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From monoclonal antibodies to small molecules: the development of inhibitors targeting the PD-1/PD-L1 pathway

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Cancer immunotherapy has made an extraordinary journey from bench to bedside. Blocking the interactions between programmed cell death protein 1 (PD-1) and its ligand, PD-L1, has emerged as a promising immunotherapy for treating cancer. Here, we review the development of drugs targeting the PD-1/PD-L1 pathway. We discuss the monoclonal antibodies (mAbs) approved or in clinical trials, peptides and patented small molecules developed against this pathway. Such compounds have the potential to treat cancer as well as chronic virological diseases. We also detail PD-1/PD-L1 interactions, an understanding of which will be useful for the rational design of small-molecule therapeutics that disrupt the PD-1/PD-L1 pathway. It is likely that more mAbs targeting the PD-1/PD-L1 pathway will be approved for the treatment of a range of cancers. By contrast, it is likely to be more difficult to successfully develop small molecules or peptides and for them to reach the clinic.

Introduction

Depending on the type, location, and grade of the cancer, and taking into account the patient's health and wishes, cancer can be treated by many ways, including surgery, radiation therapy, chemotherapy, and targeted therapy. Since 1997, when it was first used in the fight against cancer, immunotherapy has become a significant focus of research [1,2]. Cancer immunotherapy harnesses the immune system to battle tumors: that is, it directly targets the immune system rather than the tumors [3,4]. An immune checkpoint is a molecule in the immune system that either enhances (co-stimulatory molecules) or inhibits a signal [5,6]. The blockade of immune checkpoints can activate therapeutic antitumor immunity and, since 2010, immune checkpoints have been considered as novel targets for cancer immunotherapies. This active research field developed following reports of the effectiveness of two mAbs, ipilimumab (Yervoy™; Bristol-Myers Squibb) and pembrolizumab (Keytruda™, Merck), which inhibit immune checkpoints of cytotoxic T lymphocyte-associated protein 4 (CTLA4) and PD-1 (CD279), respectively. Both of these antibodies were initially indicated for advanced melanoma. Following the successes of clinical trials, cancer immunotherapy was chosen as the most important scientific breakthrough of 2013 by the journal *Science* [7].

There are currently several mAbs approved for the treatment of cancers that work by targeting different receptors or immune checkpoints, including B lymphocyte antigen CD20 (CD20), cluster of differentiation 52 (CD52), CTLA4, and PD-1. In 1997, the first mAb, rituximab (Rituxan™, MabThera™, and Zytux™), was approved for cancer immunotherapy [8], for the treatment of non-Hodgkin lymphoma. In 2010, this antibody was also approved for the treatment of chronic lymphocytic leukemia (CLL). Rituximab is a chimeric mAb against the CD20 protein, which is primarily found on the surface of B cells. By destroying B cells, rituximab can be used to treat diseases that are characterized by overactive, dysfunctional, or excessive numbers of B cells. Such diseases include many lymphomas, leukemias, transplant rejection, and autoimmune disorders. The second mAb to be approved was

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alemtuzumab [9], which is a humanized immunoglobulin (Ig)-G1 mAb that was approved in 2001 for the treatment of fludarabine-refractory CLL, cutaneous T cell lymphoma, peripheral T cell lymphoma, and T cell prolymphocytic leukemia. Alemtuzumab targets CD52, which is found on >95% of peripheral blood lymphocytes (both T cells and B cells) and monocytes. In 2009 and 2011, another two mAbs, ofatumumab [10] and ipilimumab [11], were approved for the treatment of refractory CLL and metastatic melanoma, respectively.

In 2014, nivolumab [12] and pembrolizumab [13], two mAbs binding to PD-1, were approved in succession for the treatment of metastatic melanoma. In 2015, nivolumab and pembrolizumab were further approved by the US Food and Drug Administration (FDA) to treat advanced non-small cell lung cancer (NSCLC) and squamous NSCLC, respectively. In addition to nivolumab and pembrolizumab, which are in clinical trials against other cancers, three mAbs targeting PD-1 (AMP-224, AMP-514, and pidilizumab [14]) also are in clinical trials against different cancers and have shown good anticancer results and safety profiles [15]. In addition, in China, there are at least two mAbs that have been entered clinical trials. All these mAbs have PD-1 as an effective target for cancer immunotherapy and clinic results have shown that anti-PD-1 therapy is superior to chemotherapy in the treatment of metastatic melanoma [16–18]. The successful clinical trials and marketing of nivolumab and pembrolizumab have swayed even the skeptics and, thus, immunotherapy has led to new avenue for the treatment of cancer [19,20].

PD-L1, a ligand of PD-1, is highly upregulated on many kinds of tumor cell, including melanoma, ovarian, and lung cancers [21]; therefore, a useful alternative cancer therapy is using antibodies that bind to PD-L1 on the tumor cell. Several mAbs inhibiting PD-L1 currently in different clinical stages have been hypothesized to have less immune-related toxicity than anti-PD-1 therapy, partly because of the selectivity of the immune response in the tumor microenvironment [22–25].

There are some disadvantages to antibody drugs, such as production cost, stability, and immunogenicity; thus, low-molecular-weight immune checkpoint inhibitors of PD-1 and its ligand PD-L1 have become an active research field in the drug discovery. However, unlike mAbs, which are proteins with a high molecular weight (e.g., nivolumab has a molecular weight of 143.6 kDa), the identification of PD-1/PD-L1 inhibitors with a low molecular weight has only recently begun. Currently, there are very few such inhibitors reported and, to our knowledge, only one World Intellectual Property Organization (WIPO) patent regarding small drug-like inhibitors of PD-1/PD-L1 has been disclosed. In addition, there are three patents concerning peptides and peptidomimetics as PD-1/PD-L1 inhibitors. However, no research papers concerning low-molecular-weight immune checkpoint inhibitors targeting PD-1/PD-L1 have been published, apart from a recent report of several hydrolysis-resistant D peptides that might interrupt the PD-1/PD-L1 pathway.

PD-1/PD-L1 and their interactions

PD-1 or CD279 [26–28], functioning as an immune checkpoint, is a protein on the surface of activated T cells in humans and is encoded by the *PDCD1* gene. T cells, also called T lymphocytes, are white blood cells that are essential for the immune system. When PD-L1 or PD-L2 binds to PD-1 on an immune cell surface, the T cell becomes inactive. PD-L1, a 40-kDa type I transmembrane protein, is regularly overexpressed in different cancers, including lymphoma, melanoma, lung, breast, glioblastoma, ovarian, kidney, and bladder, and has been speculated to have a major role in suppressing the immune system during events such as pregnancy, tissue allografts, autoimmune disease, and other disease states, such as hepatitis [29–31]. This enables the body to regulate the immune system by preventing the activation of T cells, which in turn reduces autoimmunity and promotes self-tolerance. Many cancer cells make PD-L1, which inhibits T cells from attacking the tumor; thus, preventing PD-L1 from binding to PD-1 can enable T cells to function correctly [32].

