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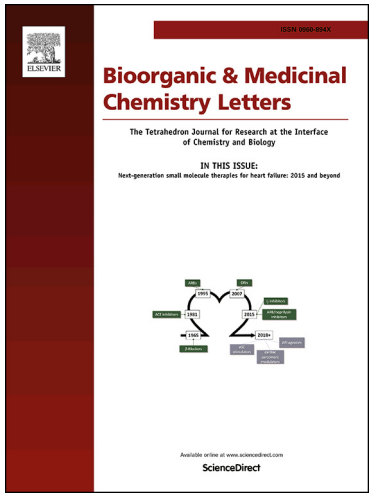

Symmetry-based ligand design and evaluation of small molecule inhibitors of programmed cell death-1/programmed death-ligand 1 interaction

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Symmetry-based ligand design and evaluation of small molecule inhibitors of programmed cell death-1/programmed death-ligand 1 interaction

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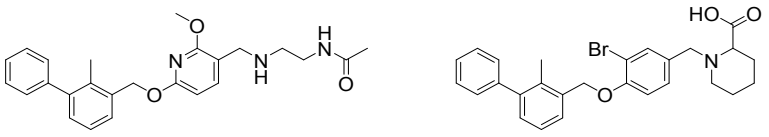
Abstract:
The development of small molecule inhibitors of PD-1/PD-L1 is eagerly anticipated for treatment of cancer. We focused on the symmetry of the ternary complex structure of reported small molecule ligands and hPD-L1 homodimers, and designed partially- or fully-symmetric compounds for more potent inhibitors. The design of the new compounds was guided by our hypothesis that the designed symmetric compound would induce a flip of sidechain of $_{A}$Tyr56 protein residue to form a new cavity. The designed compound **4** exhibited substantially increased binding affinity to hPD-L1, as well as PD-1/PD-L1 inhibitory activity in physiological conditions. Compound **4** also showed a dose-dependent increase in IFN-γ secretion levels in a mixed lymphocyte reaction assay. These results not only indicate the feasibility of targeting the PD-1/PD-L1 pathway with small molecules, but illustrate the applicability of the symmetry-based ligand design as an attractive methodology for targeting protein-protein interaction stabilizers.

Keywords: PD-1, PD-L1, Small-molecules, Immune checkpoint inhibitor, Surface plasmon resonance, Drug design

The PD-1/PD-L1 (programmed death protein 1/programmed death-ligand 1) pathway has drawn intense research interest since monoclonal-antibodies (mAbs) targeting PD-1 or PD-L1 achieved great successes in the clinic for the treatment of cancer. [1, 2, 3] Although mAbs targeting PD-1 and PD-L1 gave robust clinical outcomes, there is still room for improvement. For example, immunogenicity, immune-related adverse effects as a result of a long half-life, pharmacokinetics and high target occupancy, [4] limited routes for administration, and high cost are major shortcomings in mAbs. On the other hand, small molecule inhibitors are capable of overcoming some of these disadvantages such as immunogenicity, lack of oral bioavailability, and high cost. Therefore, the advent of small molecule inhibitors of PD-1/PD-L1 continues to be eagerly anticipated. [4, 5, 6] Recently, a series of small molecule PD-1/PD-L1 inhibitors (**1**–**2**) were disclosed in a patent application (Figure 1).[7] Later Zak and coworkers revealed in their crystallographic and biochemical studies that these biaryl compounds induce and stabilize dimerization of PD-L1s, and consequently inhibit PD-1/PD-L1 interaction. [8]

Small molecule protein-protein interaction (PPI) stabilizers have been recently highlighted in the research field as an opposing approach for modulating PPI with respect to its inhibition. [9, 10, 11, 12] According to some reviews, [13, 14] the majority of stabilized PPI complexes have simple structural features, and the main feature is their symmetry in the complex. Indeed, most stabilizers were observed in homomers and quasi homomers, and two protein monomers were virtually stabilized by single symmetric or pseudo-symmetric compounds. For example, a class of inhibitors of the bromodomain and extraterminal (BET) proteins, [15, 16] positive allosteric modulators of the AMPA receptor, [17, 18] and the Toll-like Receptor agonist Diprovocim [19] were identified as symmetric or pseudo-symmetric compounds. These examples exploit symmetry-based ligand design for discoveries of potent multivalent ligands.

Motivated by reports of compounds (**1**–**2**) that form a pseudo-C_2-symmetric complex with hPD-L1s and stabilize PD-L1 homodimer, we undertook the study of symmetric ligands for more potent PD-1/PD-L1 inhibitors.



