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Development of small-molecule immune checkpoint inhibitors of PD-1/PD-L1 as a new therapeutic strategy for tumour immunotherapy

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ABSTRACT
Cancer immunotherapy has been increasingly utilised to treat advanced malignancies. The signalling network of immune checkpoints has attracted considerable attention. Immune checkpoint inhibitors are revolutionising the treatment options and expectations for patients with cancer. The reported clinical success of targeting the T-cell immune checkpoint receptors PD-1/PD-L1 has demonstrated the importance of immune modulation. Indeed, antibodies binding to PD-1 or PD-L1 have shown remarkable efficacy. However, antibody drugs have many disadvantages, such as their production cost, stability, and immunogenicity and, therefore, small-molecule inhibitors of PD-1 and its ligand PD-L1 are being introduced. Small-molecule inhibitors could offer inherent advantages in terms of pharmacokinetics and druggability, thereby providing additional methods for cancer treatment and achieving better therapeutic effects. In this review, we first discuss how PD-1/PD-L1-targeting inhibitors modulate the relationship between immune cells and tumour cells in tumour immunotherapy. Second, we discuss how the immunomodulatory potential of these inhibitors can be exploited via rational combinations with immunotherapy and targeted therapy. Third, this review is the first to summarise the current clinical and preclinical evidence regarding small-molecule inhibitors of the PD-1/PD-L1 immune checkpoint, considering features and responses related to the tumours and to the host immune system.

Introduction
The immune system plays a very important role in fighting diseases, such as cancer. However, cancer cells usually develop a way to repress the function of the immune system to continue their own growth. One such mechanism is modifying the expression of co-stimulatory and co-inhibitory molecules expressed on immune cells.

According to the data from WHO more than 8.8 million people die of malignant tumours every year worldwide. The number is still growing, showing the serious threat to human health. Studies have shown that the immune system plays a key role in maintaining the homeostasis of the human body. Especially in the past decade, with increasing numbers of cancer immunisation drugs approved by the FDA, immunotherapy has become a current topic of intensive research, with a particular focus on cancer therapy. The basic principle is to use the immunological checkpoint inhibitors to block the transmission of the suppression signal and direct stimulation to activate cytotoxic T lymphocytes, thus achieving anti-tumour effects. Therefore, cancer immunotherapy was considered one of the four most promising scientific advances in 2015. Among the breakthroughs of tumour immunotherapy, immune checkpoint inhibitors are the fastest and most mature. Immuno-therapy via checkpoint inhibition is a different treatment approach to chemotherapy and targeted agents: instead of acting directly on the tumour to induce tumour cell death, checkpoint inhibitors enhance or initially stimulate anti-tumour immune responses to eliminate cancer cells. Immunological checkpoint molecules are mostly B7/CD28 and TNF/TNF receptor superfamily members. They are immunostimulatory or co-suppressor molecules, which are used to affect the body’s immune response upon real-time coordination with lymphocyte activity. In the body’s immune regulation, each checkpoint molecule produces a complex immunomodulatory effect. These molecules are of various types. At present, the best-studied immunological checkpoints include cytotoxic T lymphocyte antigen 4 (CTLA-4), and programmed cell death 1/programmed cell death 1 ligand (PD-1/PD-L1).

In addition to this classic checkpoint, many new checkpoints have been found as potential immune targets in recent years, for example 4–1BB [1] (cluster of differentiation 137, CD137), OX40 [2] (cluster of differentiation 134, CD134), CD27 [3] (cluster of differentiation 27), LAG3 [4] (lymphocyte activation gene 3 protein), CD224 [5] (cluster of differentiation 224), TNFRSF25 [6] (tumour necrosis factor receptor superfamily 25) and so on. Drugs targeting these checkpoints can block the transmission of the inhibitory signal and induce anti-tumour effects in T cells. Among these drugs, checkpoint protein inhibitors have occupied a larger market share than the rest; the common ones include PD-1, CTLA-4, IDO and TIM3. The checkpoint proteins PD-1 and CTLA-4, especially the former, are the two most studied in current clinical research. They have been successfully applied in clinical practice, which has greatly promoted the progress of tumour immunotherapy.

Programmed cell death-1 (PD-1), also known as CD279, is an immune checkpoint that limits excessive immune responses to antigens and guards against autoimmunity [7]. PD-1 expression can be found in all kinds of immune cells including T lymphocytes.
B lymphocytes, natural killer T cells, activated monocytes and dendritic cells (DCs) [8]. However, PD-1 is expressed only on the surface of activated T lymphocytes, not on resting T cells. Therefore, PD-1 can serve as an activation marker on T cells. It functions as an intrinsic negative feedback system to prevent the activation of T cells, which, in turn, reduces autoimmunity and promotes self-tolerance. In addition, PD-1 is known to play a critical role in the suppression of antigen-specific T cell response in diseases, such as cancer and viral infection. In tumours, PD-1 is highly expressed in dysfunctional/exhausted effector T cells (both CD8+) and in regulatory T cells (Treg) [9].

PD-1 binds to two ligands from the B7 family: PD-L1 (B7-H1, CD274) [10] and PD-L2 (B7-DC, CD273) [11]. PD-L1 has also been shown to interact with CD80 [12]. The interaction of PD-L1 and CD80 expression on immune cells has been shown to be inhibitory. While PD-L1 is basally expressed on both haematopoietic cells (macrophages, DCs, B cells and T cells) and non-haematopoietic cells (epithelial and endothelial cells), PD-L2 expression is limited to immune cells (macrophages, DCs and mast cells) [13]. When PD-1 expressed on T cells comes into contact with its ligands, its functional activities in response to antigenic stimuli are reduced, including proliferation, cytokine secretion and cytolytic activity [14]. PD-1/PD-ligand interactions down-regulate immune responses during the resolution of an infection or tumour or during the development of self-tolerance [15] (Figure 1).