Antibodies or small molecules that bind to either PD-1 or PD-L1 and, therefore, block the interactions between PD-1/PD-L1 might enable T cells to attack the tumor, resulting in tumor rejection or apoptosis. Via this mechanism, PD-1 or PD-L1 inhibitors can kill some types of cancer cells, as verified by two antibodies marketed in 2014, nivolumab and pembrolizumab, which bind to PD-1, and several other antibodies, currently in clinical trials, that bind to PD-1 or PD-L1. Additionally, in chronic viral infections, release from PD-1 inhibitory signaling revives exhausted virus-specific T cells and can augment immune responses and/or facilitate viral (e.g., HIV) eradication [33]. Thus, inhibition of the interactions between PD-1/PD-L1 is a feasible and promising therapeutic target for the treatment of cancer and/or chronic infections (Fig. 1). Some tumor types, such as melanomas and colorectal carcinomas, are more likely to respond to this therapy, because they are thought to be more immunogenic than other tumor types. On activated T cells, in addition to ligating PD-1, PD-L1 also binds Cluster of differentiation 80 (CD80) [34], which explains the differences observed in clinical activity and types of immune-related adverse events that occur between anti-PD-1 and anti-PD-L1.

The interactions between PD-1/PD-L1 are protein–protein interactions (PPIs), which control many biological processes, such as cell proliferation, growth, differentiation, signal transduction, and programmed cell death (apoptosis). Accordingly, PPIs are an important molecular target for novel human therapeutics [35]. Nevertheless, identifying small-molecule inhibitors that disrupt PPIs is challenging largely because of the typical flatness, large size, and noncontiguity of the interface between the interacting proteins and the flexibility of their surfaces. Regardless of these difficulties, several such targets have been successfully exploited, including the B cell lymphoma 2 family [36,37]. Blocking the interactions between PD-1/PD-L1 by using antibodies or small-molecule inhibitors can lower the inhibitory signaling that results in a stronger immune response.

Both PD-1 and PD-L1 are type I membrane proteins containing 268 and 290 amino acids, respectively. Crystal structures are currently available from the Protein Data Bank (PDB) for the human versions of these proteins. For example, the nuclear magnetic resonance (NMR) crystal structure with the PDB ID 2M2D [38] is the human PD-1 (hPD-1) receptor, and the X-ray crystal structure with the PDB ID 3BIS [39] is the human PD-L1 (hPD-L1). There were no hPD-1/hPD-L1 complexes available at the time of writing; however, the recombinant extracellular domains of the murine and human forms of both mPD-1 and hPD-L1 (PDB ID: 3BIK [39]) were described in 2008. Murine PD-1 binds to both

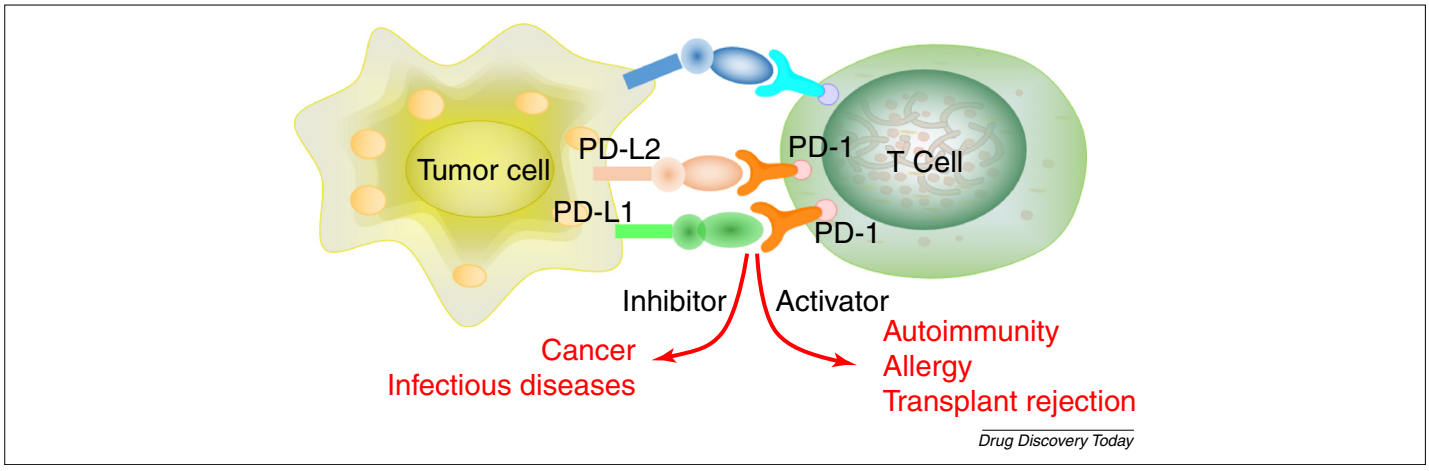

FIGURE 1

Mechanisms behind the inhibition of interactions between programmed cell death protein 1 (PD-1) and its ligand PD-L1 to treat cancer and other diseases, such as chronic infections.

murine and human PD-L1 *in vitro* and vice versa. The cross-species binding affinities are similar to those of the same-species binding. Given these results and the high level of sequence identity between mPD-1 and hPD-1 (64%) and mPD-L1 and hPD-L1 (77%), interactions between hPD-1/hPD-L1 might be similar to those between mPD-1/hPD-L1; thus, it would be useful to analyze the interactions between mPD-1/hPD-L1 by using the crystal structure of 3BIK because understanding the PPIs of PD-1/PD-L1 would provide valuable information for designing inhibitors of PD-1/PD-L1 interactions.

Based on the crystal structure of 3BIK, it is clear that mPD-1 and hPD-L1 interact using their V-domain A′GFCC′ β-sheets to form a pair of V domains in a Fv-like structure (Fig. 2a) [39]. Many hydrogen bonds can be observed in the interface, which is formed by eight residues in mPD-1 and 14 residues in hPD-L1. As with other observed PPIs, such as those from the Bcl-2 family, hydrophobic interactions occur between mPD-1/hPD-L1 (Fig. 2b): a hydrophobic interface is formed by six mPD-1 residues and five hPD-L1 residues. Overall, the interacting interface between mPD-1 and hPD-L1 is typically flat, large, and noncontiguous, which contributes to the difficulty of designing small drug-like inhibitors using computational methods [40].