1 (BMS-202)
IC_{50} = 18 nM

2 (BMS-8)
IC_{50} = 146 nM

Figure 1 Structures of the reported small molecule inhibitors of PD-1/PD-L1.
Names and IC_{50} values reported in the patent are shown.

Figure **2** illustrates the symmetry-based ligand design in this work. We selected compound **3** (BMS-1327, [20] Figure **2**) as a template compound to bring the concept of symmetry-based ligand

design into practice. Compound **3** bears the (5-cyanopyridin-3-yl)methoxy group which appeared to give high potency in our preliminary results in a cell-free assay (data not shown). The binding mode of **3** is conjectured to arise from the ternary complex of **1** with hPD-L1s (PDB ID: 5J89), which is a pseudo-symmetrical structure where the axis of symmetry is positioned at the center of the biphenyl substructure. The major structural difference between the two protein monomers is the positions of the side chain phenyl rings of both Tyr56 residues. The phenyl ring of $_{A}$Tyr56 is oriented toward the surface of the chain B, creating a hydrophobic wall close to one side of the cleft between the two proteins. In contrast, phenyl ring of $_{B}$Tyr56 is lying in a direction along the protein surface while forming a π-π interaction with the methoxypyridine ring of **1**.

On applying the concept of symmetry-based ligand design to compound **3**, the aforementioned hydrophobic wall formed by $_{A}$Tyr56 should be considered as a potential obstacle. In several reports in the literature, [21, 22, 23] crystal structures of hPD-L1, including apo-hPD-L1 (PDB ID: 5C3T) and the hPD-1/hPD-L1 complex (PDB ID: 4ZQK), [23] have been reported. The position of $_{A}$Tyr56 in the ternary complex (5J89) was different from that of the two structures apo-PD-L1 and in a complex with PD-1. Based on these facts, we hypothesized that the side chain of Tyr56 is sufficiently flexible and capable of taking both configurations. Thus, we designed dimeric ligand **4** with the aim of inducing a shift of $_{A}$Tyr56 to create a new cavity inside a large interface, while the other half of the compound would bury into the newly generated space in the same way as the original monomeric ligand does. Indeed, during our research, this assumption was experimentally confirmed with the co-crystal structure of analogue of **1** with PD-L1s (BMS-1001, [20] PDB ID: 5NIU [24]), in which the 2,3-dihydro-1,4-benzodioxinyl group induced a shift in the position of $_{A}$Tyr56 reported by Guzik et al. [25]

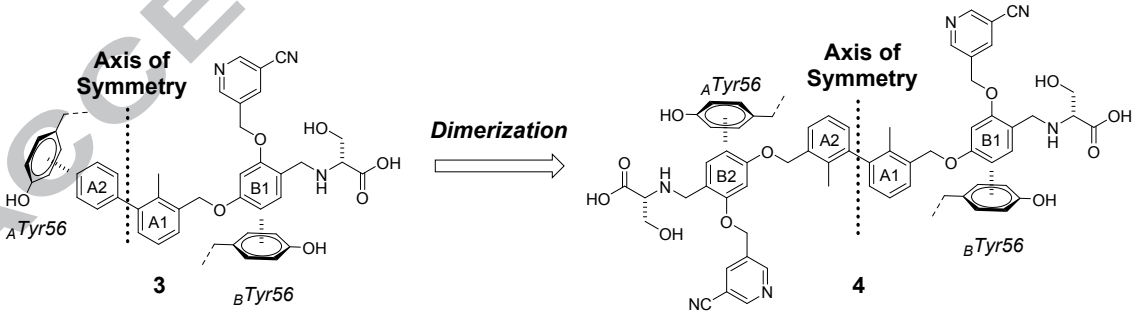
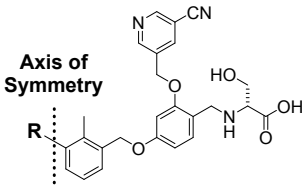


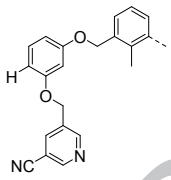
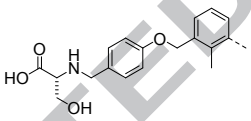
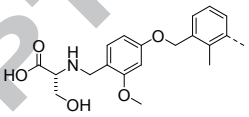
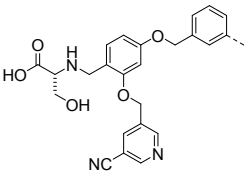
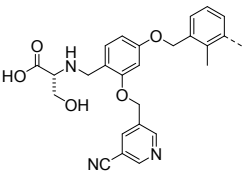
Figure 2 Schematic representation of symmetry-based ligand design

