **22**. (B) Left framed picture shows the detailed intermolecular interactions between compound **22** and the PD-L1 homodimer. The residues of PD-L1 molecules are expressed in green while compound **22** is colored by yellow. The green dash line indicates a conventional hydrogen bond and the purple dash

line indicates a π-π stacked interaction. Right framed picture shows the overlay of the BMS-37/PD-L1 and BMS-200/PD-L1 structures. Compound **21** is colored by silver and compound **22** is colored by yellow. The movement of $_{A}$Tyr56 (blue) that is induced by the 2,3-dihydro-1,4-benzdioxine group compared with the $_{A}$Tyr56 (silver) in the BMS-37/PD-L1 complex.

In the same year, another patent WO2015160641 was issued by BMS to expand the scope of patent protection[96]. In this patent, the modification focused on linking the benzonitrile or cyanopyridine to the central phenyl through an ether bond and the 2,3-dihydrobenzo[b][1,4]dioxine group was introduced to replace the distal phenyl ring in most of the patented compounds. Most compounds in this patent showed higher activity in the HTRF binding assay, with IC_{50} values being in the range of 0.6-10 nM. For example, the IC_{50} values of selected compound BMS-1001 (compound **23**) and BMS-1166 (compound **24**, Figure 15) are 2.25 nM and 1.4 nM, respectively. The Holak group resolved the crystal structures of these two compounds (PDB ID:5NIU, 5NIX) and carried out a series of experiments on them[95]. In the metabolic activity assay, compound **23** and **24** showed low toxicity to the Jurkat T cells (ECs) with EC_{50} values of 33.4 and 40.5 μM compared to the most toxic compounds (BMS-37 and -242) with EC_{50} values between 3-6 μM. Compound **23** and **24** are also able to restore the activation of effector Jurkat T-cells with higher EC_{50} values in three-digit nanomolar range and lower maximal cell activation levels compared to the clinical antibodies (EC_{50} = 0.333-1.15

nM). Moreover, compound **23** and **24** can dose-dependently abrogate the inhibitory effect of soluble PD-L1 (sPD-L1) on the activation of T cells through interacting with cell surface PD-1. The structural basis of compound **23** and **24** with PD-L1 is similar with compound **20** that provokes the rotation of $_{A}$Tyr56. The most obvious difference is the benzonitrile moiety occupies the pocket composed of $_{B}$Arg113, $_{B}$Tyr123, $_{B}$Arg125 and $_{A}$Asp61. It provides additional hydrophobic contacts and hydrogen bonds between BMS compounds and PD-L1, involving a π-π stacking interaction with $_{B}$Tyr123 and hydrogen-bond interactions with the side chain of $_{B}$Arg125. The extended moieties of compound **23** and **24** also provide additional hydrogen bonds to increase the affinity. After revealing the binding mode of BMS compounds, authors also identified a two aromatic ring system as the minimal fragment responsible for the PD-L1 binding which is helpful for future small molecule inhibitors design. Docking simulation of compound **23** and **24** at separate PD-L1 monomers showed the same energetically favorable binding mode observed in the crystal structure of $_{A}$PD-L1, suggesting a model in which BMS compounds transiently bind the first PD-L1 to form a preformed complex and recruit the second PD-L1 molecule later. If the model is verified experimentally, the development of BMS compounds would be directed to monomeric binders.

