

REVIEW

Emerging targets for anticancer vaccination: PD-1

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Among the mechanisms by which tumor cells escape the immune surveillance, one is the interaction between programmed cell death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1). Inhibition of the PD-1/PD-L1 pathway with monoclonal antibodies as immune checkpoint inhibitors targeting PD-1 or its ligand, PD-L1, represents a milestone in cancer therapy. The application of these antibodies, however, suffers from drawbacks including failure to show a response or benefit in a majority of patients following monotherapy or combination therapy, their frequent administration, and cost intensiveness. Small peptides capable of interfering with PD-1/PD-L1 interaction represent interesting alternatives to antibody-based immune checkpoint inhibitors. Moreover, peptides representing PD-1 or PD-L1 sequences can be used in active immunization approaches to induce antibodies that enhance antitumor immunity by effectively preventing PD-1-mediated inhibition in the host. Importantly, such peptides can readily be combined with peptides derived from cancer antigens to effectively induce an antitumor immune response. In this review, we have summarized the recent developments in the use of small molecules and peptides either to directly block PD-1/PD-L1 interaction, or in vaccination approaches to induce antibody responses stimulating anticancer immunity by blocking PD-1-mediated T-cell inhibition.

Key words: immune checkpoint inhibitors, PD-1, small molecules, mimotopes/peptides, vaccination

INTRODUCTION

Activation of T cells is achieved by the interaction between the T-cell receptor and the antigen in the context of major histocompatibility complex, and the interaction between costimulatory receptors like CD28 with B7 family members CD80 (B7-1) or CD86 (B7-2).¹ These stimulatory signals are, however, counterbalanced by the interaction between co-inhibitory receptors on T cells, like programmed cell death protein 1 (PD-1) which binds to its ligands programmed death-ligand 1 (PD-L1) and PD-L2, or cytotoxic T lymphocyte antigen 4 (CTLA-4) which binds to CD80 or CD86,^{2,3} functioning as a protective mechanism for the host.⁴ While CTLA-4 stops potentially autoreactive T cells at the initial stages of immune response and naive T-cell activation, typically in lymph nodes, the PD-1 pathway is involved at

the later stages of the immune response and regulates T-cell responses, primarily in peripheral tissues.⁵

Multiple lines of preclinical and clinical evidence have shown that tumors can evade the immune system, by expressing surface ligands that engage inhibitory receptors on tumor-specific T cells, resulting in immune tolerance.^{6,7} PD-L1 is expressed by a multitude of immune cells, and also on some cancer cells, and the interaction between PD-1 and PD-L1 results in activation of PD-1 and in turn attenuation of T-cell activity.⁸ Accordingly, PD-L1 overexpression on tumor cells has been linked to a poor prognosis for several types of tumors in various cancers.⁹⁻¹³

The engineering and development of therapeutic antibodies, like immune checkpoint inhibitors (ICIs), is a milestone in immunotherapy.¹⁴⁻¹⁷ Among these, the blockade of PD-1/PD-L1 interaction by various monoclonal antibodies (mAbs) targeting PD-1 (e.g. nivolumab, pembrolizumab), PD-L1 (e.g. atezolizumab, avelumab, durvalumab), or ipilimumab targeting CTLA-4,^{16,18} holds a tremendous promise for the treatment of diverse solid tumor types, including melanoma, non-small-cell lung cancer (NSCLC) and renal cell carcinoma.^{8,19,20} Although with high therapeutic efficacy,²⁰ the administration of mAbs suffers from several drawbacks including (i) the cost intensiveness of their production and administration which may not only result in a

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burden on the health system but also in limiting the access to these treatments, (ii) the unavailability for oral administration, (iii) the low permeability and diffusion into large tumors due to their high molecular weight, (iv) the strong binding to the periphery of the tumors, due to their high affinity, and consequently low capacity to reach tumor cores, and (v) the need for frequent and prolonged intravenous administration.^{21,22} Furthermore, immune-related adverse events (irAEs) are frequently observed in the patients treated with ICIs.²³⁻²⁵ In addition, the ambiguous interactions between Fc γ c and antibodies are a matter of concern in ICI therapy and require careful selection of antibodies with suitable isotypes.^{26,27} Moreover, continuous target inhibition as a result of the therapeutic mAbs' long half-lives may also contribute to irAEs.