Immune cells and certain human cancers express a ligand on their cell surface, PD-L1, that binds to its cognate receptor, PD-1 and is present on the surface of the immune system’s T cells. Cell surface interactions between tumour cells and T cells via PD-L1/PD-1 molecules result in T cell inactivation and the inability to mount an effective immune response against the tumour. It has been previously shown that the modulation of the PD-1 mediated inhibition of T cells by either anti-PD-1 antibodies or anti-PD-L1 antibodies can lead to T cell activation, which results in the observed anti-tumour effects in tumour tissues. Currently, several approved mAbs are used in cancer treatment. Those mAbs work by targeting different receptors or immune checkpoints, especially PD-1 and PD-L1. Examples include nivolumab [16] and pembrolizumab [17].

Nivolumab, developed by Bristol-Myers Squibb, is a high-affinity, fully humanised IgG4 antibody that binds to PD-1 and was developed by Merck. In September 2014, the antibody was the first PD-1 blocker approved by the FDA for the treatment of advanced, unresectable or drug-resistant melanoma and metastatic non-small cell lung cancer. A Phase I clinical trial (KEYNOTE-001) of single-use pembrolizumab treatment for non-small cell lung cancer (NSCLC) showed that the toxicity of pembrolizumab was accepted by patients, that the drug has a low rate of grade 3–5 adverse events, that patients with advanced NSCLC showed good toleration of the drug and that patients with high expression of PD-L1 exhibited a significant curative effect [20]. Pembrolizumab is the first immunotherapeutic drug approved by the FDA for non-small cell lung cancer after previous treatment with chemotherapy has failed. Pembrolizumab interferes with the binding of PD-1 to its ligand PD-L1, eventually leading to tumour tissue being recognised and killed by T cells. Therefore, patients should be tested for PD-L1 expression before treatment with pembrolizumab to determine the suitability of its use [21].

Apart from nivolumab and pembrolizumab, some anti-PD-1 inhibitors [22] have been used against different cancers in clinical trials and have exhibited good anti-cancer effects and safety profiles [23]. Examples include AMP-224, MEDI0680 (AMP-514) and Pidilizumab, among others. (1) AMP-224 is an immune checkpoint inhibitor that targets PD-1. It is a humanised IgG antibody fusion drug approved by the FDA for the treatment of advanced, unresectable or drug-resistant melanoma and metastatic non-small cell lung cancer. A Phase I clinical trial (KEYNOTE-001) of single-use pembrolizumab treatment for non-small cell lung cancer (NSCLC) showed that the toxicity of pembrolizumab was accepted by patients, that the drug has a low rate of grade 3–5 adverse events, that patients with advanced NSCLC showed good toleration of the drug and that patients with high expression of PD-L1 exhibited a significant curative effect [20]. Pembrolizumab is the first immunotherapeutic drug approved by the FDA for non-small cell lung cancer after previous treatment with chemotherapy has failed. Pembrolizumab interferes with the binding of PD-1 to its ligand PD-L1, eventually leading to tumour tissue being recognised and killed by T cells. Therefore, patients should be tested for PD-L1 expression before treatment with pembrolizumab to determine the suitability of its use [21].

Figure 1. Mechanism behind the inhibition of interaction between PD-1 and its ligand PD-L1 to treat tumour immune. Abbreviations: PD-1: programmed cell death-1; PD-L1: programmed cell death receptor ligand-1; MHC: major histocompatibility complex; TCR: T-cell receptor.
In addition, AMP-224 is well tolerated and does not show any adverse effects at the maximum dose of 30 mg/kg. Preclinical test results showed that AMP-224 exhibited good anti-tumour effects in a primary tumour model in mice. Other relevant clinical trials are still underway and have not been completed [25]. (2) MEDI0680 (AMP-S14) is a humanised IgG anti-PD-1 monoclonal antibody. It enhances T cell cytotoxicity by activating PD-1 [26]. There are three ongoing clinical trials evaluating the efficacy of MEDI0680 (AMP-S14): NCT02271945, to evaluate the use of MEDI-551 in combination with AMP-S14 in the treatment of invasive B-cell lymphoma in cases of recurrence or cases in which earlier stage chemotherapy failed [27]; NCT02013804, to evaluate the dose-dependent, safety, tolerability, immunogenicity and anti-tumour activity of AMP-S14 in adult patients with solid tumours; and NCT02118337, to evaluate the safety and tolerability of AMP-S14 in combination with MEDI4736 (PD-L1 antibody). (3) Pidilizumab, a humanised IgG1 kappa recombinant monoclonal antibody against PD-1, is currently in Phase II clinical trials. The presumed indications include large B-cell lymphoma, glioma and multiple myeloma. Studies have shown that the combination of pidilizumab and rituximab for the treatment of recurrent follicular lymphoma is effective and favourably tolerated by patients [22]. Some anti-PD-L1 inhibitors have also been used for different cancers in clinical trials and have exhibited good anticancer effect, including Avelumab (MSB0010718C), BMS-936559, Durvalumab (MEDI4736) and others. (1) Avelumab (MSB0010718C) is a fully humanised IgG1 antibody targeting PD-L1. Its main function is to inhibit the binding of PD-1 to PD-L1. Early studies suggested that its anti-tumour mechanism might also be related to antibody-dependent cell-mediated cytotoxicity and that it could be used for the treatment of ovarian and gastric cancer. Avelumab (MSB0010718C) is also being tested in the treatment of a variety of solid tumours, especially non-small cell lung cancer [28,29]. In addition, it is currently undergoing Phase I clinical trials for indications including breast cancer, colon cancer and kidney cancer [30]. (2) BMS-936559 is a PD-L1 specific, high-affinity, fully humanised IgG4 monoclonal antibody that can inhibit PD-L1 binding to PD-1 or CD80 [31]. The Phase I clinical trial of BMS-936559 began in 2008 and included 207 patients with cancers including NSCLC, melanoma, colorectal cancer, renal cell carcinoma, cervical cancer, pancreatic cancer, gastric cancer and breast cancer. The disease duration ranged from 2 to 111 weeks. The results showed that in patients who exhibited objective efficacy, the efficacy of BMS-936559 lasted for more than 1 year in eight patients. Continuing clinical data showed that BMS-936559-related adverse reactions are mild, including rash, diarrhoea, infusion reactions, allergic reactions and endocrine disorders (NCT00729664). (3) Durvalumab (MEDI4736) is an anti-PD-L1 inhibitor currently in a Phase III clinical trial for the treatment of non-small cell lung cancer and in a Phase I clinical trial for the treatment of melanoma. A Phase I clinical trial showed that Durvalumab had response rates of 39 and 5% for PD-L1-positive and PD-L1-negative patients, respectively [32]. Durvalumab is also used in the advanced squamous cell carcinoma biomarker-driven Lung-MAP test. Moreover, in China, at least four mAbs targeting PD-1 have been introduced in clinical trials and have been shown to be more effective for the treatment of metastatic melanoma and other cancers [29,33,34].