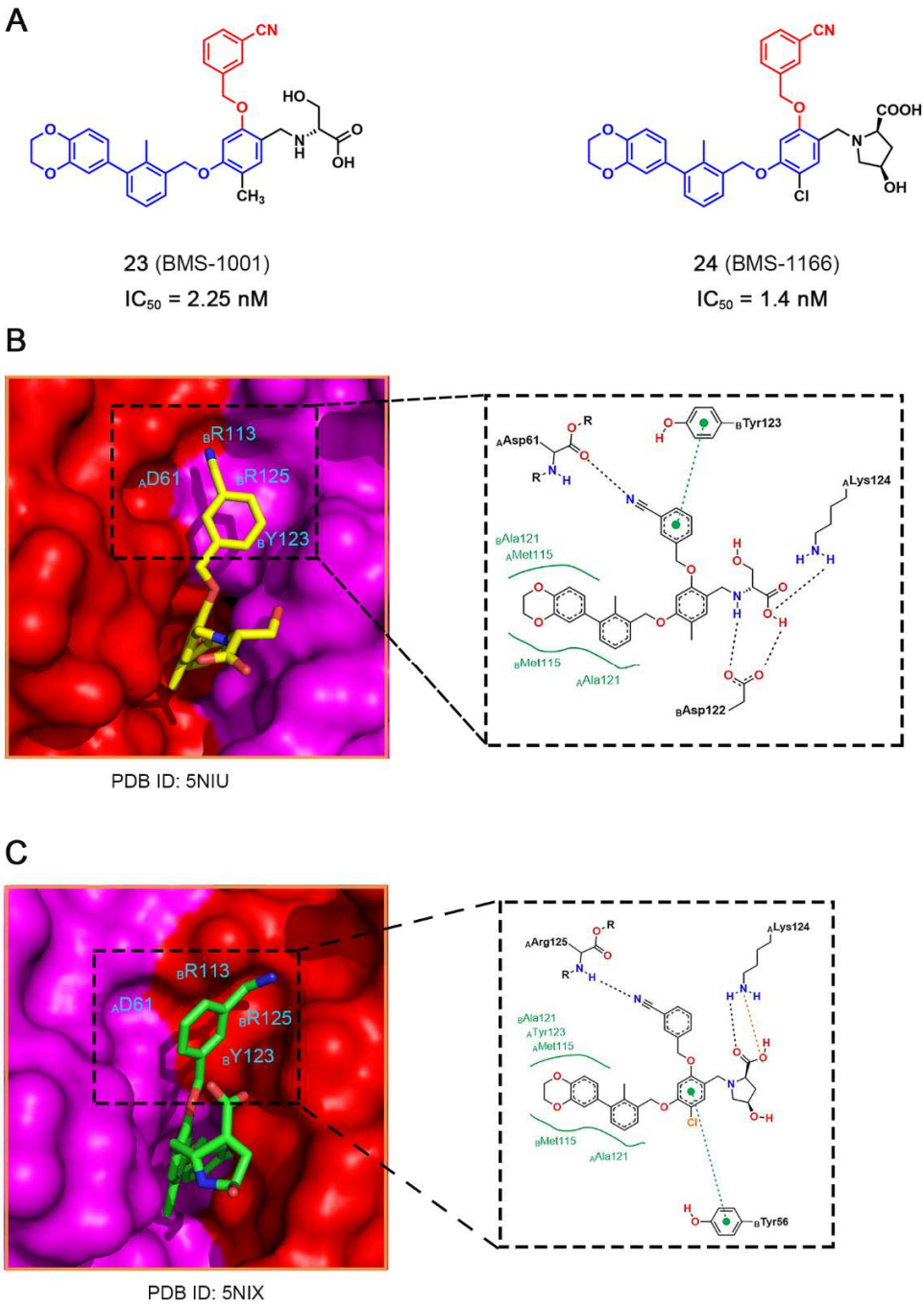


Figure 15. Detailed analysis of the crystal structures of PD-L1 and compound **23** and **24**. (A) The structures and activities of compound **23** and **24**. (B)(C) Binding pocket of benzonitrile group and detailed intermolecular interactions between compound **23** and **24**

with PD-L1. The green dash line indicates a π-π stacked interaction, the black dash line indicates a conventional hydrogen bond and the orange dash line indicates a charge-charge interaction.