BIOLOGICAL BACKGROUND

Due to their role in the reversal of tumor immunity inhibition, administration of ICIs leads to activation of the immune system by mechanisms including e.g. the reactivation of T cells after inhibition of PD-1/PD-L1 interaction, and reversion of the intrinsic PD-L1 signaling to tumor cells promoting an anti-apoptotic effect and cells growth.^{20,28} The direct exploitation of small molecules as PD-1 blockers, or the application of peptides in a formulated vaccine, are two distinct approaches that can be regarded as promising alternative strategies to counteract PD-1 inhibition. Compared with antibody-based ICIs, they would have several advantages such as low cost and potential for oral bioavailability.²⁹

The rational design of small molecules is based on the immune checkpoints' structure and physical modelling.³⁰⁻³² As small compounds,^{33,34} the direct application of such molecules is aimed to block the PD-1-mediated inhibition. The faster half-life of small compounds will necessitate a more frequent administration, but it may also result in fewer side-effects such as irAEs associated with ICIs.²⁹ The half-life of the small molecule NP-12 was shown to be approximately 90 minutes in mouse liver microsomes,³⁰ in contrast to the half-life of ICIs which is >15-20 days in humans.³⁵ Since the length of the exposure to the drug is correlated with irAEs, the short half-life of the small molecules, compared with the ICIs, would require a more frequent application. This could in turn, however, potentially result in a reduced level of the developed irAEs.³⁶

The application of peptide libraries, displayed on the surface of bacteria or bacteriophages, is an alternative strategy to identify and select the epitopes of ICIs or other therapeutic mAbs.³⁷⁻³⁹ Based on this strategy, the use of mimotopes, i.e. peptides mimicking or representing immunodominant epitopes on the target protein or the binding epitopes of the therapeutic mAb, has become a promising strategy both for infectious diseases⁴⁰⁻⁴³ and for cancer therapy.⁴¹ Opposed to the direct mode of action by small molecules, peptides representing mimotopes of PD-1 can be used in vaccination strategies to induce the generation of blocking PD-1 antibodies in the host. Such

endogenously produced immunostimulatory antibodies (ICIs) have the potential to promote antitumor responses for prolonged periods of time, i.e. immunological memory,⁴⁴ without the need for repeated administration of ICIs or other PD-1 blocking agents. More recently, we and others have exploited this approach by using mimotopes/peptides derived from PD-1 in immunization regimens.^{39,45} Importantly, both studies have demonstrated that combined immunization with PD-1 B-cell epitopes and peptides representing cancer antigens has significant antitumor activity. The significant antitumor effect observed in our study was associated with a marginal induction of PD-1-specific antibodies.³⁹ This suggests that vaccination with PD-1 derived mimotopes/peptides might potentially be associated with a reduced degree of irAEs, in comparison to the ICIs which are administered at doses to ensure their immediate bioavailability and potency in targeting the respective immune checkpoints.³⁶

The development of molecules or peptides targeting PD-L1, which can be used as blocking compounds or in vaccination approaches, is a viable alternative strategy to block the PD-1 pathway. In addition to PD-1 and PD-L1, peptides that block other immune checkpoints such as B- and T-lymphocyte attenuator (BTLA) might also have potential in immunotherapeutic approaches.⁴⁶

PD-1/PD-L1 INHIBITORS UNDER DEVELOPMENT

The abovementioned drawbacks associated with the administration of ICIs may be circumvented by the use of small compounds or peptides, to directly inhibit the PD-1/PD-L1 interaction, or by application of mimotopes/peptides to induce the production of the corresponding ICIs by the patient's own immune system following immunization/vaccination. Based on these two approaches, as depicted in Figure 1, different types of potentially applicable inhibitors have been discovered and characterized, as shown in Table 1.

Direct blockade of PD-1 with small molecules or compounds

Phage display with random amino acids has been applied to identify a cyclic peptide, C8.⁴⁷ This peptide demonstrated a high affinity to human PD-1 (hPD-1), as well as mouse PD-1 (mPD-1), and could effectively interfere with the PD-1/PD-L1 interaction. C8 suppressed the growth of CT26 and B16-OVA tumors and also showed an effect in an anti-PD-1 antibody-resistant B16 mouse model. The authors demonstrated that the antitumor effect was mediated by the activation of CD8+ T cells.