Since antibody drugs have disadvantages in aspects, such as production cost, stability and immunogenicity, there have been active drug discovery studies on low-molecular-weight immune checkpoint inhibitors of PD-1 and its ligand PD-L1. However, small-molecule PD-1/PD-L1 inhibitors with low molecular weight have only recently begun to be identified, in contrast to mAbs, which are proteins with high molecular weight (e.g. nivolumab has a molecular weight of 143.6 kDa). Until recently, few small-molecule PD-1/PD-L1 inhibitors have been reported. To our knowledge, only one patented PD-1/PD-L1 drug-like inhibitor has undergone clinical trials. In addition, there are many patents related to drug-like PD-1/PD-L1 inhibitors and to peptide and peptidomimetic PD-1/PD-L1 inhibitors that are currently undergoing preclinical research. However, no research review concerning small-molecule immune checkpoint inhibitors targeting PD-1/PD-L1 has been published until now. Therefore, we have summarised the progress in the research and development of relevant small-molecule PD-1/PD-L1 immune checkpoint inhibitors.

Role of the PD-1/PD-L1 pathway and its inhibitors in cancer

The immunosuppressive effects of the PD-1/PD-L1 signalling pathway play an important role in the pathogenesis and development of several immune disorders, such as autoimmune diseases, infectious immunity and tumour immunity. There are two hypotheses for the main mechanisms by which PD-1/PD-L1 participates in tumour immune escape. (1) In the inherent mechanism, the change in the genome or transcriptome of the tumour cells leads to high expression of PD-L1. Most commonly, the abnormal activation of the tumour-intrinsic Akt and STAT3 signalling pathways induces the high expression of PD-L1, thereby inhibiting the activation of cytotoxic T cells [35,36]. (2) The adaptability mechanism relies on a protective mechanism of tumour cells against the scavenging effect of immune cells in the immune microenvironment. Tumour cells overexpress PD-L1 in response to IFN-γ, which is secreted by a series of cells involved in tumour clearance (CD4+ Th1 cells, activated T cells, activated natural killer cells, etc.) [37]. In addition, IFN-γ increases indoleamine 2,3-dioxygenase (IDO) content, which has immunosuppressive effects in the TME and promotes PD-L1 expression in the tumour. Except for the tumour cell surface, TILs in TME also detect PD-L1 expression [14,38,39]. PD-1/PD-L1 is not only involved in the regulation of tumour immunity but also plays a role in the progress of tumourigenesis, growth and metastasis. In vivo studies show that high expression of PD-L1 can enhance tumour metastasis, leading to increased mortality in cancer patients. Studies have found the PD-1/PD-L1-related signalling pathway in solid and haematological malignancies including breast cancer and non-Hodgkin lymphoma [40–42].