BMS researchers further explored the biphenyl scaffold in patent WO2017066227 aiming to obtain more active compounds[97]. Based on the structures of the above compounds, explicit examples in this patent extend a pyrrolidine ring from the distal phenyl ring via an ether bond. The purpose of this modification may be similar with the benzonitrile to interact with the residues exposed to solvent on the other side of the hydrophobic channel. The selected highly active compounds **25** (Figure 16) have excellent IC_{50} values of 0.5 nM in the HTRF binding assays. The selectivity assays of BMS compounds on PD-1/PD-L2 and CD80/PD-L1 interactions demonstrated these compounds work as both PD-1/PD-L1 and CD80/PD-L1 interaction blockers. In addition to adding long-chain substituents to the distal phenyl ring, various heteroaromatic rings are introduced to replace the distal or proximal phenyl ring of the biphenyl group in the recent BMS patent[98] As a selected compound, compound **26** showed the IC_{50} value less than 10 nM in the HTRF binding assays. No further biological assays have been reported to support their activities.

Figure 16. The structures and activities of compound **25** and **26**.

Recently, Incyte company published several novel series of immunomodulating inhibitors of the PD-1/PD-L1 interaction. As shown in Figure 17, the core scaffold structure of these compounds is a biphenyl group which is similar to the BMS compound, and the difference lies in the aromatic ring and the connecting chain attached to biphenyl. Compound **27** (Figure 17B) from patent WO2017106634[99] obtains a central pyridine ring and a characteristic amide linker instead of the ether bond of the above compounds. The IC_{50} value of the compound **27** in the HTRF assay is less than 10 nM. SAR shows the proximal phenyl ring of the biphenyl group favors substitution of cyano group while the distal phenyl ring can be replaced by the 1,4-benzodioxan. Moreover, the 4-position of the central pyridine ring can tolerate small substituents. Compound **28** (Figure 17B) from patent WO2018013789 obtains a pyridone ring and showed IC_{50} value less than 100 nM in the HTRF assay. Activity data shows methylation of the amino group in the pyridine is well tolerated while ethylation could result in the decline of activity[100].