The structure of the complex of hPD-1/hPD-L1 (PDB ID: 4ZQK) is now available [41]. The interactions between these molecules resemble those between the lg V domains within antibodies and T cell receptors, which are mediated by the strands from the front surfaces of the interacting domains. hPD-1 and hPD-L1 form a 1:1 complex in the crystal, whereas 2:1 stoichiometry is observed for the mPD-1/hPD-L1 complex. This structure also demonstrates that there are differences in the details of the binding modes between hPD-1/hPD-L1 and mPD-1/hPD-L1.

When the complex of hPD-1/hPD-L1 forms, significant structural flexibility can be observed, especially within hPD-1: the CC′ loop (Met70–Asp77) in the apo-hPD-1 is in an open conformation and all the side chains within this loop direct away from the binding site, whereas this loop in the complex is rearranged in a 90° twist and a few residues have big movements, leading to a close conformation of the CC′ loop (Fig. 3a). This rearrangement results in four hydrogen bonds between hPD-1 and hPD-L1: Gln75 of hPD-1 forms three hydrogen bonds with Asp26 and Arg125 of hPD-L1; and Thr76 of hPD-1 forms one hydrogen bond with Tyr123 of hPD-L1. Evidence shows that this CC′ loop is specific to human PD-1. By contrast, hPD-L1 involves only minor adjustments to the binding surface and there are no noteworthy changes within its backbone.

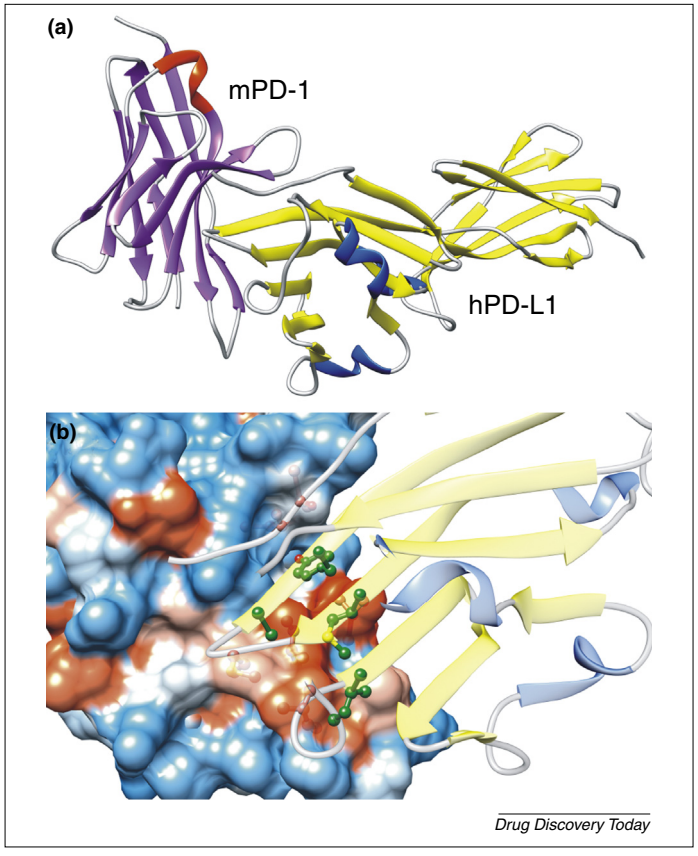

FIGURE 2

(a) The murine/human programmed cell death protein 1 (mPD-1; purple ribbon)/hPD-L1 (yellow ribbon) complex. **(b)** The binding surface of murine PD-1. Blue, hydrophilic surface; orange, hydrophobic surface. Several hydrophobic residues of PD-L1 that have hydrophobic interactions with PD-1 are shown as green ball and stick models.

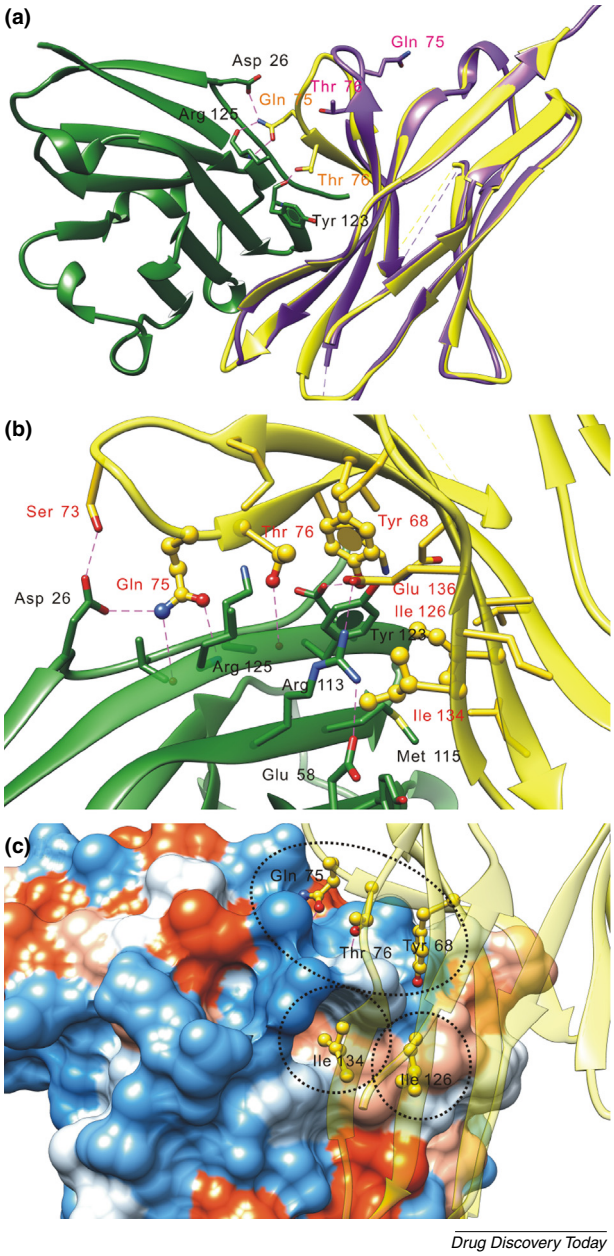

FIGURE 3

(a) The human programmed cell death protein 1 (hPD-1; yellow ribbon)/hPD-L1 (green ribbon) complex [Protein Data Bank (PDB) ID: 4ZQK]. Significant structural rearrangements can be observed when apo-hPD-1 (purple ribbon, PDB ID: 3RRQ) binds to hPD-L1. **(b)** The binding network between hPD-1/hPD-L1. Hydrogen bonds are shown in magenta dashed lines. **(c)** The three hotspots on the surface of hPD-L1. Residues belonging to PD-1 and inserting into these hotspots are shown as yellow ball and stick models. Blue, hydrophilic surface; orange, hydrophobic surface.