In breast cancers, the PD-L1 signalling pathway may be involved in the negative regulation of anti-tumour immune responses due to the high expression of PD-L1 molecules in tumour-infiltrating lymphocytes (TILs). The researchers Ghebeh et al. [43] found that breast cancer patients exhibited PD-L1 up-regulation in tumour cells and that the expression levels of PD-L1 were positively correlated with tumour size and clinicopathological stage. Soliman et al. [44] monitored PD-L1 expression in breast cancer cell lines and confirmed that the expression of PD-L1 was highest in triple-negative breast cancer. Soliman et al. [44] reported the detection of significantly higher amounts of invasive, metastatic and drug-resistant factors in breast cancer cell lines overexpressing PD-L1 than in breast cancer cell lines expressing lower levels of PD-L1. Hasan et al. [45] found that the up-regulation of PD-L1 expression in breast cancer is also related to the tumour microenvironment. It was shown that the activation of the JAK/STAT signalling pathway by IFN-γ can affect the tumour cell expression of PD-L1 and, via MEK/ERK and PI3K/AKT, promote a proliferation pathway with increased expression of PD-L1.
In addition, the surface expression of PD-L1 on cancer cells has been described in several haematological cancers. For example it was demonstrated in primary non-Hodgkin lymphoma [46]. Unlike solid tumours, lymphomas are derived from the immune system, so the PD-1/PD-L1 pathway is more complex. The various subtypes of the lymphoma tumour microenvironment are also different. In NHL, abnormal expression of PD-L1 and PD-1 molecules was detected in diffuse large B-cell lymphoma (DLBCLs) and in T cell lymphoma [47,48]. Approximately, 20–30% of DLBCLs express PD-L1. It is expressed both in tumour cells and in non-tumour cells in the tumour microenvironment. According to dual staining for PD-L1 and PAX5, 10.5% of DLBCL tumour cells expressed PD-L1, and 15.3% of DLBCL tumour microenvironments expressed PD-L1. Accordingly, the expression of PD-L1 has some implications for the prognosis of DLBCL. A large study of 1200 DLBCL pathology specimens found that patients with PD-L1+ had a shorter overall survival than patients with PD-L1− [49]. The 3-year overall survival of DLBCL patients was short when the levels of soluble PD-L1 in the plasma were elevated [50,51]. Among T cell lymphomas, anaplastic large cell lymphoma (ALCL) and extranodal NK/T cell lymphoma express PD-L1 the most stable. ALK+ anaplastic large cell lymphoma (ALCL) cells overexpress PD-L1, and pathological specimens exhibit a PD-L1 positive rate of 34–100%. The up-regulation of PD-L1 expression in ALK+ anaplastic large cell lymphoma (ALCL) is due to the activation of the transcription factor STAT3 by the NPM-ALK fusion gene [52]. The NPM-ALK fusion gene also induces the production of IL-10 and TGF-β, while IL-10 activates the JAK/STAT signalling pathway and up-regulates the expression of PD-L1.

Tumour cells express PD-L1 under the action of various cytokines, which is related to tumour immune escape. Because PD-L1 expression is closely related to tumourigenesis and invasion in vivo and in vitro resistance to T cell-mediated lysis, it acts as an anti-apoptotic factor in the development of cancer cells [53]. During cancer development, activation of the PD-1/PD-L1 immune pathway can indicate that the tumour cells are exhibiting an adaptive mechanism of resistance to tumour infiltration lymphocytes involved in the anti-tumour T cell immune response. A molecule that binds to the PD-1 receptor can block its interaction with the ligands PD-L1 and PD-L2, releasing the PD-1 pathway-mediated inhibition of tumour immune responses. Therefore, PD-1/PD-L1 immune checkpoint inhibitors that inhibit the interaction between the PD-1 expressed on activated T cells and the ligand PD-L1/PD-L2 expressed on tumour cells are believed to inhibit tumour T cell immune surveillance [54,55], leading to the T cell-mediated death of malignant cells.

**Structures of PD-1/PD-L1 and their interaction**

PD-1 is a 55 kDa type I surface transmembrane glycoprotein receptor consisting of 288 amino acids [56]. The protein contains four domains: the extracellular domain is an IgV-like immunoglobulin superfamily domain, the cytoplasmic domain consisting of 95 residues, a handle consisting of 22 residues and a transmembrane domain. The cytoplasmic domain contains two tyrosine-dependent immune receptor signalling motifs, the immunoreceptor tyrosine-inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM). Unlike other family members of B7-CD28, PD-1 is usually present as a monomer, not a homodimer. The two ligands PD-L1 and PD-L2 of PD-1 are both transmembrane proteins consisting of 290 and 268 amino acid residues and belong to the B7 ligand family, with 34% homology. In addition, the two have a common structural basis, that is, their extracellular domain consists of two side-by-side domains, one IgV-like region and one IgC-like region, which binds directly to PD-1. The binding of PD-L1 or PD-L2 to PD-1 initiates antigen receptor signalling transduction, leading to the phosphorylation of two tyrosine signal motifs in the PD-1 cytoplasmic domain and thereby activating downstream signalling pathways and destroying the T cell metabolism and releasing other signal factors, ultimately undoing the immune function of T cells [57]. In addition, PD-L1/PD-L2 has a conserved intracellular domain consisting of 30 residues whose function is unknown [58].

The PD-1/PD-L1 interactions are protein–protein interactions (PPIs), which can control many biological processes, such as cell proliferation and growth, cell differentiation, signal transduction and programmed cell death. Therefore, PPIs are important and novel therapeutic targets [59]. However, the identification of small-molecule inhibitors that disrupt PPIs is a challenge, mainly because of the typical flatness, large size and non-contiguity of the interface between the interacting proteins and the flexibility of their surfaces. Despite the difficulties, several such targets have been successfully developed, including the B cell lymphoma 2 family [60,61]. Using antibodies or small-molecule inhibitors to block the PD-1/PD-L1 interaction can reduce inhibitory signalling, leading to a stronger immune response.