In addition to the biphenyl-core group (A1 and A2) which forms a T-shaped π-π stacking with $_{A}$Tyr56 and CH-π interactions with $_{A}$Met115 and $_{B}$Ala121, three other structural components of compound **3** were taken into account in design of the compounds: 1) the methyl group on the A1 ring which is buried in the lipophilic cavity surrounded by lipophilic residues; 2) an aromatic ring B1

which forms π-π interaction with the phenyl ring of $_B$Tyr56; and 3) a terminal amino acid moiety which forms multiple hydrophilic interactions such as water-mediated or direct hydrogen bonds and electrostatic interactions with both chains. For the purpose of elucidating the contribution of each substructure in compound **3**, both the fully dimerized compound (**4**) and partially dimerized compounds **5**–**9** (Table 1) were designed and synthesized.

Table 1 Binding affinities and PD-1/PD-L1 inhibitory activities of designed compounds

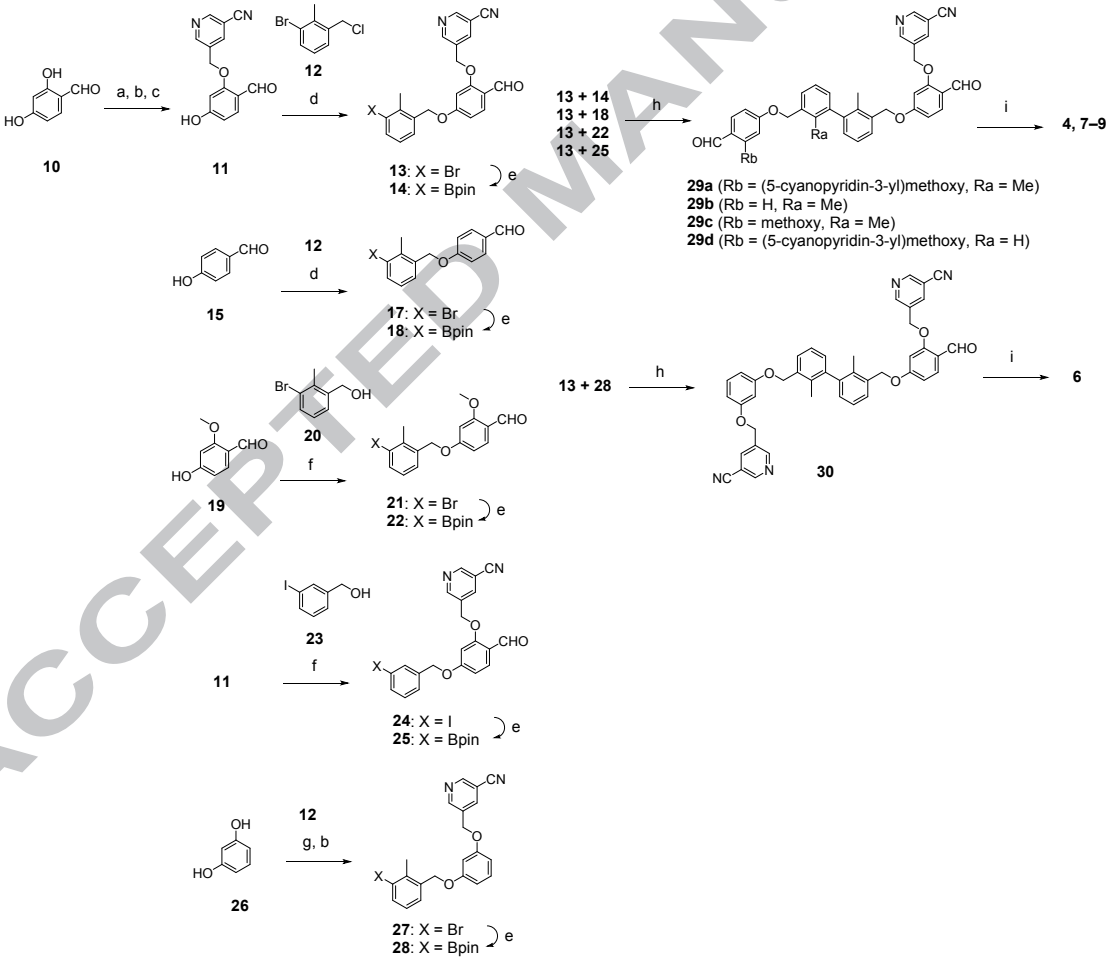


Entry	R	K_D (nM)[a]	EC_{50} (μM)[b]
3 (BMS-1327)		1.1	> 30
5		3.0	NT
6		3.7	> 100
7		0.29	NT
8		0.21	9.5
9		0.20	8.4
4		0.019	1.0

[a] Binding affinities (K_D) were determined by Surface Plasmon Resonance analysis. [b] PD-1/PD-L1 inhibitory activities were measured by blockade bioassay (for details see Supporting Information).