A total surface area of 1970 $Å^2$ is buried in the binding surface, and both hydrophobic and polar interactions can be observed between the surfaces of hPD-1 and hPD-L1 (Fig. 3b). The hydrophobic interactions formed between hPD-1 and hPD-L1 mainly come from nonpolar residues in the front sheets of hPD-1 (Val64, Ile126, Leu128, Ala132, and Ile134) and hPD-L1 (Ile54, Tyr56, Met115, Ala121, and Tyr123). Many polar residues surround these nonpolar residues, providing additional hydrogen bond interactions between hPD-1 and hPD-L1. Three major hot spots in the surface of hPD-L1 have been identified (Fig. 3c). The first accommodates Ile134 of hPD-1 and its size perfectly accommodates a six-membered aromatic ring. The second hot spot, close to the first one, accommodates Ile126 of hPD-1 and can be effectively filled by an aliphatic moiety. The third is an extended groove accommodating three polar residues, Tyr68, Gln75, and Thr 76. of hPD-1. This groove provides multiple hydrogen bond donors and acceptors but might be hard to competently target using small-molecule probes.

As many targets involving PPIs, fragment-based drug discovery [42,43] techniques, as a powerful approach in the structure-based drug discovery, may be applicable for identifying small drug-like inhibitors to interrupt the interactions between hPD-1/hPD-L1. However, the authors who resolved the structure of hPD-1/hPD-L1 (PDB ID: 4ZQK) attempted to use this method to test a limited number of tyrosine derivatives, but were unable to identify any active inhibitors. As discussed above, the protein surface between hPD-1/hPD-L1 is flexible, and the interface is large and noncontiguous. All these features contribute to the challenges of developing small inhibitors that block PD-1/PD-L1 interactions. Using fragment-based drug discovery and screening more fragments might be a feasible way to identify small inhibitors that can block PD-1/PD-L1 interactions.

Inhibitors targeting the PD-1/PD-L1 pathway

mAb targeting the PD-1/PD-L1 pathway

To date, two mAbs (nivolumab [12] and pembrolizumab [13]) targeting the PD-1/PD-L1 pathway have been approved for the treatment of metastatic melanoma and NSCLC, and several others are in clinical trials (Table 1). Currently, nivolumab and pembrolizumab also are in clinical trials for the treatment of other cancers. More approvals of mAbs targeting the PD-1/PD-L1 pathway are expected in the near future.

Additionally, initial advances have made in the targeting of the PD-1/PD-L2 pathway for medical purposes: an anti-PD-L2 mAb, rHIgM12B7, which is co-developed by the Mayo Clinic and the National Cancer Institute (NCI) of the US Government, is currently in a Phase I clinical trial for the treatment of metastatic melanoma. However, no published literature is available relating to this mAb.

Nivolumab [44,45] (also called BMS 936558, MDX 1106, or ONO 4538) is a fully humanized IgG4 antibody that blocks the PD-1 receptor and selectively blocks its interaction with its ligands PD-L1 and PD-L2. In 2014, nivolumab was approved by the FDA for treatment of patients with unresectable or metastatic melanoma who no longer responded to other drugs; in 2015, the FDA expanded the approved use of nivolumab for the treatment of squamous NSCLC. Currently, this mAb is under clinical trials to evaluate its treatment effects on Hodgkin's lymphoma, kidney cancer, and hepatitis C virus (HCV). Nivolumab was the first mAb to be used in a Phase I clinical trial for 39 patients with solid tumors, including advanced melanoma, NSCLC, renal cell carcinoma (RCC), prostate, and colorectal cancer. For this agent, a half-life longer than anticipated and prolonged disease stabilization were observed [46]. In a Phase III trial comparing nivolumab monotherapy to treatment with traditional chemotherapy, nivolumab exhibited better results in terms of response rate (40% vs 13.9%), progression-free survival (5.1 months vs 2.2 months), and

TABLE 1

mAbs currently on the market and in clinical trials targeting the PD-1/PD-L1 and PD-1/PD-L2 pathways for the treatment of cancer and other diseases.

Name	Trade name	Developed by	Target	Approved treatment(s) and year	Treatments in clinical trials
Nivolumab	Opdivo	Bristol-Myers Squibb	PD-1	Unresectable or metastatic melanoma (2014), squamous NSCLC (2015)	Colorectal cancer, prostate cancer, RCC, Hodgkin's lymphoma, kidney cancer, HCV
Pembrolizumab	Keytruda	Merck	PD-1	Metastatic melanoma (2014), advanced NSCLC (2015)	Advanced refractory malignancies, hematologic malignancies
AMP-224	–	GlaxoSmithKline	PD-1	–	Colorectal cancer, colorectal neoplasms, colorectal carcinoma
AMP-514	–	GlaxoSmithKline	PD-1	–	Aggressive B cell lymphomas
Pidilizumab	–	CureTech	PD-1	–	Diffuse large B cell lymphoma, follicular lymphoma, multiple myeloma
BGB-A317	–	BeiGene (China)	PD-1	–	Advanced cancer
SHR-1210	–	Jiangsu Hengrui Medicine	PD-1	–	Advanced solid tumors
BMS-936559	–	Bristol-Myers Squibb	PD-L1	–	Advanced solid tumors, severe sepsis, HIV
Atezolizumab	–	Genentech/Roche	PD-L1	–	Melanoma, RCC, NSCLC, breast cancer, multiple myeloma, bladder cancer, etc.
MEDI4736	–	AstraZeneca	PD-L1	–	Advanced solid tumors, NSCLC
Avelumab	–	Pfizer/Merck	PD-L1	–	Advanced solid tumors, Merkel cell carcinoma, renal cell cancer
rHIgM12B7	–	Mayo Clinic/NCI	PD-L2	–	Metastatic melanoma

percentage of patients still alive after 1 year (72.1% vs 42.1%) [47]. To examine the use of nivolumab as a single agent or as part of combination therapy for patients with NSCLC, a multi-arm Phase I trial was executed, with encouraging results. For example, 56 chemotherapy-naive patients were treated with nivolumab plus one of three chemotherapy regimens (cisplatin/gemcitabine, cisplatin/pemetrexed, or carboplatin/paclitaxel). The response rates ranged from 33% to 50%, and 59–87% of the patients were still alive after 1 year [48]. Nivolumab was granted breakthrough designations for resistant Hodgkin lymphoma. The antiviral potential of nivolumab has also been explored in a proof-of-concept, placebo-controlled single-ascending-dose study in 54 patients with chronic HCV infection [49].