The diffraction resolution obtained for the PD-1/PD-L1 complex is 2.45 Å, with 1970 Å² embedded in the binding surface, as shown in the asymmetric unit of the complex crystal. Stoichiometric studies confirmed that PD-1 and PD-L1 form a 1:1 complex crystal in solution [62]. The PD-1/PD-L1 interaction domain is similar to that of the antibody IgV region and to that of T cell receptors, which is mediated by the GFCCβ-folded orthogonal structure region, and the two interacting structural regions achieve orthogonal binding by antigen-antibody binding sites, inducing conformational changes in PD-1 and PD-L1. PD-1 presents a β-sheet topology immunoglobulin (IgV) conformation with a unique disulphide bridge structure formed by Cys54 and Cys123, but lacking disulphide bonds present in other protein family members (CD28, CTLA-4 and ICOS). As with PD-1, the N-terminal domain of PD-L1 also has immunoglobulin IgV topology. The hydrophobic interactions between PD-1 and PD-L1 are mainly derived from the facing non-polar residues, including Val64, Ile126, Leu128, Ala132, Ile134 (PD-1) and Ile54, Tyr56, Met115, Ala121 and Tyr123 (PD-L1) [63].

**Small-molecule inhibitors under clinical development**

The small-molecule inhibitors currently in clinical trials – including their efficacy, therapeutic indications, sponsors and status – are shown in Table 1.

**Table 1. Small molecular inhibitor in clinical trials.**

<table>
<thead>
<tr>
<th>Small molecular inhibitor</th>
<th>Target</th>
<th>EC50</th>
<th>Indications</th>
<th>Company</th>
<th>Clinical Phase</th>
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<tr>
<td>CA-170</td>
<td>PD-1/PD-L1/PD-L2</td>
<td>17 nM</td>
<td>Advanced solid tumours or lymphomas</td>
<td>Curis/Aurigene</td>
<td>Phase I</td>
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interaction surface simulant library, and it has been used as an oral antagonist of the PD-L1/PD-L2 and VISTA/PD-1H checkpoint protein signalling pathways. In vitro studies showed that CA-170 exhibited cross-species antagonistic effects on the PD-1/PD-L1/PD-L2 or VISTA/PD-1H signalling pathways, and oral administration promoted tumour infiltration and peripheral T cell viability in a dose-dependent manner, thus, inhibiting syngeneic tumour growth in mice. Its EC50 was 17 nM. Preclinical in vivo data demonstrated that CA-170 can induce effective proliferation and IFN-γ production by T cells that are specifically suppressed by PD-L1 or VISTA. In addition, CA-170 also exhibits anti-tumour effects similar to those of anti-PD-1 or anti-VISTA antibodies in multiple tumour models in vivo. In preclinical toxicity studies, CA-170 exhibited safety when administered on a once daily oral dosing schedule at multiple dose levels. Curis is currently investigating CA-170 for the treatment of advanced solid tumours and lymphomas in patients in a Phase 1 trial. However, the structure of compound CA-170 has not yet been reported (ClinicalTrials.gov Identifier: NCT02812875).

Small-molecule inhibitors in preclinical development

There are several small-molecule inhibitors in preclinical development, including drug-like inhibitors and peptide or peptidomimetic inhibitors (Table 2). Their structures are shown in Figure 2.

**Compound 1**

A series of small-molecule drug-like inhibitors have been reported to target immunological checkpoint proteins. Patent WO 2015/034820 A1 was filed by Bristol-Myers Squibb in 2015 [64]. Bristol-Myers Squibb claimed that compounds having the formula detailed in Figure 2 are capable of inhibiting PD-1/PD-L1 interactions and could, therefore, be useful in treating, preventing or slowing the progression of virological diseases or disorders, such as HCV and cancer. The structures of four chosen compounds (BMS-1/BMS-2/BMS-79/BMS-196) of the 297 examples of inhibitors of PD-1/PD-L1 interactions are shown in Figure 3. All four inhibitors have IC50 values between 0.006 and 0.10 μM, as evaluated by the homogenous time-resolved fluorescence (HTRF) binding assay.

<table>
<thead>
<tr>
<th>Table 2. Small molecular inhibitors in preclinical development.</th>
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<td>Inhibitors</td>
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<tr>
<td>Small molecular drug-like inhibitors</td>
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<td>Small molecular peptides or peptidomimetics inhibitors</td>
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Zak et al. [65] revealed that, because of a high degree of overlap with the PD-1/PD-L1 protein–protein interaction surface, these inhibitors render PD-1 and PD-L1 unable to perform their normal mutual function, blocking the signal pathway. Then, based on the putative binding modes, structure-based drug design techniques can be used to identify inhibitors with novel scaffolds. BMS202 and BMS-8 (Figure 3) were used as model compounds to elucidate the principle of action of these small-molecule inhibitors. This study indicates that these small-molecule inhibitors act on the PD-L1 protein surface, causing PD-L1 to form dimers, and BMS202 and BMS-8 are bound to the column-shaped hydrophobic chamber created by the two dimers of PD-L1 molecules.

**Compound 2**

Saksukar et al. [66] applied to patent the small-molecule compounds 1,2,4-oxadiazole and 1,2,4-thiadiazine and their derivatives, which are capable of inhibiting the PD-1/PD-L1/PD-L2 signalling pathway (Figure 2 Compound 2). Their research found that such small molecule drugs can inhibit the PD-1/PD-L1/PD-L2 signalling pathway effectively in a variety of cancers, such as skin cancer, head and neck cancer, pancreatic cancer, or intraocular malignant, and endometrial cancer, in addition to bacterial, fungal and viral infectious diseases, such as, influenza, malaria and herpes virus, thereby reversing the inhibitory status of the immune system. Based on the above compounds containing diaclyhydrazine and urea structures, Aurigene researchers also designed and synthesised a series of nucleus-based structures, including triazole and thiodiazole that contained diaclyhydrazine and/or urea tripeptide derivatives (Figure 2 Compounds 3–6). In a splenocyte proliferation assay, the researchers studied the effect of stimulating splenocytes with anti-CD3/CD28 at compound concentrations ranging from 100 nmol/L to test the anti-PD-L1 activity of the compounds in the presence of the recombinant ligand PD-L1 [67]. The results showed that the proliferation rates of splenocytes treated with these compounds were 93, 89, 60 and 92% [68].