Phage display was also applied in another study, resulting in the identification of four peptides that bound to distinct sites on PD-1.⁴⁸ The combination of the four peptides was examined *in vivo* in the B16 F10 syngeneic mouse melanoma model and was shown to induce antitumor activity comparable to that of a PD-1 antibody. The peptides were found to improve overall survival with enhanced bacterial clearance and increased macrophage function in a sepsis

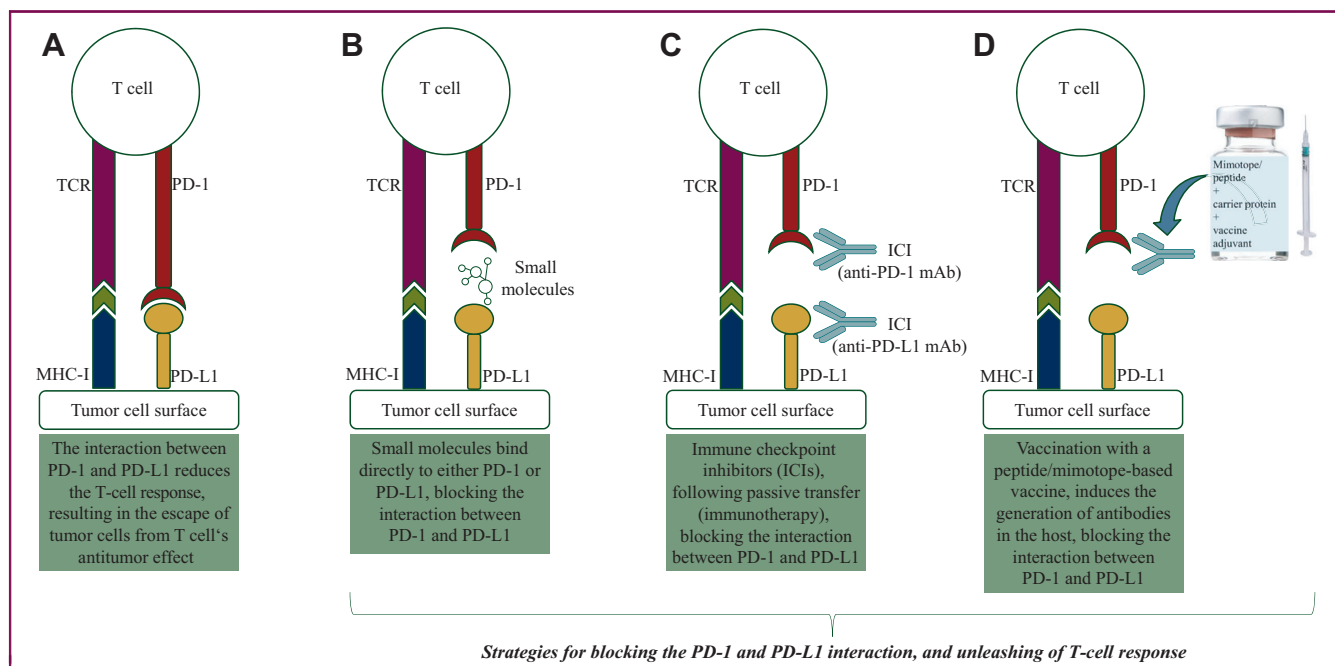


Figure 1. A diagram depicting strategies to inhibit the interaction between PD-1 and PD-L1, to consequently result in increased T-cell response and tumor cell death.

(A) Normal condition involving no blockade of PD-1 and PD-L1 interaction. With the blockade of the interaction by (B) small molecules, (C) ICIs, or (D) antibodies generated following vaccination with a mimotope/peptide.