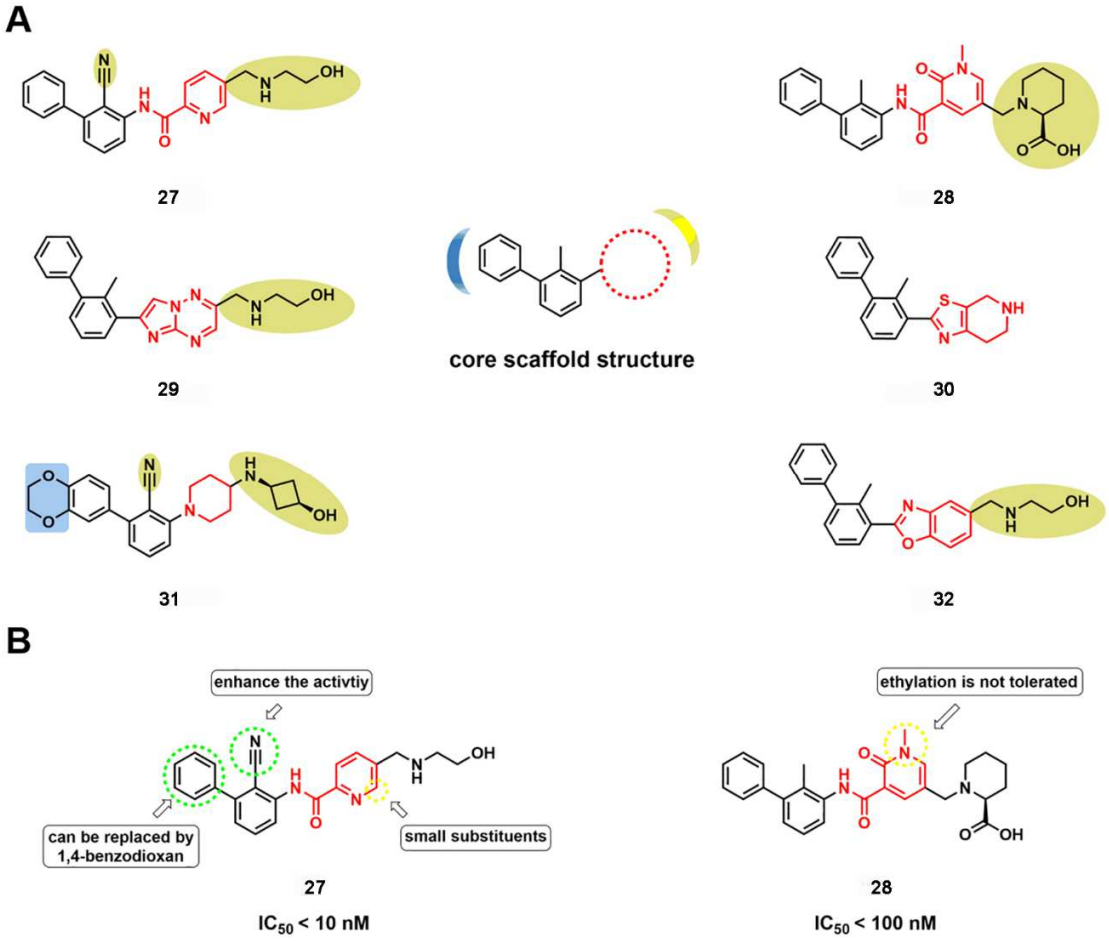


Figure 17. (A) The core scaffold structure and selected examples of Incyte compounds. (B) The structures and SAR of compounds **27** and **28**.

In their second approach, Incyte researchers rigidified the linker between the biphenyl group and the aromatic ring. Several series of fused-heterocycle were introduced to replace the aromatic ring and the amide chain. Some highly active compounds showed IC_{50} values less than 100 nM in the HTRF binding assay. For example, compound **29** (Figure 18) obtains a [1,2,4]triazolo[1,5-a]pyridine scaffold[101]. SAR showed the halogen atom can be replaced by the cyano-containing aliphatic chains.

The 4,5,6,7-tetrahydrothiazolo[5,4-c]pyridine scaffold of compound **30** (Figure 18) can extend hydroxyl or carboxyl-containing aliphatic chains[102]. Further analysis showed the proximal phenyl ring of the biphenyl group can be replace by pyridine ring and the distal one tolerates small substituents. Compound **31** (Figure 18) obtains a central piperidine ring which link another cycloalkane or heterocycle via an amino group[103]. Substituents can be introduce into the 6- and 7-positons of the benzo[d]oxazole moiety in compound **32** (Figure 18)[104]. Similar with the modification of Incyte compounds, ChemoCentryx researchers rigidified the linker through forming fused-heterocycles with the proximal phenyl ring of the biphenyl group. They have applied to patent another series of compounds with a 2,3-dihydrobenzodioxin structure[105]. In the same time, a novel enzyme-linked immunosorbent assay (ELISA) has been established to measure the inhibitory activity of compounds targeting PD-L1. The IC_{50} values of these compounds were reported to be in the nanomolar range. For example, compound **33** (Figure 18) exhibits high *in vivo* efficacy (IC_{50}<100 nM) as a PD-L1 antagonist. SAR favors the cyano group on the pyridine ring and the S-configuration of the chiral carbon in the dihydrobenzodioxin. Moreover, small substituents like halogen are well tolerated in the distal phenyl ring and the amino group can be replaced by N-containing heterocyclic group.

