Solubilizer tag effect on PD-L1/inhibitor binding properties for m-terphenyl derivatives

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Abstract

Although heavily studied, the subject of anti-PD-L1 small molecular inhibitors is still elusive. Here, we present a systematic overview of principles behind the successful anti-PD-L1 small molecule inhibitor design on the example of the m-terphenyl scaffold with a particular focus on the neglected influence of the solubilizer tag on the overall affinity towards PD-L1. The inhibitor developed according to the proposed guidelines was characterized through its potency in blocking PD-1/PD-L1 complex formation in HTRF and cell-based assays. The affinity is also explained based on the crystal structure of the inhibitor itself, its co-structure with PD-L1 as well as molecular modeling study. Our results structuralize the knowledge related to the strong pharmacophore feature of the m-terphenyl scaffold preferential geometry and the more complex role of the solubilizer tag in PD-L1 homodimer stabilization.

1. Introduction

PD-1 (programmed cell death protein 1) is a 55-kDa transmembrane protein constituted of an IgV-like N-terminal extracellular domain, a transmembrane domain, and a cytoplasmic domain. PD-1 is mostly expressed on surfaces of T cells, natural killer cells, and B cells. PD-1 binds two natural ligands: PD-L1 and PD-L2 which are both transmembrane proteins belonging to the immunoglobulin superfamily. In a healthy system, the PD-1 engagement by its natural ligands inhibits a T-cell response, resulting in reduced effector functions, leading to cancer cell protection but also chronic infections, and decreased autoimmunity (Han et al., 2020; Keir et al., 2008).

Dysfunctions of the regulatory effect of the PD-1/PD-L1 checkpoint toward the immune system can lead to several diseases related to autoimmunity, infections, and cancer (Chen & Han, 2015; Sharpe et al., 2007). In cancer cells, overexpression of PD-L1 leads to the progression of T cells into an exhausted state and decreased tumor cell apoptosis. The disruption of the PD-1/PD-L1 interaction leads to the reactivation of T cells laying the foundations for cancer treatments coined (Alsaab et al., 2017; Ribas & Wolchok, 2018; Sharma & Allison, 2015, 2020; Sunshine & Taube, 2015). Since its discovery, the PD-1/PD-L1 blockade has proven to be an efficient treatment of several cancer types, such as: non-small cell lung cancer, Hodgkin lymphoma, breast cancer, etc. (Lin et al., 2020; Sukari et al., 2016). Currently, all clinically approved anti-PD-1/PD-L1 therapeutics rely on highly selective monoclonal antibodies (mAbs), such as nivolumab against PD-1, durvalumab against PD-L1 (Ribas & Wolchok, 2018; Twomey & Zhang, 2021). Despite their superb efficacy, there is a constant urge to develop alternative therapeutic classes, overcoming the limitations of mAbs related to their poor pharmacokinetic profile, high manufacturing costs, oral unavailability, and also observed adverse effects (Hutchinson et al., 2021; Johansen et al., 2019). Those additional classes comprise mainly small molecular inhibitors (SMIs) and macrocyclic peptides (Butera et al., 2021; Konieczny et al., 2020b; Magiera-Mularz et al., 2017a; Muszak et al., 2021a; Shaabani et al., 2018). Moreover, several studies have shown that synergistic effects were observed in therapies combining SMIs and mAbs, leading to a new wave of anti-PD-L1-oriented therapies (Sun et al., 2021).

Although several postulated and promising SMIs have been designed and tested, only a few reached the clinical trials stage, while to date none have been approved for cancer treatment. Amongst all the discovered putative small-molecular drugs, the most promising results have been shown for PD-L1 binding inhibitors
belonging to macrocyclic peptides and peptidomimetics (Guzik et al., 2019a; Lin et al., 2020; Yang & Hu, 2019). Amongst the small-molecule inhibitors, the first class and the most significant breakthrough were compounds based on biphenyl core, disclosed by Bristol Myers Squibb in 2015 (L. Chupak et al., 2015; L. S. Chupak & Zheng, 2015). Since then, the biphenyl-core structures have been highly developed, with the PD-1/PD-L1 complex inhibition results reaching up to the nanomolar scale (Guzik et al., 2019a; Lin et al., 2020; Wu et al., 2020). However, except for the C2-symmetrical structures, such as the most prominent compound A presented by Park et al. in 2021 (Park et al., 2021), this class of compounds still lacks the level of activity displayed by mAbs in the in vitro assays (Surmiak et al., 2021).

Despite biphenyl core being a well-established and crucial pharmacophore fragment of anti-PD-L1 active SMI agents, its solubility remains a challenge. Thus, several solubilizing tags were tested in order to modulate the designed molecule's physicochemical properties (Konieczny et al., 2020b; Muszak et al., 2021a). Nevertheless, the function of this molecular fragment (apart from increasing the compound's solubility) on ligand-protein complex stabilization is poorly understood. The question arises whether it is possible to rationally design the solubilizer tag to increase ligand's affinity and anti-PD-L1 activity by allowing additional protein-ligand interaction formation. Herein we present a systematic structure-activity study for newly synthesized and biologically tested compounds, based on the recently discovered m-terphenyl core decorated with cyclic amino acid derivatives as one of the most reported solubilizer tags in anti-PD-L1 SMIs. Compounds were initially tested for their potency in disrupting the PD-1/PD-L1 complex using standardized Homogeneous Time-Resolved Fluorescence (HTRF) assay followed by the cell-based Immune Checkpoint Blockade (ICB) assay. The understanding of the protein-ligand interactions, including the role of the solubilizer tag, was assessed via computational methods and crystal structures analysis.

