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In Vitro Assessment of Putative PD-1/PD-L1 Inhibitors: Suggestions of an Alternative Mode of Action

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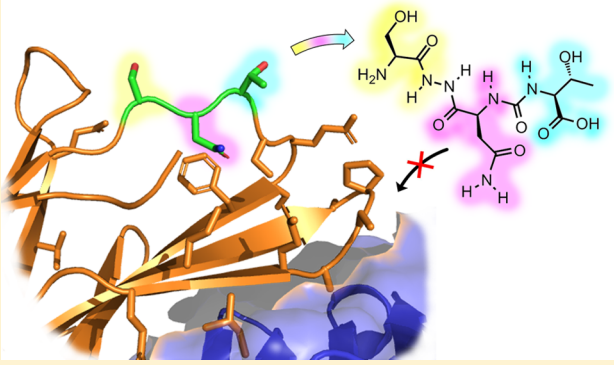
Supporting Information

ABSTRACT: The programmed cell death protein 1 (PD-1) signaling axis is among the most important therapeutic targets in modern oncology. Aurigene Discovery Technologies Ltd. (Aurigene) has patented a series of peptidomimetic small molecules derived from the PD-1 protein sequence for use in targeting the interaction between PD-1 and its ligand, PD-L1. We evaluated three of Aurigene's most potent compounds in SPR binding assays. Our results showed that these compounds—each of which is known to be potently effective in a splenocyte recovery assay—do not directly inhibit the PD-1/PD-L1 interaction nor do they appear to bind to either of the constituent proteins, indicating that another mechanism is at play. As a result of these studies and upon consideration of structural features within the PD-1/PD-L1 complex, we hypothesize that the Aurigene molecules may interact with a currently unknown protein capable of regulating the PD-1 axis.



KEYWORDS: *PD-1, PD-L1, protein−protein interaction inhibitors, SPR*

Programmed cell death protein 1 (PD-1) and its ligand (PD-L1) are transmembrane immunosuppressive checkpoint proteins.[1] PD-1 is expressed on activated T cells, and PD-L1 is expressed on somatic tissue and antigen-presenting cells.[2,3] The binding interaction between PD-1 and PD-L1 causes a down-regulation of T cell proliferative gene expression, thus disrupting cytotoxic activity. Certain aggressive cancers, such as pancreatic cancer, breast cancers, and nonsmall lung carcinomas, have evolved to overexpress PD-L1 as a means of immune evasion.[3,4] In these cases, PD-L1 expresses constitutively, allowing the tumor to masquerade as immune privileged tissue and evade detection.[5−8] Overexpression of PD-L1 is therefore a strong prognostic biomarker in oncology.[2−12]

Pharmaceutical companies have sought to develop modulators for the PD-1/PD-L1 interaction to recover lymphocyte activity. Current treatments are antibody-based therapies targeting either PD-1 or PD-L1 (Table 1). Despite the remarkable therapeutic success of these antibodies,[13,17−25] there are considerable drawbacks such as poor bioavailability, immunogenicity, and the high cost of large-scale production.[13,26,27] Small molecule inhibitors have the potential to overcome these obstacles, and multiple research groups are pursuing this objective.[28−30] However, there are currently no FDA-approved small molecule inhibitors capable of blocking the PD-1/PD-L1 interaction.[31,32]

Table 1. List of Current FDA-Approved Immunotherapeutics That Block the PD-1/PD-L1 Interaction[13−17]

name	company	target	FDA approval year
nivolumab	Bristol-Myers Squibb	PD-1	2014
pembrolizumab	Merck	PD-1	2014
atezolizumab	Genentech/Roche	PD-L1	2016
avelumab	Merck	PD-L1	2017
durvalumab	AstraZeneca	PD-L1	2017
cemiplimab	Regeneron/Sanofi	PD-1	2018