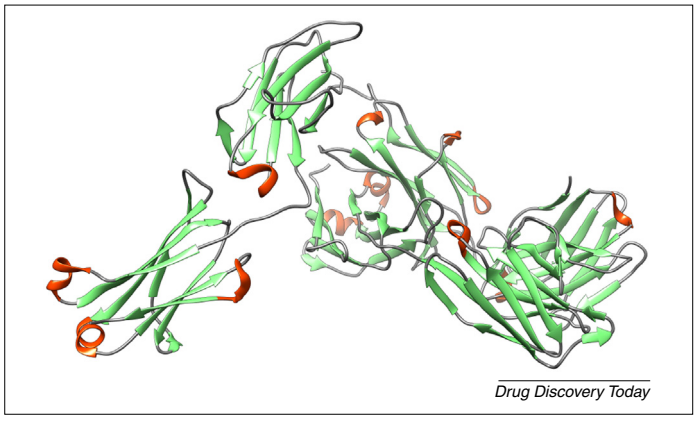

FIGURE 4

The crystal structure of pembrolizumab [Protein Data Bank (PDB) ID: 5DK3]. The β sheets are shown in green.

Pembrolizumab (formerly MK-3475 and lambrolizumab) is a humanized monoclonal lgG4 anti-PD-1 antibody [50,51]. In September 2014, the FDA approved this antibody under the FDA Fast Track Development Program for use following treatment with ipilimumab, or after treatment with ipilimumab and a BRAF inhibitor in patients with advanced melanoma carrying a BRAF mutation. In 2015, pembrolizumab was approved for the treatment of metastatic NSCLC in patients whose tumors expressed PD-L1 and who had failed treatment with other chemotherapeutic agents. A large Phase I trial led to a response rate of 37–38% in patients with advanced melanoma and an overall response rate of 26% in patients who had progressive disease after treatment with ipilimumab [52]. Currently, this drug is in clinical trials for advanced refractory malignancies and hematologic malignancies. Recently, the X-ray crystal structure of this antibody was disclosed (PDB ID: 5DK3, Fig. 4), showing that its tertiary structure comprises β sheets.

AMP-224, developed by GlaxoSmithKline, is a PD-L2 lgG2a fusion protein that targets PD-1. The Phase I clinical study was finished in January 2014 in 44 patients with advanced cancer. Currently, this agent is in Phase II trials in combination with stereotactic body radiation therapy in patients with metastatic colorectal cancer.

AMP-514 (MEDI0680), also developed by GlaxoSmithKline, is a PD-L2 fusion protein that targets PD-1. A Phase I multicenter open-label study to evaluate the safety tolerability and pharmacokinetics of AMP-514 in patients with advanced malignancies began in

December 2013. Another Phase I study of AMP-514 in combination with MEDI4736 in patients with advanced malignancies currently is recruiting participants. In addition, there is a Phase Ib/II open-label study to evaluate the safety and/or efficacy of MEDI-551 in combination with AMP-514 in participants with relapsed or refractory aggressive B cell lymphomas who have failed one or two prior lines of therapy.

Pidilizumab (formerly CT-011), also targeting PD-1, is a mAb developed by CureTech for the treatment of cancer and infectious diseases [14,53,54]. This antibody is currently in clinical trials for diffuse large B cell lymphoma, follicular lymphoma, and multiple myeloma, and has shown encouraging results and favorable toxicity.

BMS-936559 (MDX-1106/ONO-4538), a fully humanized IgG4 antibody developed by Bristol-Myers Squibb, inhibits the binding of PD-L1 to PD-1 and CD80 with high affinity [23]. Currently, it is in a clinical trial in patients with advanced solid tumors. The Phase I study of safety and immune response of BMS-936559 in patients with HIV taking combination antiretroviral therapy was performed and completed in November 2015. The study of the safety, pharmacokinetics, and pharmacodynamics of BMS-936559 in severe sepsis is currently under way.

Atezolizumab (also known as MPDL3280A/RG7446) [55,56], investigated by Genentech/Roche, is a fully humanized, engineered mAb of IgG1 isotype against PD-L1. Currently, atezolizumab is in clinical trials as an immunotherapy for several types of solid tumor, such as bladder cancer and NSCLC, alone or in combination with other agents. In February 2015, atezolizumab was classified as a 'breakthrough therapy' by the FDA because of its encouraging clinical results. It is likely that atezolizumab will be approved by the FDA in 2016 and would be the first marketed mAb targeting PD-L1.

MEDI4736 [57,58], another human IgG1 antibody, also binds specifically to PD-L1. This drug was developed by AstraZeneca and is now in clinical trials for advanced solid tumors as a mono- or combination therapy. The results are promising and its toxicity profile is favorable.

Avelumab (also named as MSB0010718C) [59] is a fully humanized monoclonal PD-L1 antibody of isotype IgG1, currently co-developed by Pfizer and Merck for use in immunotherapy, especially for the treatment of NSCLC. Avelumab has been in Phase I clinical trials for several cancers, including bladder cancer, gastric cancer, head and neck cancer, mesothelioma, NSCLC, ovarian cancer, and renal cancer. There are a Phase II trial underway for Merkel cell carcinoma and a Phase III trial for NSCLC.

In China, drug development targeting the PD-1/PD-L1 pathway has made remarkable progress. A mAb named BGB-A317, developed by BeiGene, currently is in a Phase Ia/Ib clinical trial in subjects with advanced tumors. SHR-1210, another anti-PD-1 mAb, is in an open-label, multicenter, nonrandomized, dose escalation Phase I trial to evaluate its safety and tolerability. This antibody was developed by Jiangsu Hengrui Medicine, and its exclusive development and commercialization rights outside of China were sold to Incyte. Another mAb, JS001, from Shanghai Junshi Biosciences Co., Ltd. will soon be in clinical trials in China.

To improve the efficacy or position the treatment regimen of these therapies, these antibody drugs discussed above can be dosed individually or be combined with other checkpoint inhibitors, such as ipilimumab, immunostimulatory cytokines, such as interferon (IFN)-γ, cytotoxic chemotherapy, antiangiogenic inhibitors, and small-molecule molecularly targeted therapies [60]. Evidence has shown that cytotoxic drugs have the ability to stimulate the immune system and can result in antitumor immune responses that contribute to the clinical efficacy of antibody drugs targeting the PD-1/PD-L1 pathway [61].