**Compounds 7, 8**

Harvard et al. [69] constructed a mouse model with transgenic expression of PD-L1 (PD-1⁻) and with PD-L1 gene knockout (PD-1⁻⁻). The synthesised sulfamethoxine and sulfamethoxazole oxadiazoles (Figure 2 Compounds 7 and 8) were tested for potency by using wild-type PD-1⁺ T cells and PD-1⁻⁻ T cells in vitro. The results showed that these compounds have inhibitory activity in the concentration range of 0−10 mmol/L and both of them are expected to be useful to correct immune disorders. Additional activity testing by monitoring the release of IFN-γ in transgenic mouse T cells expressing PD-1 showed that these two compounds antagonise PD-1, with activity in the micromolar concentration range. Above 400 nM, specific effects indicating the rescue of T cells from the PD-1 mediated inhibition of IFN-γ production are observed. Therefore, this structure can be used as a lead compound for the design of the pharmacodynamic and pharmacological properties of appropriate PD-1 inhibitors.

**Compound 9**

Researchers at Jilin University in China used resorcinol and 3-hydroxythiophenol as the core and linked them with N,N-dimethylcarbamate and other alkyl-substituted amines to afford 13 amine-appended phenyl dimethylcarbamates (AAPDs), which exhibited the potential to inhibit PD-1/PD-L1 signalling
Based on the PD-1/PD-L1 complex structure reported by Lin et al. [71] and Zak et al. [72] and on the results reported by Harvard et al. [69] on sulfamoxol and sulfamethoxypyridazine, these researchers used V-type domain complete resorcinol and mercapto-substituted resorcinol as the raw materials to synthesise 13 compounds. Then, activity screening was performed to obtain amino- or dimethylcarbamate-substituted resorcinol with the ability to bind to PD-1, thereby efficaciously...
blocking the binding of PD-1 to PD-L1. One of the most active structures was Compound 9 (Figure 2).

**Compound 10**

The Incyte researchers published a patent showing that the company had made progress with a series of small-molecule inhibitors targeting the PD-1 and PD-L1 immune checkpoint (based on Compound 10 Figure 2) in 2017. This small molecule inhibitor is designed to block the PD-1 signalling pathway, thereby blocking the interaction between PD-1 and PD-L1, rescuing T cell responses, and treating or overcoming diseases caused by the activation of this signalling pathway, such as cancer or infectious diseases. HTRF showed that all of the 10 compounds based on this scaffold showed good activity, with IC50 values of less than 10 nM [73].

**AUNP-12**

Aurigen and Laboratoires Pierre Fabre researchers have announced that the joint development of the compound AUNP-12 as a PD-1/PD-L1 pathway inhibitor. This compound is the first peptide PD-1/PD-L1 signalling pathway inhibitor and has the special significance of surpassing the pharmacokinetic limitations of PD-1/PD-L1 antibody drugs with shorter half-lives and significantly reducing the incidence of immune-related adverse events. The researchers took the important role of the binding of the PD-1 extracellular domain with the ligand into full consideration and designed peptide derivatives 7–30 amino acids in length by
simulating the amino acid sequences of the human and mouse PD-1 extracellular domain. AUNP-12 [74], one of the most active compounds, showed excellent anti-PD-L1 activity in animal experiments and was able to effectively inhibit the tumour growth and metastasis of cancers including melanoma, breast cancer and kidney cancer. A rat PBMC proliferation assay in the context of the PD-1/PD-L1 pathway indicated that the EC50 of AUNP-12 was 0.72 nM. When tested on HEK293 cells expressing human PD-L2, AUNP-12 showed an EC50 value of 0.72 nM in the inhibition of the binding of PD-1 to PD-L2. In addition, it is noteworthy that AUNP-12 is well tolerated and shows no significant toxicity at all doses tested. The specific structure of AUNP-12 is shown in Figure 2 [75].

Compounds 11, 12

The continued work of Aurigene researchers on cyclic peptide inhibitors of the PD-1 signalling pathway has disclosed two new classes of peptide derivative structures, namely, cyclic peptide derivatives and open-chain peptide derivatives, or Compounds 11 and 12 [76] (Figure 2). The researchers designed a mouse CFSE proliferation assay to validate the activity of these compounds. CFSE-labelled $1 \times 10^5$ splenocytes were added to wells containing either MDA-MB231 cells ($1 \times 10^5$ cells cultured in glucose DMEM medium) or recombinant human PD-L1/PD-L2 (100 ng/mL) and the test compounds. Splenocytes were stimulated with anti-mouse CD3 and CD28 antibody (1 μg/mL). The results showed that compounds 11 and 12 resulted in splenocyte proliferation rates of 82 and 81%, respectively, indicating that the patented compounds have a significant inhibitory effect on both PD-L1 and PD-L2.