Aurigene has an extensive patent portfolio of peptides and peptidomimetic small molecules that mimic various regions of the PD-1 protein sequence. The most promising peptidomimetic compounds are reported to exhibit nanomolar potency in a phenotypic cell-based splenocyte recovery assay that Aurigene uses to scout for PD-1/PD-L1 inhibitors.[33−37] However, no direct protein binding experiments have been reported for this family of small molecules. We selected three of the most promising compounds (Figure 1) from three recent patents[35−37] and aimed to characterize them using new surface

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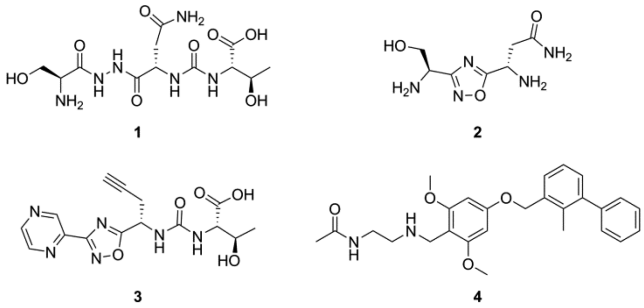

Figure 1. Compounds used for the present study. Compounds **1**−**3** are patented immunomodulators from Aurigene, hypothesized to disrupt the PD-1/PD-L1 interaction. Compound **4** is a known inhibitor developed by Bristol-Myers Squibb, employed here as a positive control.

plasmon resonance (SPR)-based methods. Each test compound was chosen with an eye toward maximizing both potency and drug-like properties, while optimally representing the compounds claimed within each patent.

Using SPR, we developed inhibition assays to detect how binding between the extracellular domain of PD-1 and the extracellular domain of PD-L1 is affected in the presence of the Aurigene compounds (refer to Figures S1 and S2 in the Supporting Information for sensorgrams showing PD-1/PD-L1 binding). In our first inhibition assay (Figure 2), biotinylated PD-L1 was adsorbed on a streptavidin-coated gold chip (SA chip). Varied concentrations (ranging from 30 nM to 30 μM) of Aurigene compounds **1**−**3** were titrated over a flow cell in the presence of recombinant soluble PD-1 (corresponding to residues P34−E150 of the native protein).

The binding responses were normalized to PD-1 binding to PD-L1 (control). Compounds acting as inhibitors of the PD-1/PD-L1 interaction would decrease the binding response relative to the control. To validate the assay, we used a known PD-1/PD-L1 inhibitor from Bristol-Myers Squibb as a positive control (compound **4**, data shown in yellow).[29] Previous studies have shown that compound **4** induces a dimerization of the PD-L1 proteins, preventing PD-1 from binding.[38−40] As expected, compound **4** showed effective *in vitro* inhibition at both low concentrations (30 nM) and high concentrations (30 μM). For titration data associated with **4**, see Figures S3 and S4.

When compounds **1**−**3** were tested in this assay, we observed no change in binding between the two proteins (Figure 2). Moreover, in the absence of PD-1 protein (data shown in hashed columns) the Aurigene compounds **1**−**3** also elicited little signal variance between 30 nM and 30 μM, suggesting that there is no directly observable binding to PD-L1. While a very small signal was likewise observed for **4** in the absence of PD-1, this is consistent with the known 2:1 binding mode between PD-L1 and compound **4**.[38] Such behavior would effectively double the molecular weight of the receptor (since **4** binds to a PD-L1 homodimer, rather than to monomeric PD-L1), while reducing the density of receptor on the surface of the chip (since not all surface-bound proteins would be able to homodimerize). The result would be very weak signal since SPR response is proportional to both the ratios of the molecular weights of the two interacting species and to the immobilization density.