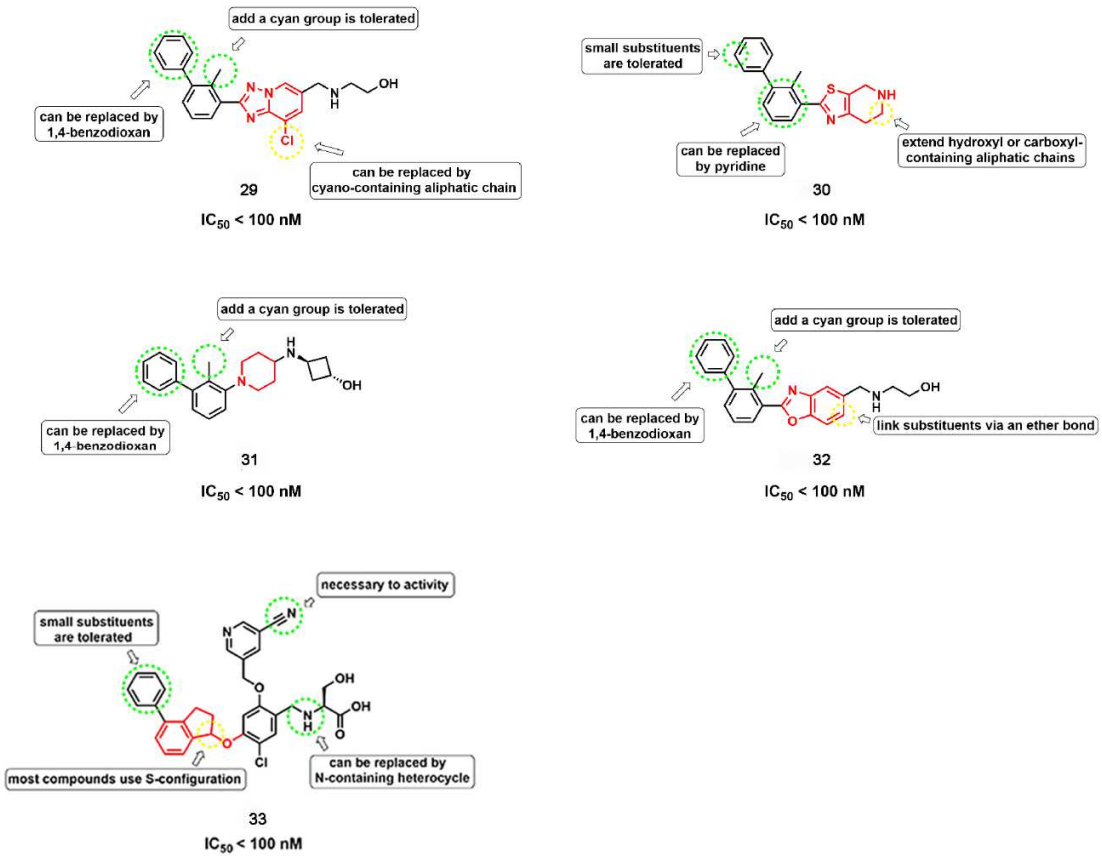
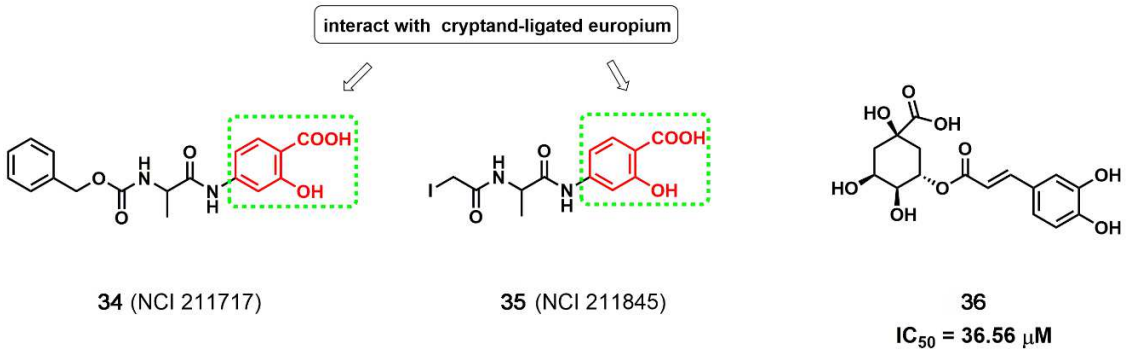


Figure 18. The structures and SAR of compounds **29**, **30**, **31, 32** and **33**.