ICIs, immune checkpoint inhibitors; mAb, monoclonal antibody; MHC-I, major histocompatibility complex I; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TCR, T-cell receptor.

model. Further, the peptides were shown to act as an adjuvant in combination with a prophylactic malaria vaccine in increasing T-cell immunogenicity and the vaccine's protective efficacy.⁴⁸

Sasikumar et al.^{30,49} have developed the first rationally designed peptide antagonist (NP-12) of the PD-1 signaling pathway, using computational tools for the prediction of the naturally folded structure of proteins. The peptide NP-12 is a decoy PD-1 receptor comprising a branched peptide containing 29 amino acids with structural elements derived from the PD-1 protein.³⁰ Although it has a shorter pharmacokinetic exposure than an examined anti-PD1 antibody, the peptide was found to result in a comparable antitumor effect in various syngeneic *in vivo* tumor models, also indicating that a sustained pharmacokinetic exposure is not needed to achieve efficacy.^{30,49} Bristol-Myers Squibb (BMS) have characterized macrocyclic peptides displaying nanomolar activities in dissociating the PD-1/PD-L1 interaction by the homogeneous time-resolved fluorescence (HTRF) assay.⁴⁹⁻⁵²

Peptides targeting PD-L1, and consequently inhibiting the interaction between PD-1 and PD-L1, have also been identified and characterized (Table 1). A peptide, PL120131, which like PD-L1 binds to the shallow binding pocket of PD-1, was rationally designed and developed.^{49,53} As a PD-L1 peptide mimetic, PL120131 was shown to inhibit PD-1-mediated apoptotic signaling and induced antitumor activity *in vitro*.⁵³

The PD-L1-targeting peptide (TPP-1),⁵⁴ with high affinity to human PD-L1 (hPD-L1), was also identified and shown to inhibit tumor growth in a xenograft mouse model using the

human lung cancer cell line H460.⁵⁴ Ahmed et al.⁵⁵ described PDLong1, a 19-mer peptide harboring an HLA-A2-restricted and PD-L1-derived CD8+ T-cell epitope.⁵⁵ Incubation of PDLong1 with peripheral blood mononuclear cells from patients with malignant melanoma who had received a dendritic cell-based vaccine was shown to result in a significantly higher number of T cells that reacted to a dendritic cell-based vaccine. By a computational *de novo* peptide design method, the peptide Ar5Y_4 was identified and shown to have a strong affinity to hPD1, comparable to hPD-L1. By a surface plasmon resonance (SPR) competitive binding assay, the peptide was shown to inhibit the interaction of hPD-1 and hPD-L1, and restore the function of Jurkat T cells which had been suppressed by the human colon carcinoma cell line HCT116.⁵⁶ A novel biopanning strategy to discover anti-PD-L1 inhibitors led to the discovery of peptide CLP002 exhibiting high binding to human PD-L1 protein as well as PD-L1-positive human cancer cells MDA-MB-231 and DU-145. CLP002 was demonstrated to bind to the residues involved in the interaction between PD-L1 and PD-1.⁵⁷ The small molecules BMS-1001 and BMS-1166, with a capacity of inhibiting the interaction between hPD-1 and hPD-L1, by binding to the latter, were developed by BMS.⁵⁸ The molecules were shown to antagonize T-cell inhibition by interfering with the PD-1/PD-L1 interaction, to block the binding of soluble PD-L1 T cells, and to induce PD-L1 dimerization in solution.⁵⁸

Although the mode of action and the CA-170-binding epitope need further verification,⁵⁹ this rationally designed molecule, which was reported to function as an antagonist of the immune checkpoints PD-L1, PD-L2, and

Table 1. Targeted agents under development.