We repeated the inhibition experiment, this time attaching biotinylated PD-1 to the gold surface and flowing recombinant PD-L1 protein (corresponding to residues A18-T239, chosen to represent both extracellular domains of the native protein) across the chip along with various concentrations of **1**−**4** (Figure 3). This type of reciprocal binding assay serves as an important control for binding artifacts in SPR experiments. Once again, the positive control molecule (**4**) was observed to inhibit the interaction (data shown in yellow), although in this case higher concentrations of **4** were required because of the high concentration of PD-L1 required in the experiment. These data provide additional support for the earlier study determining PD-L1 as the biological target for compound **4**.[39]

As in the previous assay, however, the Aurigene compounds **1**−**3** showed no inhibition of the PD-1/PD-L1 interaction (Figure 3) nor did they appear to elicit any statistically significant binding to adsorbed PD-1. At very high concentrations (300 μM), Aurigene compounds **1** and **2** gave an increase in SPR response (i.e., negative inhibition), but this is likely due either to molecules nonspecifically adsorbing to the attached protein or else to compound precipitation. The same results were observed in our assay for soluble PD-1 flowed over another adsorbed binding partner, PD-L2 (Figure S6). Compound **4** was also shown to not inhibit the PD-1/PD-L2

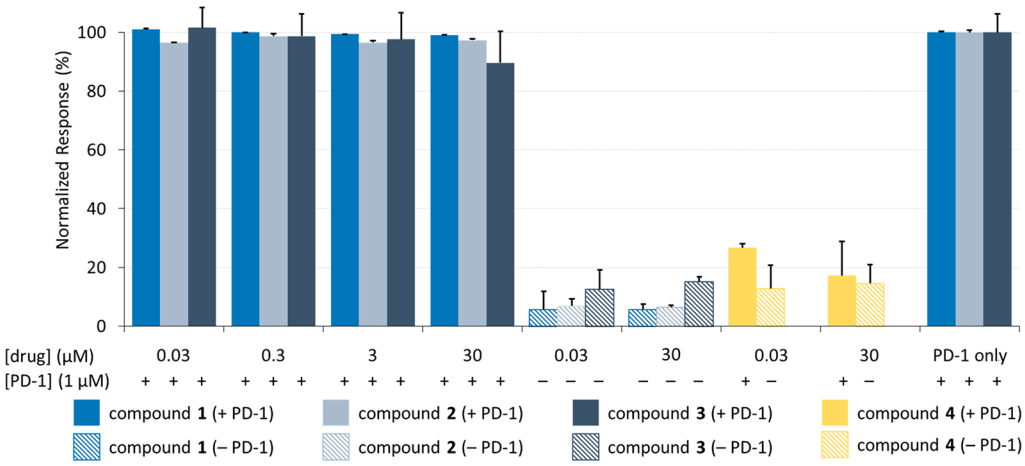

Figure 2. Aurigene compounds (**1**−**3**) show no inhibition between 30 nM and 30 μM for PD-1 flowing over PD-L1. Soluble PD-1 was flowed across surface-bound PD-L1 with and without test compounds at varying concentrations. The response is normalized to the control protein interaction (PD-1 only). Responses were measured in triplicate, and error bars represent standard deviation.

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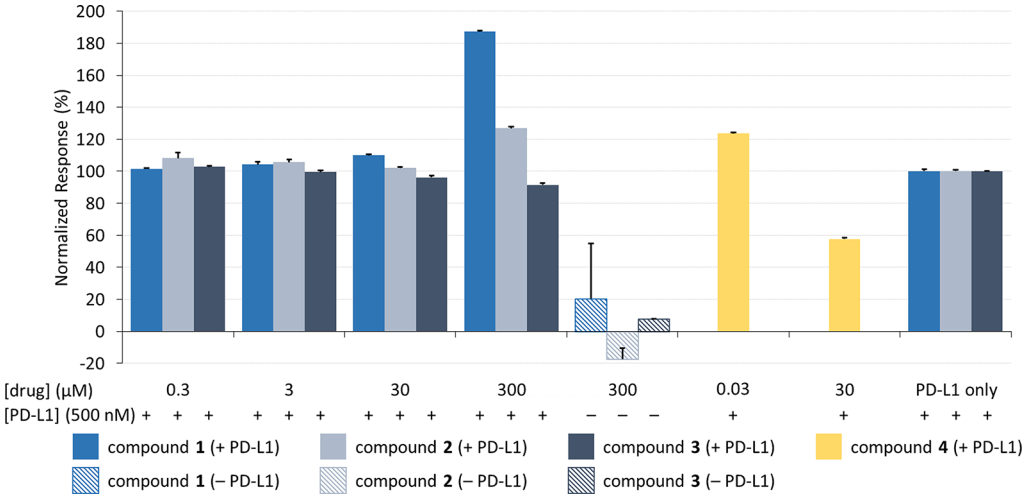