Targeting the immune checkpoints in patients with cancer has led to long-lasting tumor responses. However, by unbalancing the immune system, these new immunotherapies also cause immune-related adverse events that mainly involve the gut, skin, endocrine glands, liver, and lung, but that can potentially affect any tissue [62,63]. The use of mAbs can be related to severe and potentially fatal immunological adverse effects owing to T cell activation and proliferation. Indeed, adverse effects and immune-related adverse events have been observed in the clinic. For example, toxicities, including pneumonitis, low-grade fatigue, diarrhea, pruritus, nausea, and decreased appetite, were observed in a Phase I dose-optimization trial of nivolumab performed in patients with melanoma, lung cancer, kidney cancer, and other cancers. Pneumonitis was the chief adverse effect, and led to three deaths; 22% of patients in the trial experienced a treatment-related Grade 3 or Grade 4 toxicity [31,47,64]. Antibodies have inherent limitations, including poor tissue and/or tumor penetrance and detrimental Fc-effector functions that deplete immune cells. Peptides, peptidomimetics, or small drug-like molecules might avoid the immune-related adverse events, and have additional advantages over antibodies. Recently, the Ring group from the Stanford University School of Medicine published a paper that corroborated the idea that PD-1:PD-L1-directed immunotherapy could be improved with smaller, nonantibody therapeutics [65]. A high-affinity (110 pM) competitive antagonist of PD-L1 was engineered from the PD-1 ectodomain by directed evolution and a yeast-surface display. In contrast to anti-PD-L1 mAbs, this high-affinity competitive antagonist of PD-L1 displayed superior tumor penetration without inducing the depletion of peripheral effector T cells. In syngeneic CT26 tumor models, it was effective in treating both small (50 mm^3) and large tumors (150 mm^3), while the activity of anti-PD-L1 antibodies was completely abrogated against large tumors. These results highlight the promising pharmacology of small, nonantibody therapeutics for future drug development targeting the PD-1/PD-L1 pathway [65].

Peptides and peptidomimetics as PD-1/PD-L1 inhibitors

Although mAbs have shown impressive clinical activity, it is evident that they have severe immune-related adverse effects. To address these limitations of mAbs, peptide-based immune checkpoint blockers have been developed. AUNP-12 (AUR-12/Aurigene-012), a peptide therapeutic targeting the PD-1 immune checkpoint pathway for cancer immunotherapy, was co-developed by Aurigene Discovery Technologies and Pierre Fabre Laboratories and is currently in a preclinical study. This peptide showed potent antitumor activity but a shorter pharmacokinetic profile. US Patent Application 2011/0318373 shows that derived from human and murine PD-1 sequences and combined in a non-linear manner, many 7- to 30-mer peptides were investigated, leading to the discovery of AUNP-12, a 29-mer peptide. The sequence of AUNP-12 has not yet been disclosed; however, it is believed that

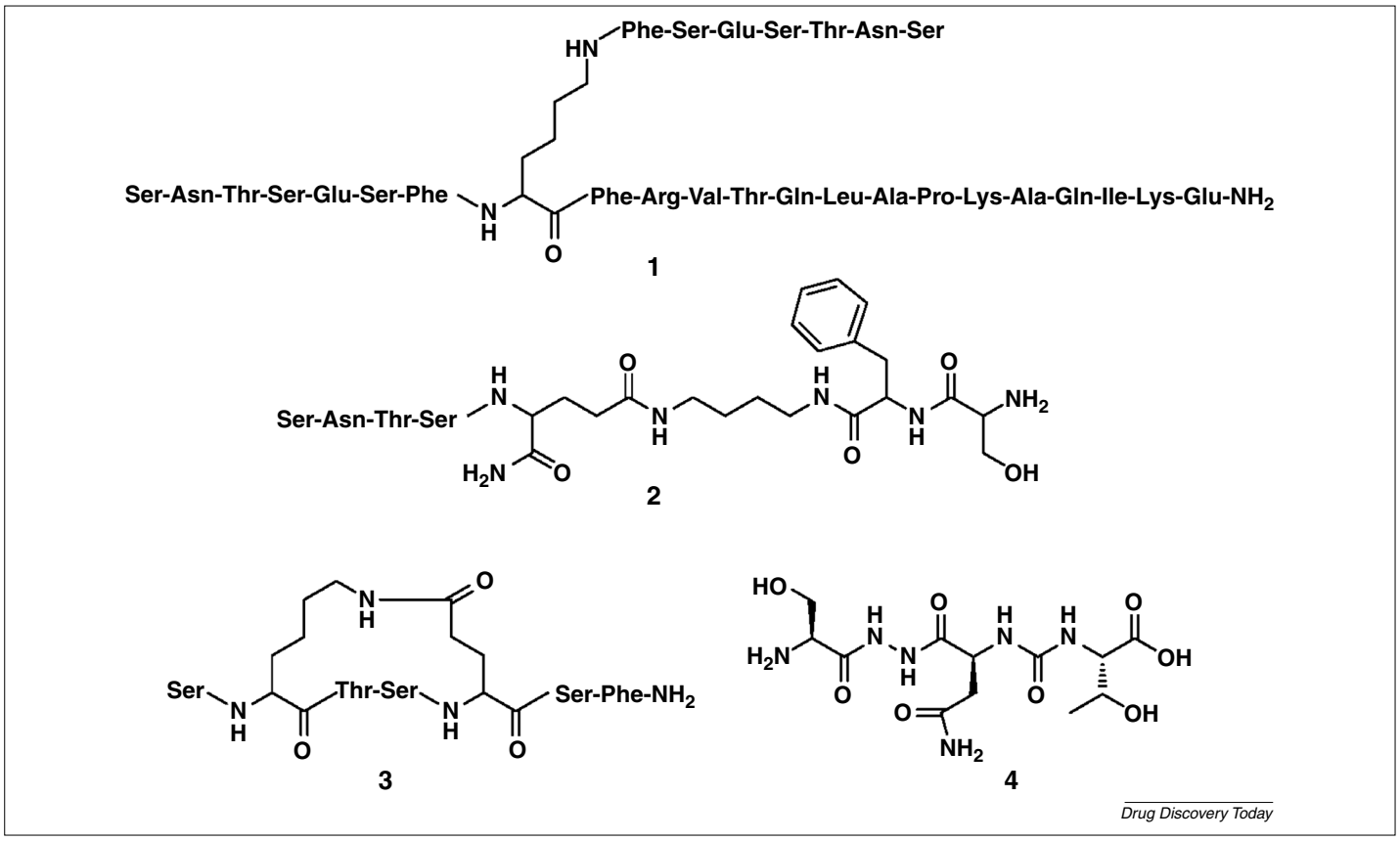

FIGURE 5

Structures of four patented peptides and peptidomimetics as programmed cell death protein 1 (PD-1)/PD-L1 inhibitors.

it has the chemical structure of compound **1** (Fig. 5) or is the *N*-acylated derivative therefore.