Compound (designation)	Source/identification method	Mechanism of action	Reference
Cyclic peptide (C8)	Phage display	Binding to hPD-1 and mPD-1, and inhibiting the interaction between PD-1 and PD-L1	47
Combi vaccine, containing 4 peptides (QP-20, HD-20, BQ-20, WQ-20)	Phage display	Binding to hPD-1 and mPD-1. Combi vaccine With <i>in vivo</i> antitumor effect, comparable to the effect of an anti-PD1 antibody, in B16 F10 syngeneic mouse melanoma model	48
NP-12	Native sequences of protein interacting interfaces, decoy PD-1 receptor containing 29 amino acids with the structural elements derived from PD-1 protein	Peptide antagonist (NP-12) of the PD-1 signaling pathway	30,49
Macrocyclic peptides	Multiple hydrophobic amino acids, N-methyl and unnatural amino acids	Inhibiting the interaction between PD-1 and PD-L1	49-52
PL120131	Rationally designed peptide, as a PD-L1 peptide mimetic	Binds to the shallow binding pocket of PD-1	53
Targeting PD-L1 peptide (TPP)	Bacterial phage display	With a high binding affinity to hPD-L1	54
PDLong1	Amino acids 'FMTYWHLNNAFTVTPKDL', containing a hPDL-1-derived CD8+ T-cell epitope	Containing an HLA-A2-restricted, PD-L1-derived CD8+ T-cell epitope (PDL115–23, LLNAFTVTV)	55
Ar5Y_4	Computational <i>de novo</i> peptide design	Strongly inhibiting the interaction of hPD-1 and hPD-L1, and restoring the function of Jurkat T cells which had been suppressed by HCT116 cells	56
CLP002	Biopanning strategy	High binding to hPD-L1 protein as well as PD-L1-positive human cancer cells MDA-MB-231 and DU-145 Binding to PD-L1 at the residues where PD-L1 interacts with PD-1	57
BMS-1001 and BMS-1166	Chemical structures along all modifications of distal, flexible moieties exposed to the solvent	Binding to PD-L1NMR-based AIDA was carried out	58
CA-170	Rationally designed molecule	Targets the PD-L1, PD-L2, and the V-domain Ig suppressor of T-cell activation (VISTA) immune checkpoints, and results in activation of T-cell proliferation and cytokine production.	59
JT-N1	Amino acids 'PGWFLDSDRPNWPP' of hPD-1, using overlapping bio-peptides	Reverts the inhibitory capacity of nivolumab, in both ELISA and T cell-based cellular assay	39
JT-mPD-1	Amino acids 'ISLHPKAKIEESPGA' of mPD-1, using overlapping bio-peptides	Reverts the inhibitory capacity of the anti-mouse functional mAb clone 29F.1A12, in both ELISA and T cell-based cellular assay; induces antibodies reducing tumor growth, and enhances the antitumor effect of the Her-2/neu vaccine HerVaxx, in a syngeneic BALB/c model with mammary carcinoma cells expressing human Her-2/neu	39
PD-1-Vaxx	Amino acids 'GAISLAPKAQIKESLRAEL' of hPD-1	Induces antibodies reducing tumor growth in a syngeneic BALB/c model with CT26 colon carcinoma cells	45,61

ELISA, enzyme-linked immunosorbent assay; hPD-1, human programmed cell death protein 1; hPD-L1, human programmed death-ligand 1; Ig, immunoglobulin; mAb, monoclonal antibody; mPD-1, mouse programmed cell death protein 1; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1.

V-domain Ig suppressor of T cell activation (VISTA), has been shown to enhance T-cell proliferation and interferon- γ production. Further, the molecule was shown to be orally bioavailable and safe in preclinical studies, and to have an antitumor effect, *in vivo*, similar to anti-PD-1 or anti-VISTA antibodies.⁶⁰

Blockade of PD-1 following vaccination with mimotopes/peptides

With the aim for use as peptide-based vaccines, the peptides/mimotopes JT-mPD1, JT-N1, from mPD-1 and hPD-1, respectively, and PD-1-Vaxx were identified based on the sequence of PD-1, and were shown to induce an antitumor effect *in vivo*^{39,61} (Table 1). The antitumor effect by mimotope JT-mPD1 was shown to be associated with a significant reduction of proliferation and increased apoptotic

rates in the tumors in the employed Her-2/neu-expressing syngeneic tumor mouse model. Further, the antitumor effect of our Her-2/neu vaccine (HerVaxx)^{62,63} was shown to be potentiated when combined with JT-mPD1.³⁹ Our ongoing investigations have further indicated that active immunization has not led to increased inflammatory responses. Furthermore, using an influenza infection mouse model, no indication of enhanced inflammatory responses or cytokine storm were observed, indicating the potential safety of such a vaccination approach (submitted manuscript).