Figure 3. Aurigene compounds (**1**−**3**) show no inhibition between 300 nM and 300 μM for PD-L1 flowing over PD-1. Soluble PD-L1 was flowed across surface-bound PD-1 with and without test compounds at varying concentrations. The response is normalized to the control protein interaction (PD-L1 only). Responses were measured in triplicate, and error bars represent standard deviation.

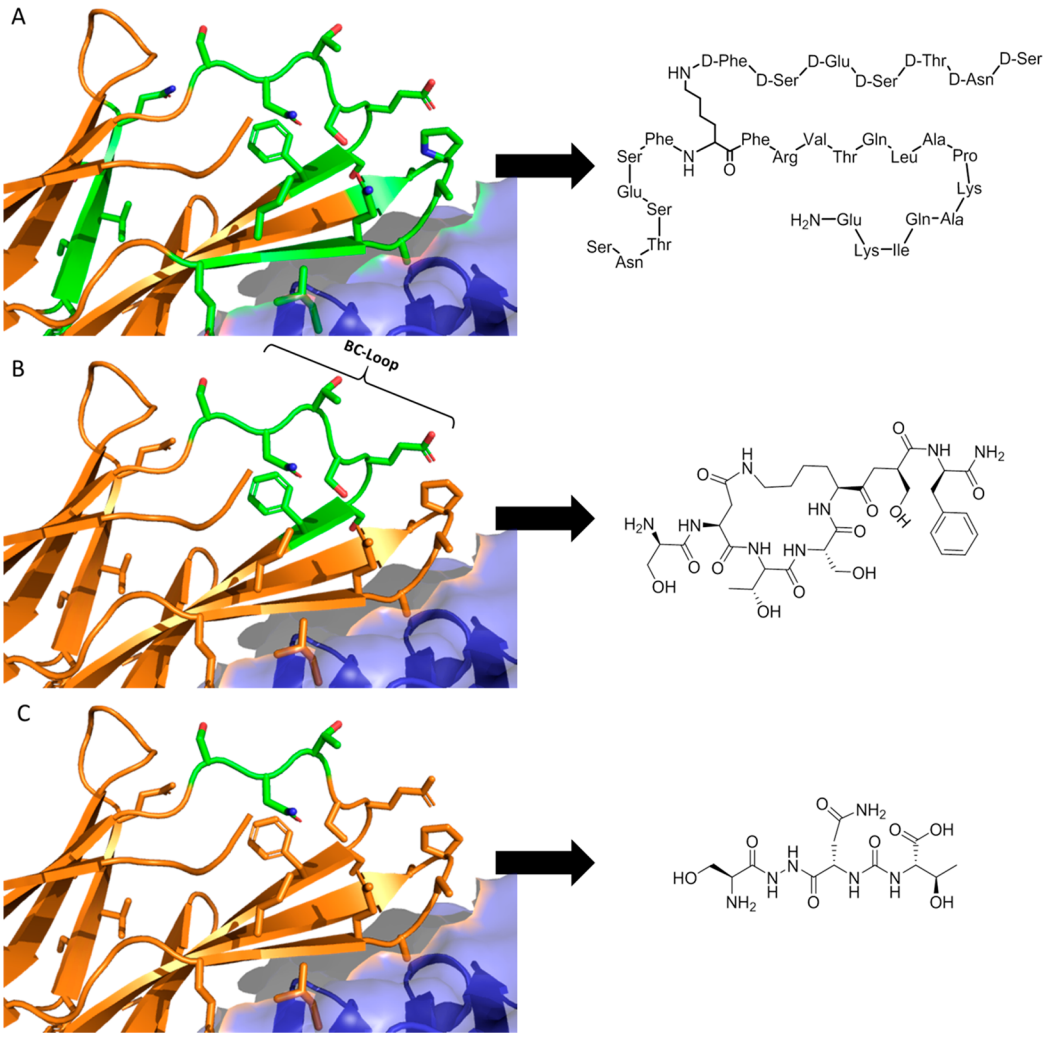

Figure 4. Binding interface between PD-1 and PD-L1 (from PDB: 4ZQK), together with an illustration of the discovery process leading to Aurigene's lead compounds. PD-1 and PD-L1 are shown in cartoon representation (orange) and surface representation (blue), respectively. Residues highlighted in sticks are the residues the patent compounds are derived from. (A) Left: Residues (57−63, 95−99, 127−136) of the PD-1 protein. Right: Patented peptide derived as a structural analogue to the region. (B) Left: Residues (57−63) of the PD-1 protein. Right: Patented macrocyclic compound derived as a structural analogue to the region. (C) Left: Residues (57−59) of the PD-1 protein. Right: Small molecule peptidomimetic derived as a structural analogue to the region.

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interaction and showed no sign of binding directly to PD-L2, highlighting the specificity of the BMS compound.

Seeking confirmation of these results in an orthogonal assay type, we also briefly explored the function of compound **1** in a commercial ELISA assay for PD-1/PD-L1 inhibitors (Figure S8). Once again, however, we saw no significant inhibitory activity. Positive controls (including compound **4** and two related inhibitors from Bristol-Myers-Squibb, as well as a known antibody inhibitor) all worked as expected, once again validating the assay.