Although traditional high-throughput screen and fragment-based lead discovery are helpful to identify lead compounds, the large and hydrophobic interface of PD-1/PD-L1 considerably increase the chances of discovering false positive hits. Recently Hanley *et al.* have described the salicylic acids, an extremely common compound subclass in screening libraries, interfere with the time-resolved fluorescence resonance energy transfer (TR-FRET) for the PD-1/PD-L1 assay in a high-throughput screening. The result indicated this scaffold was promising to inhibit the PD-1/PD-L1 interaction and NCI 211717 (compound **34**) and NCI 211845 (compound **35**, Figure 19) are representative

examples. However, later researches revealed that the large signal enhancement was principally induced by the interaction of the salicylate chelator moiety with the cryptand-ligated europium FRET donor, which indicated salicylates appeared as false positive hits[106]. Han *et al.* also utilized the appropriate screening method, surface plasmon resonance (SPR) technology, to evaluate the affinity and competitive inhibition of caffeoylquinic acid compounds (CQAs) against PD-1/PD-L1. Kinetic models demonstrated the affinities of CQAs with PD-1 and PD-L1 ranged from 0.50 to 81 μM, which were weaker but equivalent to the affinity between PD-1 and PD-L1 (0.17 μM) and were similar with the $_{D}$-peptide antagonists (0.51–22 μM). The maximum inhibition rates and IC_{50} values of CQAs were also determined, suggesting mono-CQAs (compound **36**, Figure 19) with IC_{50} values of 36.56 μM were more effective than the di-CQAs to inhibit PD-1/PD-L1 pathway. These results suggested CQAs have the potential to block PD-1/PD-L1 interaction. It is worth noting that this study firstly used SPR technology to screen the small molecule inhibitors targeting PD-1/PD-L1 pathway. In contrast to the NMR-based AIDA and HTRF, where the proteins had to be modified in advance, SPR avoids these problems and is efficient to evaluate the binding affinities of small molecule inhibitors[107].



34 (NCI 211717) **35** (NCI 211845) **36**

IC_{50} = 36.56 μM

Figure 19. The structures of compound **34**, **35** and **36**.

In China, small molecule drug development targeting the PD-1/PD-L1 pathway has made remarkable progress. Institute of Materia Medica of Beijing disclosed a series of bromine substituted small molecules as PD-L1 inhibitors[108]. As a representative compound, compound **37** (Figure 20) inhib3its PD-1/PD-L1 interactions in the femtomolar range (IC_{50}=8×10^{-14} M) in the HTRF assay using Cisbio PD-1/PD-L1 binding assay kit. These compounds also work as active antagonists in the IFNγ-release assay in PBMC expressing PD-1. The results showed particular implementations can partially relieve the inhibitory effect of PD-L1 on IFNy at 10 nM. In preclinical models of allograft (xenograft) breast, colon, lung, melanoma and metastatic cancers, these small molecules exhibited promising anti-tumor activity, increased the proportion of several lymphocytes and had synergistic effects with chemotherapy. In addition, Sun *et al*. from China pharmaceutical university has discovered the derivatives of 2-substituted isonicotinic acid. Compound **38** (Figure 20) showed a 36% inhibition ratio at 10 μM in the PD-1/PD-L1 HTRF assay[109]. Guangzhou Maxinovel Pharmaceuticals also has

disclosed an aromatic acetylene or aromatic ethylene compound. Compound **39** (Figure 20) has a significant inhibitory effect (IC_{50}= 18 nM) on PD-1/PD-L1 interaction in the HTRF assay[110].