Compounds 13, 14, 15

Aurigene researchers also found that diacylhydrazine and urea-linked tripeptide derivatives could inhibit the PD-L1 and PD-L2 signalling pathway. According to the patent reports, these compounds have IC50 values at the nanomolar level. Taking Compound 13 (Figure 2) as an example, in vivo pharmacodynamic tests in a CT-26 colon cancer mouse model showed that the inhibitory rate of compound 13 was 46% (3 mg/kg, 25 d). In addition, its IC50 values for PD-L1 and PD-L2 were 30 and 40 nM, respectively [77]. Aurigene further investigated the structures of these compounds and designed a series of tripeptide derivatives that contain more amino acid residues along with the diacylhydrazine and urea structures (Figure 2 Compounds 14, 15) [78]. In mouse splenocyte proliferation rescue assays, compounds 14 and 15 gave splenocyte proliferation rates of 87 and 84%, respectively, indicating that the patented compounds have significant inhibitory effects on both PD-L1 and PD-L2.

Compound 16

Aurigene researchers also found another series of cyclic peptide derivatives containing 7–9 amino acids, characterised by the linkage of the N-terminal and the C-terminal of the amino acid residues by an amide bond to form a fixed ring structure. The anti-PD-L1 activity of these compounds has been demonstrated in mouse splenocyte proliferation assays, resulting in splenocyte proliferation rates was 85%. Of these compounds, the best activity belonged to Compound 16 (Figure 2), which reduced the rate of tumour metastases by 54%, according to in vivo activity assays in a B16F10 lung metastatic melanoma mouse model [79].
**Compound 17**

Aurigen researchers used the mouse splenocyte proliferation test, the human peripheral blood mononuclear cell (PBMC) proliferation test and other biological testing methods to screen many other types of peptide inhibitors of the PD-1/PD-L1 signalling pathway. One of the series is a straight-chain peptide derivative with 7–8 amino acids, in which Compound 17 (Figure 2) shows the optimal activity. It reduced the incidence of metastasis by 64% in a B16F10 lung metastatic melanoma mouse model (sc, qd, 5 mg/kg and 14 d) *in vivo* [80].

**Compounds 18 and 19**

In recent years, BMS researchers have announced a series of macrocyclic peptide inhibitors that immunomodulate the PD-1/PD-L1 signalling pathway. The best representatives are Compounds 18 and 19 (Figure 2): in an HTRF binding assay, the IC50 values were 5.6 and 1.03 nM, respectively, and in a cell binding assay, the IC50 values were 12 and 1 nM, respectively, all clearly lower than those of the control group [81].