Compound **1** showed an EC_{50} value of 0.72 nM in the inhibition of PD-1 binding to PD-L2 using hPDL2-expressing HEK293 cells, and an EC_{50} value of 0.7241 nM in a rat peripheral blood mononuclear cells proliferation assay using hPDL1-expressing MDA-MB231 cells. In preclinical models of melanoma, breast, and kidney cancers, this peptide demonstrated better efficacy compared with therapeutic agents currently used in the clinic in the inhibition of both primary tumor growth and metastasis. It also had a safer toxicological profile.

In three patents, Aurigene disclosed various peptides or peptidomimetics as PD-1 inhibitors. Compounds **2**, **3**, and **4** are from patent applications WO 2012/168944, WO 2013/144704, and US 2013/0237580, respectively. Compound **2** is a short heptapeptide, whose sequence corresponds to the so-called BC loop (amino acids 24–30) of the extracellular domain of PD-1. Compounds **2** and **3** inhibit PD-1/PD-L1 interactions and displayed high percentages of inhibition in the mouse splenocyte proliferation assay. Compound **4** is a tripeptide peptidomimetic with diacylhydrazine and urea liner moieties. This compound inhibits both PD-L1 and PD-L2 binding to PD-1 with EC_{50} values in the nanomolar range as measured in the splenocyte effector function assay by monitoring IFNγ release. In a CT-26 colon cancer model in mice at a dose of 3 mg/kg (25 days), this compound demonstrated *in vivo* efficacy by reducing tumor growth by 46%.

Recently, hydrolysis-resistant D-peptides were reported as PD-L1 antagonists that were developed by using mirror-image phage display [66]. The optimized peptide ^{D}PPA-1 has the sequence of NYSKPTDRQYHF, and binds to PD-L1 at an affinity of K_D = 0.51 μM *in vitro*. Experiments with tumor-bearing mice models demonstrated that the peptides disclosed could inhibit tumor growth and prolong animal survival. Further experiments indicated that ^{D}PPA-1 could effectively disrupt the PD-1/PD-L1 PPIs *in vivo* and ^{D}PPA-1 could not directly kill tumor cells, although the activity might come from the activation of the antitumor immune system, which could provide a way to develop low-molecular-weight drug candidates for cancer immunotherapy. This is the first report of the development of hydrolysis-resistant peptide inhibitors of immune checkpoint proteins. Two other peptides, ^{D}PPA-2 and ^{D}PPA-4, were also reported to demonstrate inhibitory activities against PD-L1, with K_D values of 1.13 and 22.0 μM, respectively.

By comparison with mAbs, such as pembrolizumab and nivolumab, peptides as drug candidates have several advantages, such as lower manufacturing costs, higher stability, reduced immunogenicity, and better organ or tumor penetration [67]. However,

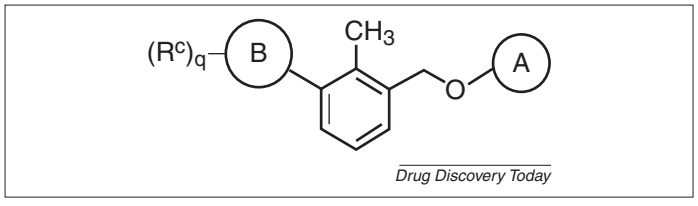

FIGURE 6

The formula of Patent WO 2015/034820 A1.

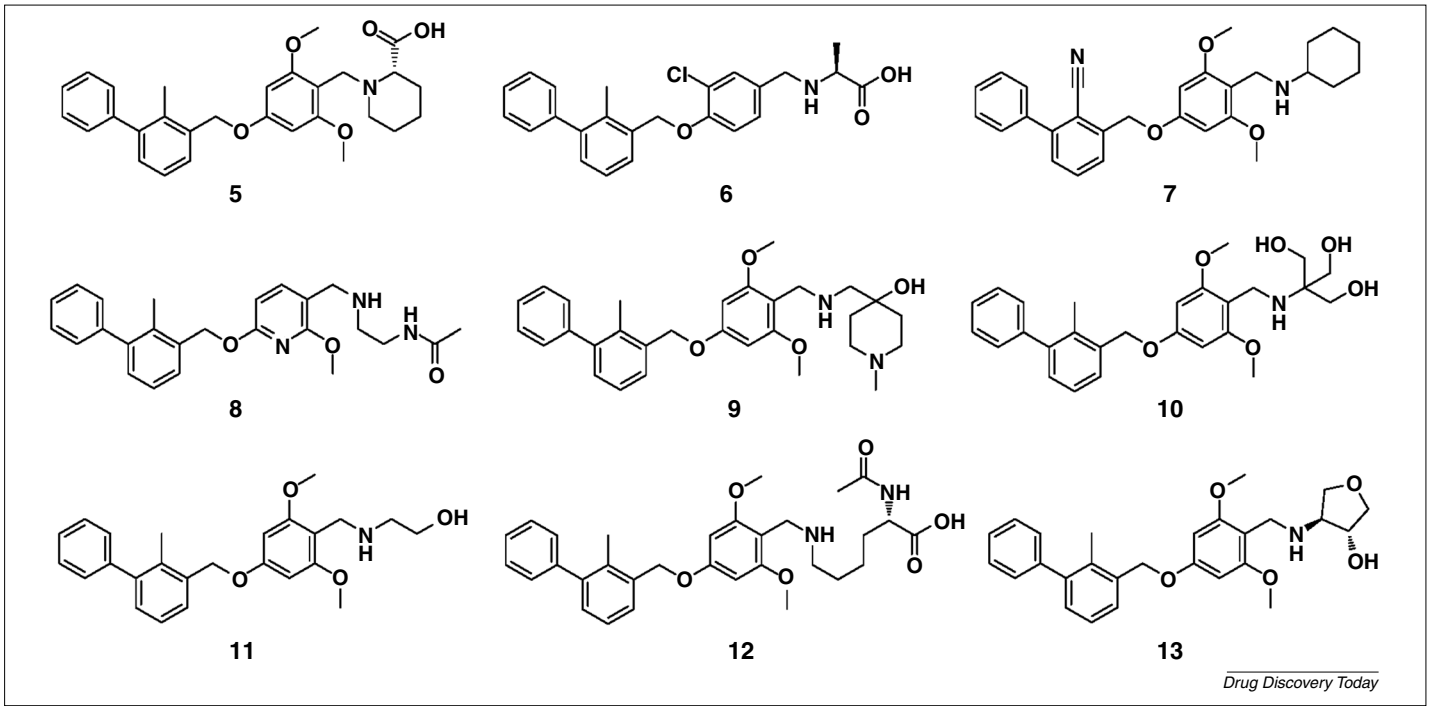

FIGURE 7

Structures of nine randomly chosen compounds from the 297 exampled inhibitors targeting the programmed cell death protein 1 (PD-1)/PD-L1 pathway from the WO 2015/034820 A1 patent.