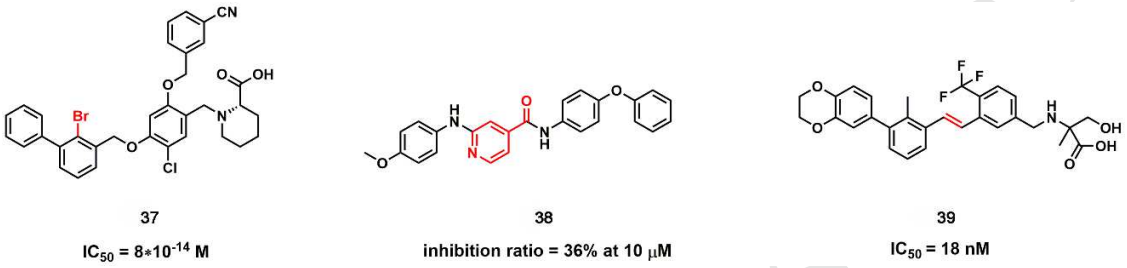


Figure 20. The structures of compound **37**, **38** and **39**.

5. Perspective

PD-1/PD-L1 inhibitors have become a highly popular research topic and achieved exciting results in the field of cancer treatment. So far, five mAbs targeting PD-1 or PD-L1 are already available and more antibody drugs are in clinic trials. They have showed unprecedented clinical efficacy to more than 20 cancer types. However, in contrast to the excellent clinical performance, the defects of antibody drugs such as poor oral bioavailability, low tumor permeability, low target selectivity and intractable irAEs limit its development. These defects mainly come from the nature of the antibody itself and its poor pharmacokinetic properties. Although the binding affinity of peptide-based and small molecule inhibitors on targets may be inferior to antibody drugs, their steerable pharmacokinetic properties and mature research system make it possible to overcome the existing problems of antibody drugs and become an alternative or complementary method

to regulate the PD-1/PD-L1 pathway.

With more and more crystal structures published, the design of small molecule inhibitors targeting the PD-1/PD-L1 pathway has a more solid foundation. First, the binding modes of mAbs and peptides can provide a blueprint for the design of small molecule inhibitors. Simplification from macromolecules to small molecules has become a more mature method for inhibitor design. Then, the published small molecule compounds can be roughly divided into two categories, one is small molecules/peptidomimetics containing oxadiazole/thiadiazole scaffold discovered by Harvard College and Aurigene Discovery Technologies, and the other containing a biphenyl scaffold has been widely researched. Designing derivatives and performing screening according to these important pharmacophores (oxadiazole and biphenyl) provide a promising direction in small molecule inhibitor design. Considering that cancer immunity is an extremely complicated process, single-target therapy may not be able to prevent cancer cells escaping from the immune system through other pathways and may even activate rapid amplification of other tumor genes, which in turn leads to drug resistance and the development, recurrence, and metastasis of tumors. Therefore, multiple-target therapy plays an increasingly important role in cancer treatment. Currently, the combination of anti-PD-1/PD-L1 antibodies and other therapies (immune checkpoint inhibitors, chemotherapy, radiotherapy, and vaccines) are ongoing in about 1,000 clinical trials to explore potentially synergistic therapeutic strategies[111]. The results of some

drug combinations are encouraging such as the combination of Pidilizumab and Rituximab for the treatment of recurrent follicular lymphoma is well tolerated and effective[112]. It is also worth noting that some combination therapies seem to lead to increased toxicity. For example, the combination of Ipilimumab and Nivolumab showed significantly increased toxicity compared to monotherapy[113, 114]. In this respect, small-molecule inhibitors targeting multiple signaling pathways have unique advantages and can display different therapeutic mechanisms compared to mAbs. It is possible to select another target based on the results of clinical drug combination, the research situation of targets and whether inhibitors have similar pharmacokinetic properties. For example, VISTA and PD-L1 are simultaneously expressed on the surface of some cancer cells and CA-170 in clinical trials is able to inhibit both of them, which demonstrates the potential of dual-target inhibitors.

Currently, the development of small-molecular inhibitors lags far behind antibody drugs. The lack of target structure information restricted the reasonable design of small molecule inhibitors targeting PD-1/PD-L1 pathway. PD-1/PD-L1 interaction is a protein-protein interaction with a large interaction surface and few conventional small molecule binding pockets. In order to discover hot spots on this paired protein, a more detailed analysis of target structures needs to be carried out. For this purpose, the combination of the structural biology and computer simulation techniques is of crucial importance and is necessary for the subsequent rational design of small molecules.