**Conclusion**

Since 1997, cancer immunotherapy has become a highly popular research topic and amazing progress has been made. The topic receiving the greatest focus is inhibiting the blocking signal at immune checkpoints to regulate T cell activity in order to enhance anti-tumour effects in cancer treatment. For example the use of antagonists of CTLA-4/IFN-1/PD-L1 and other drugs to interfere with immune checkpoints can directly stimulate the activation of cytotoxic T lymphocytes and thereby initiate anti-tumour immunity, mediating a sustained tumour immune process. Targeting the PD-1/PD-L1/PD-L2 signalling pathway and identifying inhibitors, such as mAbs, peptides and peptidomimetics and small-molecular compounds have proved to have clear effects in treating cancer. To date, tumour immunotherapy inhibiting the PD-1/PD-L1 signalling pathway has achieved encouraging clinical effects. Patients receiving anti-PD-1 or anti-PD-L1 drugs approved for clinical and preclinical trials have achieved long-term tumour response and controlled cytotoxicity [82]. The best therapeutic effects have been achieved in cases of melanoma, non-small cell lung cancer, colorectal cancer, kidney cancer, urothelial cancer and haematological malignancies. (1) Melanoma: In a Phase III clinical trial conducted by Robert et al. [83] in 2014, the one-year survival rates of the nivolumab group and the dacarbazine group were 73 and 42%, respectively. The objective remission rates (ORRs) were 40 and 14%. In July 2014, nivolumab was first approved for the treatment of advanced melanoma in Japan; in December 2014, nivolumab was also approved for the treatment of advanced melanoma patients by the FDA [84]. At 2014, ASCO melanoma conference, the results of several clinical trials were presented and showed that pembrolizumab treatment significantly improved the progression-free survival (PFS) of patients with melanoma compared to chemotherapy; with better objective remission rates and that the patients often have better quality of life [85]. In September 2014, pembrolizumab was approved as a breakthrough drug for the treatment of unresectable or metastatic melanoma patients by the FDA [86]. (2) NSCLC: Recently, the data from many clinical trials have shown that anti-PD-1 antibody can achieve significant effects in the treatment of NSCLC. In March 2015, nivolumab was approved by the FDA for the treatment of squamous NSCLC. At the 2015 ASCO conference, Professor Spigel and Professor Poddubskaya presented a trial of nivolumab in advanced nonsmall cell lung squamous cell carcinoma and showed that nivolumab is superior to docetaxel as a second-line treatment of advanced non-small cell lung squamous cell carcinoma [87]. Another Phase III clinical study in patients with advanced non-small cell lung cancer non-squamous cell carcinoma also showed that nivolumab was superior to docetaxel. In treatment-related adverse events, the nivolumab group was superior to the docetaxel group. However, it is noteworthy that, although the overall efficacy of nivolumab was superior to that of docetaxel, the researchers found that the efficacy in PD-L1-positive patients was better than that in PD-L1 negative patients during the trial. Therefore, PD-L1 expression may be set as a valid predictor for non-small cell lung cancer non-squamous cell carcinoma immunotherapy. In advanced NSCLC patients, clinical trials have shown that nivolumab can provide good clinical benefit for progressive NSCLC. Therefore, the NCCN guidelines recommend nivolumab as a standard second-line treatment for advanced NSCLC [88]. (3) Colorectal cancer: immunotherapy has also made significant breakthroughs for the treatment of colorectal cancer in recent years. The results of a Phase II clinical trial in colorectal cancer were released at the 2015 ASCO conference. It is widely noticed that clinical studies on anti-PD-1 drugs in the treatment of tumours with mismatch repair deficiency tumours, result have shown that treatment with anti-PD-1 antibodies exhibits a significantly higher curative effect on colorectal tumours with MMR deficiency than on colorectal tumours with MMR. Thus, the different MMR expression in colorectal cancer patients provides an effective predictor of the success of immunotherapy. This clinical trial offers innovative significance in the treatment of colorectal cancer [89]. (4) Renal carcinoma: A Phase I clinical study by Herbst et al. [38] included 53 patients with renal cell carcinoma (RCC) and evaluated the safety and efficacy of MPDL3280A drug treatment. The objective remission rate of the whole RCC group was 13%. In 32% of the patients, the disease was stabilised for 24 weeks or longer and 53% of patients in the RCC group had progression-free survival of up to 24 weeks. More importantly, some PD-L1-negative tumours were also effectively treated with MPDL3280A. In the Phase I study of nivolumab for the treatment of RCC, the objective remission rate was 27% for 33 patients with renal cancer. The objective remission rates of the 1 and 10 mg/kg groups were 24 and 31%, respectively. In eight patients with objective remission, five patients remained in remission for 1 year or longer and another nine patients (27%) had the disease stabilised for 24 weeks or longer [90]. (5) Haematological malignancies: In a Phase I clinical trial in which 23 patients with recurrent or refractory Hodgkin's lymphoma were treated with nivolumab, four patients achieved complete remission, 16 patients achieved partial remission and three patients maintained a stable disease; and 86% of patients had progression-free survival for up to 24 weeks. In the final data analysis, 11 patients continued the trial; 12 patients had degree 3–4 adverse reactions; and no drug-related degree 4–5 adverse reactions occurred in all patients [91]. Therefore, nivolumab exhibits safety and therapeutic value in the treatment of malignant Hodgkin's lymphoma. In another Phase II trial of 66 patients with diffuse large B-cell lymphoma who received autologous stem cell transplantation and treatment with pidilizumab, 72% of patients had no disease progression up to 16 months and 85% patients had survival of 16 months. Common degree 3–4 adverse reactions were neutropenia (19%) and thrombocytopenia (8%). No significant autoimmune toxicity, transfusion infections or treatment-related deaths occurred, indicating that autologous stem cell transplantation after receiving the PD-1 blocker pidilizumab has clinical value for patients with diffuse large B-cell lymphoma [92].
(6) Urothelial carcinoma: On 18 May 2016, the FDA approved atezolizumab for the treatment of the most common type of urothelial carcinoma for bladder cancer. This is the first PD-1/PD-L1 inhibitor for bladder cancer to be approved by FDA. Atezolizumab was approved for locally advanced or metastatic urothelial carcinoma in patients, expanding the family of PD-1/PD-L1 immunotherapeutic treatments. Although anti-PD-1/PD-L1 inhibitors have achieved good clinical efficacy in tumour immunotherapy, research on biological predictors of the safety and efficacy of tumour immunotherapeutic drugs remains slow. The complete anti-tumour spectrum needs confirmation via further study, which is very important for the individualised treatment of cancer patients.

Although there are none yet on the market, scientists are paying increasing attention to research on small-molecule inhibitors of the PD-1/PD-L1/PD-L2 signalling pathway. The development of small-molecule inhibitors of the PD-1/PD-L1/PD-L2 signalling pathway lags behind that of monoclonal antibodies mainly due to the lack of PD-1/PD-L1/PD-L2 interaction information. Currently, one small-molecule PD-1/PD-L1/PD-L2 signalling pathway inhibitor has entered a Phase I clinical trial, namely, the previously mentioned CA-170, and other potentially biologically active compounds are in preclinical studies. Although monoclonal antibodies against certain cancers have been successfully developed, their high cost, instability and potential immunogenic side effects have caused researchers to turn their attention to the investigation of peptides and small-molecule inhibitors, especially small-molecule inhibitors. First, compared with antibody drugs, traditional small-molecule drugs can achieve higher levels of exposure in the tumour microenvironment. Second, small-molecule drugs can be administered orally, with a generally short half-life and no immune rejection, which helps the doctor to adjust the medication and control adverse drug reactions according to the patient’s condition. In addition, it should be noted that the manufacturing costs and transportation costs of small-molecule drugs are also lower than those of biological drugs, enabling pharmaceutical manufacturers and distributors to gain higher profit margins. At the same time, along with the interaction between PD-1 and its ligands PD-L1/PD-L2, the formation of dimer surface binding hot spots is attracting increasingly in-depth investigation, providing an important structural basis for the rational design of small-molecule drugs to inhibit this signalling pathway. In addition, as far as tumour immunotherapies are concerned, the immune system has many tumour-related regulator factors and pathways, which also provide rich targets for small-molecule compounds. For drug chemists, the development of small-molecule immunotherapeutic drugs will gradually become an important direction in the development of new drugs. Therefore, in the search for immunotherapy that can be expected to cure cancer, the application scope of immune checkpoint protein immunosuppressants in tumours will certainly be further expanded. Targeted small-molecule inhibitors for cancer immunotherapy will bring brighter treatment prospects for patients.

Disclosure statement

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