Compounds **3** and **5** were synthesized according to the method described in the patent [20]. Scheme 1 shows the general synthetic route for designed compounds **4** and **6–9.** 2,4-

Dihydroxybenzaldehyde **10** was used as a starting material. After protection of the 4-hydroxy moiety of **10** with THP, the 2-hydroxy group was converted to the (5-cyano-3-pyridine)methoxy group, and subsequent deprotection gave phenol **11**. Alkylation of phenol **11** with benzyl chloride **12** afforded key intermediate **13**. Similarly, intermediate **17**, **21**, **24**, and **27** were prepared in one or two steps from 4-hydroxybenzaldehyde **15**, 4-hydroxy-2-methoxybenzaldehyde **19**, phenol **11**, or resorcinol **26**, respectively. Each halogenated intermediate (**13**, **17**, **21**, **24**, and **27)** was subjected to palladium-catalyzed borylation to give aryl boranes (**14**, **18**, **22**, **25**, and **28)**. Formation of dimeric biphenyl core products was conducted with halogenated product **13** and aryl boranes (**14**, **18**, **22**, **25**, and **28)** via palladium-catalyzed Suzuki coupling reaction to give **29a–d** and **30.** Reductive amination of mono- or bis-aldehyde product **29a–d** and **30** with D-serine finally afforded compounds **4** and **6–9**.



Scheme 1 Synthetic route of dimeric compounds 4 and 6–9.

a) DHP, PPTS, CH_2Cl_2, rt. b) 5-Chloromethylnicotinonitrile, K_2CO_3, DMF, rt. c) *p*-TsOH, MeOH-H_2O, rt. d) K_2CO_3, DMF, rt. e) Bis(pinacolato)diboron, AcOK, $Pd_2(dba)_3$, Pcy_3, Dioxane, 100 °C. f) DIAD, PPh_3, THF, rt. g) NaH, DMF, rt. h) method A: $Pd(PPh_3)_4$, Na_2CO_3, CPME/H_2O, 100 °C.,

method B: Sphos Pd G1, K_3PO_4, DMF, 80 °C. i) D-Serine, AcOH, $NaBH(OAc)_3$, DMF or DMSO, rt.

Binding affinities of **3–9** against hPD-L1 were evaluated with surface plasmon resonance (SPR). As shown in Table 1, it is evident that there are marked increases in binding affinity with compounds bearing two amino acid moieties (**4** and **7–9)**, while partially-dimerized compounds such as **5** or **6** did not show significant increases in K_D from monomeric ligand **3**. Additionally, compound **4** exhibited 50 times smaller K_D as compared to **3**. Compounds **7–8**, bearing single replacements of the (5-cyanopyridin-3-yl)methoxy group of **4** with hydrogen or methoxy group, also showed subnanomolar binding affinity. However, 10-fold decreases in K_D from **4** were observed in both compounds. Similarly, compound **9**, with the single displacement of the methyl substituent on the phenyl ring A2 also resulted in 10-fold drop in K_D from **4**. These results revealed that terminal amino acids groups have a great impact on binding affinity. The (5-cyano-3-pyridine)methoxy group on ring B and the methyl substituent on the ring A also appeared to contribute significantly in binding to hPD-L1. To investigate whether compound **4** induces the PD-L1 dimerization in solution, a size-exclusion chromatography was conducted in the presence and absence of **4**. The retention time of PD-L1 was significantly shortened in the presence of **4** and the change in retention time was indicative of dimer formation (Supplementary Figure 1).

PD-1/PD-L1 inhibitory activities were evaluated with a blockade bioassay (Table 1). Monomer or partially-dimerized compound **3** and **6** did not show EC_{50} values of less than 10 μM, while anti-PD-1 antibody as a positive control exhibited EC_{50} of 5.7-14 nM. In contrast, fully-dimerized compounds **4**, **8**, and **9** showed significantly lower EC_{50} values. Especially, compound **4**, EC_{50} = 1.0 μM, showed the highest potency among all compounds tested. We were able to show the efficacy of dimerized compounds in the cell assay, but the EC_{50} was approximately 40,000 ~ 50,000-fold less potent than K_D values (Table 1). Since non-specific binding of compounds to serum protein was considered as the reason of the discrepancy, cell-free PD-1/PD-L1 inhibitory activities of several compounds were measured in the presence or absence of serum protein. However, no remarkable effect of serum protein on inhibitory activity was observed (data not shown). Therefore, the reason for the discrepancy is still unclear, but may result from non-specific binding to other factors such as cell surface protein, cell membrane.