Computer-aided drug design (CADD) strategies may play an important role at this stage, but their methods must also be changed according to the characteristics of the PPI and cannot be rigidly adhered to traditional ways. According to the characteristics of PD-1/PD-L1 interactions, build focused libraries has important implications for improving the hit rate of compound screening because conventional small molecule databases may be difficult to find well-structured lead compounds. In addition, as several hot spot residues on both PD-1 and PD-L1 have been identified, fragment-based drug design and other methods may also play a unique role in this aspect.

Build efficient and accurate screening systems is also of great importance for small molecules design. As a protein-protein interaction, the *in vitro* screening strategy of PD-1/PD-L1 is different from kinase, GPCR and other traditional targets. The use of biophysical methods, such as SPR, isothermal titration calorimetry (ITC), HTRF, TR-FRET and other methods to build a rapid screening system is critical. The screening at the cell level also needs to highlight the core status of immune regulation, which also depends on the cooperation of cell biology, structural biology and other disciplines.

Researches on small molecule inhibitors targeting the PD-1/PD-L1 pathway is still in the early stage, but it has good prospects. Small-molecule inhibitors can not only improve oral bioavailability, but also reduce production costs and irAEs. In addition, small molecule inhibitors targeting multiple signaling pathways can provide a novel mechanism for preventing immune evasion. As more and more crystal structures of

PD-1- and PD-L1-directed inhibitors have been published, the structures of the PD-1 and PD-L1 have been widely studied and several hot spots have been discovered. This laid a solid foundation for drug discovery, and the combination of structural biology and computer simulation technology can accelerate this progress. However, there are still problems to be resolved. For example, the mechanism of PD-1/PD-L1 pathway and the cooperation and antagonism between the PD-1/PD-L1 and other targets has not been thoroughly studied. In terms of drug design, the flat and hydrophobic β-stands also bring difficulties in drug design. Therefore, establishing a reasonable research system, ensuring the activity and selectivity of compounds and exploring the possibility of targeting multiple targets will be critical tasks for the successful development of potent PD-1/PD-L1 inhibitors.

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Notes

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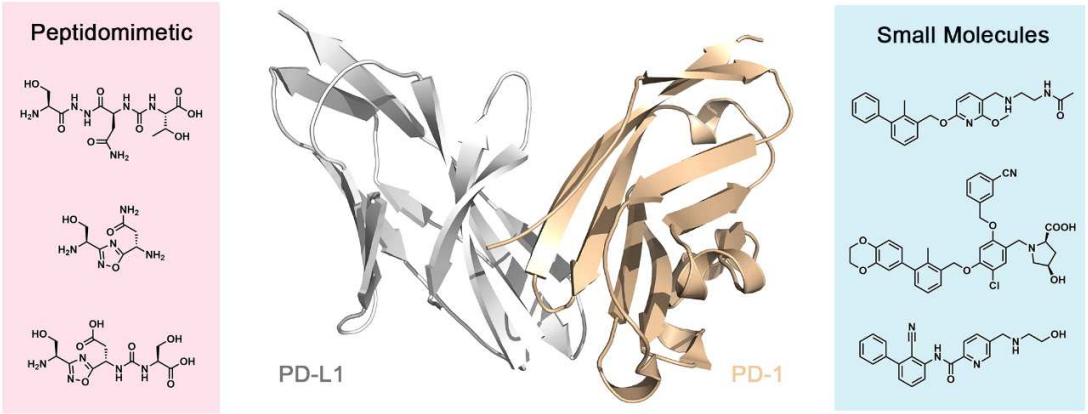
Graphical abstract



Highlights:

- Cancer immunotherapy has achieved great success in recent years.
- The discussed monoclonal antibodies, peptidomimetics and patented small molecules.
- Analysis of the crystal structures of monoclonal antibodies and small molecules.
- The structure and activity relationship of representative small molecules.