CURRENT ONGOING CLINICAL TRIALS

We have recently shown, for the first time, the concept of vaccination with mimotopes (B-cell epitope) of anti-PD-1 mAbs.³⁹ Active immunization with a mimotope, as

Table 2. Current ongoing clinical trials

Drug	Identifier number	Tumor types	Setting	Phase	Status	Reference
BMS-986189	NCT02739373	Healthy individuals	To measure the drug's amount in the blood and urine, and to evaluate its safety	I	Completed	⁵²
CA-170	NCT02812875	Patients with advanced tumors and lymphomas	Open-label, dose-escalation and dose-expansion	I	Completed	⁶⁴
IMU-201 (PD-1-Vaxx)	NCT04432207	Non-small-cell lung cancer	Multiple arms, monotherapy (dose-escalation)	I	Recruiting	^{45,61}

monotherapy or as a combination therapy together with our anti-Her-2/neu vaccine (HerVaxx),^{62,63} was shown to induce a strong antitumor effect³⁹ *in vivo*, similar to the corresponding mAb (Table 1). Along these lines, a peptide (PD-1-Vaxx) residing at the position 92-101 of hPD-1 was identified and shown to have a strong antitumor effect *in vivo* (Table 1).⁶¹ This peptide is currently being evaluated in a phase I clinical trial (Table 2), as an open-label, multicenter, non-randomized, dose-escalation and expansion study, to evaluate its safety, tolerability, and immunogenicity as monotherapy in patients with PD-L1-expressing NSCLC.⁴⁵

A multicenter, open-label, phase I trial involving the evaluation of the orally administered PD-L1/PD-L2/VISTA antagonist CA-170 (Table 2) was recently completed. The compound was evaluated in adult patients with advanced solid tumors or lymphomas which had progressed, or patients non-responsive to available therapies, or when no standard therapies were available.⁶⁴ At the time of this review's preparation, the results of the trial are pending publication. In addition to the small molecule CA-170, the macrocyclic peptide BMS-986189, inhibiting the interaction between PD-1 and PD-L1, has also proceeded to a clinical trial. This completed trial, also with results pending publication, was a randomized, double-blinded, placebo-controlled, single ascending dose study to evaluate the pharmacokinetics, safety, tolerability, and pharmacodynamics of the drug in healthy subjects.

CONCLUSION

Although there is a boom in the development of therapeutic antibodies functioning as ICIs, alternative therapeutics targeting PD-1 or PD-L1 are still needed.¹⁵ While the PD-1 and PD-L1 immune checkpoint pathways reduce T cell activation, to maintain peripheral tolerance, they can be exploited by tumors to induce an immunosuppressive state allowing the tumors' growth and immune escape. The discovery of the therapeutics based on small-molecule inhibitors of the PD-1/PD-L1 interaction, or immunogenic peptides inducing antibodies inhibiting the interaction, represents a promising immunotherapeutic approach, which could potentially overcome some of the disadvantages associated with the administration of ICIs. Although vaccination with peptides and the consequent immunological memory may result in the continuous inhibition of the PD-1/PD-L1 interaction and irAEs, also affected by the isotype of the generated peptide-specific Abs, the use of

proper vaccine adjuvants such as Alum in conjunction with the peptide may potentially skew the immune system towards the production of the peptide-specific antibodies with an isotype with low capacity for engaging with Fc γ c, i.e. IgG4.^{27,65} As mentioned previously, we have reported that active immunization of mice with the mimotope from mouse PD1 results in an increased antitumor effect. This effect, however, was shown to be associated with a marginal level of the induced mimotope/PD-1-specific antibodies which was clearly lower than the level of the passively transferred respective antibody.³⁹ This suggests that active immunization induces broader immunological and cellular effects, which have resulted in the observed antitumor effect in the employed mouse model. However, in-depth analyses including long-term experiments and eventually evaluating the safety of active immunization with the mimotope in clinical trials are essential. Such studies should also address how re- or co-treatment with chemotherapeutic agents impact the generation of PD-1 blocking antibodies. Importantly, the immunogenicity, safety, and tolerability of active immunization with a PD1 peptide (PD-1-Vaxx) are already being evaluated in a clinical trial (NCT04432207; Table 2). The various small molecules and peptides reviewed here, including a number of them being evaluated in clinical trials, have the potential to be used as inhibitors to disrupt the interaction between PD-1 and PD-L1 and result in the activation of T cells. The opportunity of using such inhibitors, possibly adapted to the stage and progression phase of the disease and tumor in addition to the existing ICIs, may potentially pave the way for treatments overcoming the disadvantages of the use of ICIs in non-responsive settings or patients receiving the currently conventional ICI treatments. Furthermore, as an especially promising strategy, such inhibitory small molecules or peptides may also serve as 'adjuvants', when vaccinating with tumor-associated or tumor-specific antigens, to induce potent anticancer responses.

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