Aurigene characterized the efficacy of their lead compounds using a phenotypic cell-based assay built around splenocyte recovery as a proxy for immune activation. Compounds **1**−**3** triggered high recovery in this experiment (68%, 93%, and 92%, respectively, relative to an uninhibited positive control) at 100 nM.[35−37] The limited experimental data in Aurigene's patents (including controls with PD-L1 in the absence of small molecule) suggest that the phenotype was PD-L1 specific. But as we have shown above, the results from Aurigene's cell-based assays cannot be attributed to direct inhibition of PD-1/PD-L1 binding. Instead, it would appear that an alternative mechanism is responsible for the observed phenotypic effect.

Despite the recent appearance of a number of papers related to small molecule modulation of the PD-1/PDL-1 interaction,[14,28,31,41−44] there is little explanation of how the Aurigene compounds exert their function. However, a close reading of Aurigene's patent portfolio provides some insight into the development of this class of small molecules. Aurigene's early patent filings claimed large peptides that mimic a significant portion of the PD-1 extracellular domain (Figure 4A).[33] This includes much of the PD-L1 binding interface, and so it is likely that these large peptides would be competitive inhibitors of the PD-1/PD-L1 interaction. Attempts to achieve more drug-like properties, however, led to subsequent filings describing the development of macrocyclic peptides that mimic the 7-residue BC-loop (Figure 4B).[34] The most recent filings further refine the structure (and, presumably, improve the pharmacokinetic properties) by developing small tripeptides and peptidomimetic analogues that mimic the central serine-asparagine-threonine tripeptide of this loop (Figure 4C).[35−37] This is the strategy that ultimately led to small molecules **1**−**3**.