small drug-like inhibitors have more advantages; therefore, developing drug-like inhibitors to block PD-1/PD-L1 interactions appears to be more appealing than focusing on either mAbs and synthetic peptides.

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

The successful and exciting development of mAbs targeting PD-1/PD-L1 to treat cancer has stimulated the development of small drug-like inhibitors because these could overcome the typical disadvantages of antibody-based immunotherapies, including certain adverse effects and immune-related adverse events, as discussed above. Moreover, the route of administration of most mAbs is intravenous injection with high dosage. Small drug-like molecule drugs, which are more amenable for oral administration than mAbs, could reduce severe immune-related adverse events resulting from prolonged target occupancy by modulating the half-life of the drug. When compared with mAbs, small drug-like inhibitors have many other benefits; for example, they are cheaper, more stable, better for organ or tumor penetration, and more economic for manufacturing [68].

Nevertheless, identifying small drug-like inhibitors targeting the PD-1/PD-L1 pathway is hindered by several factors, including incomplete structural information about these proteins. We found only one patent and no research articles relating to small drug-like inhibitors in this field: Patent WO 2015/034820 A1 (priority to US 61/873,398) was filed by Bristol-Myers Squibb in 2015 [69] (Fig. 6).

Bristol-Myers Squibb claimed that compounds having the formula detailed in Fig. 6 were inhibitors of PD-1/PD-L1 interactions, and, therefore, could be useful in treating, preventing, or slowing the progression of virological diseases or disorders such as HCV, and cancer. The structures of nine randomly chosen compounds (compounds **5**–**13**) of the 297 exampled inhibitors of PD-1/PD-L1 interactions are shown in Fig. 7. All nine inhibitors have IC_{50} values between 0.006 and 0.10 mM, as evaluated by the homogenous time-resolved fluorescence (HTRF) binding assay.

It is possible to explore the binding modes of these compounds against the hPD-1/hPD-L1 target using the crystal structure recently resolved (PDB ID: 4ZQK) by employing docking methods, especially induced fit docking. Then, based on the putative binding modes, structure-based drug design techniques can be used to identify inhibitors with novel scaffolds. However, experimental X-ray structural information derived from the complexes of PD-1 or PD-L1 with small drug-like inhibitors (or peptides) should be more valuable for inhibitor design, and we expect that, in the future, such crystal structures will be deposited into the PDB.

No more patents or research articles regarding small drug-like molecules as inhibitors of PD-1/PD-L1 pathway could be found from the published literature. However, given the huge potential market of the PD-1/PD-L1 pathway and the advantages of small drug-like molecules, it is likely that pharmaceutical and/or biotech companies and academia are trying to identifying such inhibitors and we expect that more patents or research articles will be disclosed or published in the near future.

Concluding remarks

Cancer immunotherapy has attracted much interest since the approval of rituximab in 1997 and much progress has been made over the past decade. Targeting the PD-1/PD-L1 pathway and identifying inhibitors, including mAbs, peptides, and small drug-like compounds, have been proved an impactful way to fight cancer. This has resulted in two approved mAbs, nivolumab and pembrolizumab, to treat metastatic melanoma and NSCLC.

The marketing and clinical trials of inhibitors targeting the PD-1/PD-L1 pathway have revealed impressive results in different cancers, which supports the fact that immunotherapy is one of the most encouraging strategies for cancer treatment. However, there are no small drug-like inhibitors approved to block the PD-1/PD-L1 pathway or to treat cancer. For peptides or peptidomimetics as PD-1/PD-L1 inhibitors, which might mimic the interactions between PD-1/PD-L1, only three patents have been disclosed and one research article published, as far as we are aware.

PD-1 is the prevailing pathway by which cancer protects itself. The upregulation of PD-1 ligands occurs in some tumors and signaling through this pathway can inhibit active T cell immune surveillance of tumor tissue. This is why PD-1 ligands can kill cancer cells and extend patients' survival. For example, the clinical study of nivolumab showed that patients with untreated metastatic melanoma had an overall survival rate of 72.9%, compared with 42.1% for patients who took decarbazine, the standard-of-care chemotherapy [70]. Additionally, it was revealed that nivolumab has a good adverse effect profile. In addition to metastatic melanoma, mAbs in clinical trials also are showing good activity in other cancers and might work best in virus-associated cancers, such as Merkel cell and human papillomavirus-associated head and neck cancers, because such cancers are more visible to the immune system. Targeting PD-L1 is another effective alternate for cancer immunotherapy, and several antibodies binding to PD-L1 in different stages of clinical trials have been assumed to have less immune-related toxicity, in part by regulating the immune response selectively in the tumor microenvironment [22,29].

Despite their long-lasting effects and promising outcomes, the disadvantages of inhibitors targeting the PD-1/PD-L1 pathway is that only a proportion of patients respond to the treatments. Further understanding of the regulation of PD-L1 expression could be helpful for the improvement of anti-PD-L1 and PD-1 treatments.

To improve the efficiency of anti PD-1/PD-L1 immunotherapies, combination therapy can be used, and several approaches for combining PD-1/PD-L1 pathway inhibitors with other chemotherapies have been explored. Results have shown that combination therapy might improve survival and delayed tumor growth more than either drug alone [71–74].

Although PD-L1 immunotherapy targeting PD-1/PD-L1 is in the early stage of clinical development, the clinical results demonstrated by the several antibodies tested are encouraging. There is no doubt that, in the near future, more mAbs (either as mono- or combination therapies) targeting the PD-1/PD-L1 pathway will be approved for the treatment for more cancers. However, for small drug-like molecules or peptides, there is likely to be a longer road to the clinic.

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