For further evaluation of the most potent compound **4**, cellular function was evaluated with a mixed lymphocyte reaction assay (MLR assay). [26] For comparison, anti-PD-1 antibody was also tested. Results are presented in Figure 3.

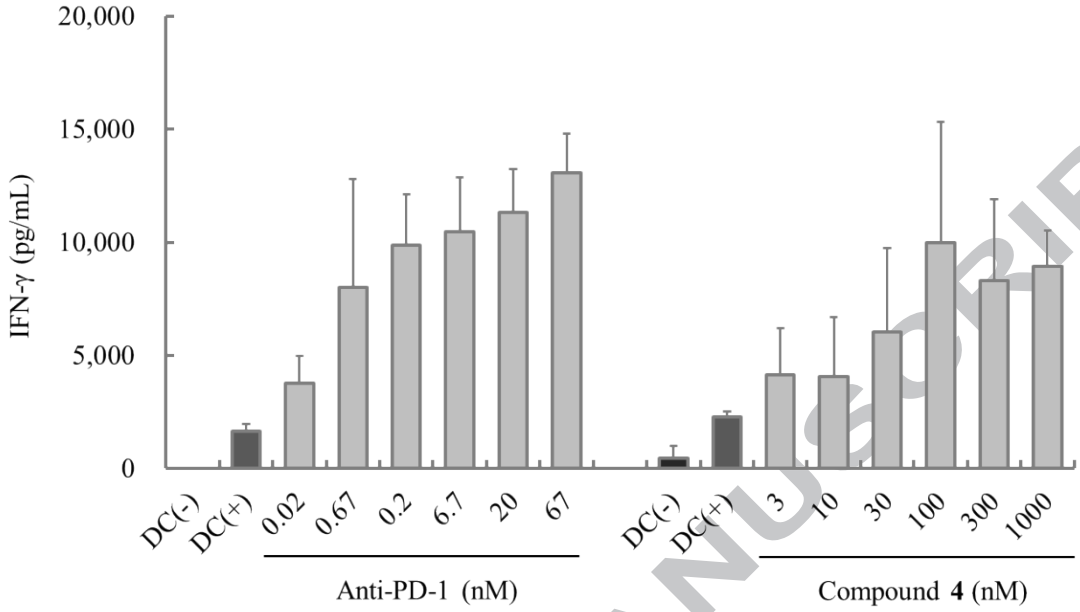


Figure 3 MLR assay

CD4+ T cells were cultured for 7 days alone or in the presence of monocyte-derived dendritic cells (DCs) with or without anti-PD-1 or compound **4**. IFN-γ secretion in culture supernatants was determined as an indicator of cell activation. The graphs present the mean ± SD from experiments performed in triplicate.

As shown in Figure 3, compound **4** exhibited a dose-dependent increase in IFN-γ secretion levels. At 100 nM, **4** showed similar levels of IFN-γ as compared to the maximum efficacy of anti-PD-1 antibody exhibited. From these results, compound **4** appeared to function as a promising small molecule PD-1/PD-L1 inhibitor to restore T-cell function. However, there is a large gap in potency between **4** and anti-PD-1 antibody. Thus, further optimization and exploration remains for future work.

In summary, we designed novel PD-1/PD-L1 inhibitors bearing a C_2-symmetric scaffold. Our ligand design is unique in terms of the consideration of both symmetricity of homodimer and a potential shift of a protein residue which was projected from co-crystal structures. Designed compound **4** induced dimerization of PD-L1, and showed significantly increased binding affinity to hPD-L1, as well as inhibitory activity of PD-1/PD-L1 interaction under physiological conditions compared to the original monomeric ligand. The most potent compound **4** also exhibited a dose-dependent increase in IFN-γ production in the MLR assay. To our knowledge, this is the first example of small molecule PD-1/PD-L1 inhibitors that verify cellular function with the MLR assay. Therefore, we believe our results will lead to the development of small-molecule modulators based

on dimeric scaffold targeting the PD-1/PD-L1 pathway. Moreover, this work illustrates the applicability of the symmetry-based ligand design as an attractive methodology for targeting protein-protein interaction stabilizers.

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