Critically, however, the BC-loop (and particularly the region mimicked by **1**−**3**) points away from the PD-L1 binding interface (Figure 4B), and so it is unclear why these molecules would be expected to be direct binding inhibitors, notwithstanding their apparent potency in cell-based assays and the fact that they are referred to (with little supporting data) in Aurigene's patents[35−37] and subsequent reviews[28,42,45] as inhibitors of the PD-1/PD-L1 interaction.

While it is possible that there is some key difference (e.g., glycosylation state) between our *in vitro* system and the "real-life" PD-1 and PD-L1 proteins expressed on the surface of T cells and antigen presenting cells, a more likely scenario is that the compounds do mimic the PD-1 surface as intended, but that this serves not to directly disrupt binding with the PD-L1 protein as has been assumed, but perhaps to modulate the function of some other PD-1 binding partner. Both PD-1 and PD-L1 are thought to participate in regulatory binding interactions with other proteins,[46,47] and we hypothesize that one or more of these might be the true biological target of **1**−**3**.

In 2015, Curis Inc. initiated clinical trials with a small molecule called CA-170, which had been developed at Aurigene.[48] The exact structure of CA-170 has yet to be disclosed, but this lead candidate apparently emerged from a focused library at Aurigene that was designed to exploit hotspots within the PD-1/PD-L1 complex.[49] In a recent review by two of the inventors of the Aurigene molecules **1**−**3**, it is stated that CA-170 was designed to target one or more conserved pockets found in PD-L1 and VISTA, a nonredundant immunosuppressing protein of the B7-superfamily.[28] These authors are careful to not indicate whether CA-170 is structurally related to compounds **1**−**3**, but two subsequent reviews (from two different groups) both speculate that CA-170 is related to Aurigene's earlier disclosed compounds.[50,51] One of these recent reviews asserts that CA-170 is an oxadiazole (like compounds **2** and **3**),[51] while the other describes CA-170 as a molecule capable of disrupting the PD-1/PD-L1 complex.[50] The existence of a clinical candidate that is likely related to compounds **1**−**3** and that is thought to function through direct blockade of PD-1/PD-L1 binding provides motivation to better understand the function for this series of small molecules. In this study, we performed surface-plasmon resonance assays to test the hypothesis of direct protein binding inhibition and found that none of the compounds tested can disrupt the interaction between soluble PD-1 and adhered PD-L1 or between soluble PD-L1 and adhered PD-1. Preliminary testing also did not reveal binding of **1**−**3** to surface-bound VISTA (Figure S7). Based on these data and an analysis of structural features of the PD-1/PD-L1 interaction (and particularly the region of the PD-1 BC-loop from which Aurigene's lead compounds were derived), we hypothesize that this family of small molecules may regulate the function of some other PD-1 binding partner.[52]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00221.

Supplementary Figures S1−S8, together with experimental details for protein expression, analytical methods, and compound synthesis, as well as NMR spectra for synthesized compounds **1**−**4** (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare the following competing financial interest(s): D.A.P. is an employee of Inception Sciences Canada. The remaining authors declare no competing financial interest.

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■ ABBREVIATIONS

PD-1, programmed cell death protein 1 (CD279);; PD-L1, programmed death-ligand 1 (CD274 or B7−H1); VISTA, V-domain Ig suppressor of T cell activation; SPR, surface plasmon resonance

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(52) Following the submission of this manuscript, a study on the *in vitro* function of a likely CA-170 candidate (structurally related to **1**−**3**) was disclosed. This study likewise finds that the candidate is inactive in a variety of protein-binding experiments and concludes that an alternative mechanism may be responsible for its biological activity. See: Musielak, B.; Kocik, J.; Skalniak, L.; Magiera-Mularz, K.; Sala, D.; Czub, M.; Holak, T. A.; Plewka, J. CA-170 - a Potent Small-Molecule PD-L1 Inhibitor or Not? *bioRxiv 662668*; DOI: 10.1101/662668.

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