2. Results and Discussion

2.1 Chemistry

We started by developing further the scaffold described in (Muszak et al., 2021a) to investigate if we can improve its biological properties by modifying the solubilizer tag and how it influences the results in cells. The synthesis of the m-terphenyl-based parent inhibitors presented herein is shown in Scheme 1. The initial m-terphenyl core was synthesized using Suzuki-Miyaura coupling reactions as described previously (Muszak et al., 2021a). An m-terphenyl precursor was then reacted with thionyl chloride, leading to a reactive benzyl chloride which is suitable for the subsequent nucleophilic substitution with one of the ester-protected cyclic amino acids: proline (1), β-proline (2), pippecolinic acid (3), nipepicotic acid (4) and isonipeptic acid (5). Obtained esters were then directly transferred into final compounds using an aminolysis reaction or were hydrolyzed and coupled with different amines.

2.2 The PD-L1 binding affinity determination

Synthesized compounds were tested for their potency in disrupting the PD-1/PD-L1 complex using HTRF assay in a scouting mode, validating the percentage of undissociated PD-1/PD-L1 complex at 5 nM concentration of inhibitor (Table 1). At this condition, the BMS-1166 compound, used herein as a reference, gave the value of 42.1 ± 6.4% of undissociated complex. The IC50 of BMS-1166 in the HTRF assay, with a
value of 3.89 ± 0.19 nM, was reported in our previous paper (Muszak et al., 2021b). The vast majority of the tested compounds effectively disrupted the PD-1/PD-L1 complex at a concentration of 5 nM. The most prominent solubilizing tags amongst all the tested amino acid fragments are β-proline (2a-2h) and isonipecotic acid (5a-5h), while pipercolinic acid (3a-3h) and proline (1a-1h) derivatives, which are often used as PD-L1 SMIs’ solubilizers were generally the worst-performing. Additionally, the acidic form of the designed inhibitors (1a-1b) was, in most cases, one of the weakest inhibitor in each series. The effect is especially noticeable in the direct comparison of carboxylic acid derivatives with its less acidic bioisoster: hydroxamic acids (1b-5b) (Lassalas et al., 2016). For the carboxylic acid series, only the proline derivative 1b resulted in improved activity compared to the hydroxamic acid analog 2b, yet both are rather mediocre inhibitors. Therefore, we have decided to reduce the acidity and moved to the more neutral amides or acyl hydrazides. However, the latter one’s acyl hydrazides (1c-5c) and N,N-dimethyl acyl hydrazides (1d-5d) have not shown satisfactory results, except for β-proline derivatives 2c and 2d. For the amide series, the weakest activity was observed for the ethanolamine derivatives (2f-5f). Increased molecular size and solubility of the amide by introducing serinol (1g-5g) and tris(hydroxymethyl)aminomethane (TRIS) (1h-5h) resulted in an improvement in the activity of the tested inhibitors. Interestingly, the methylenediamine derivatives (1e, 2e, and 5e), yielded similar results to the corresponding TRIS derivatives. Nevertheless, such compounds proved to be less stable and, therefore, more difficult to synthesize. Altogether, the highest inhibitory activity was shown for the β-proline derivative with the TRIS solubilizer (2h) which resulted in almost complete complex dissociation at the 5 nM concentration. Significantly, one of the most prominent small-molecule inhibitors of PD-L1, compound A (J.-J. Park et al., 2021) produced similar results in our laboratory reference test performed at the same condition (18.0 ± 1.3% for 5 nM inhibitor concentration).
2.3. Cell-based assay

Following the HTRF analysis, the activity of the selected molecules was verified in the well-exploited cell-based PD-1/PD-L1 ICB assay (Cheng et al., 2015; Konieczny et al., 2020a; Magiera-Mularz et al., 2017b; Muszak et al., 2021b). For the analysis, the compounds displaying the most striking activity in the HTRF analysis were chosen, with a cutoff of 20% of the undissociated PD-1/PD-L1 complex. All the analyzed compounds increased the activation of the effector Jurkat T cells in the assay, where the activation thereof is blocked by the PD-1/PD-L1 immune checkpoint (Fig. 1A). This bioactivity was observed at the concentration of 1 µM for the β-proline derivatives 2b, 2e, 2g, and 2h (Fig. 1B). For compounds 2d and 2g at the concentration of 6.4 µM, this activity was retained, while for compounds 2e and 2h the T cell activation dropped down most probably due to toxic effects on the cells used in the assay. The observation proves the PD-L1-blocking activity of the compounds in the cellular context, although it has to be acknowledged that the observed effect is considerably lower than the one observed for the control anti-PD-L1 antibody durvalumab (Fig. 1B).

2.4. Crystal structure of PD-L1 with 2f

Diffraction-quality crystals of the PD-L1/2f complex were obtained using a sitting-drop setup. The final resolution of the obtained co-crystal structure was 2.1 Å (crystallographic parameters are shown in Table S1).
The asymmetric unit contains one molecule of inhibitor 2f and two molecules of PD-L1, which form a homodimer (Fig. 2A). This type of dimerization upon the interaction with the inhibitor has been previously observed for biphenyl-based scaffold inhibitors of PD-L1 (Guzik et al., 2017). The terphenyl moiety of 2f provides strong stabilizing π interaction with A_Tyr56 as well as numerous hydrophobic interactions with both PD-L1 subunits’ amino acids including A_Tyr56, A_Met115, B_Met115, A_Ala121, B_Ala121, B_Tyr123 (Fig. 2B). A strong salt bridge between B_Asp122 carboxylic group and the protonated amine of the 2f molecule is also observed. Additionally, a hydrogen bond between B_Arg125 and the terminal hydroxyl group of 2f, is observed. However, it should be noted that the electron density of this terminal part of the inhibitor is poor, suggesting a high flexibility of the 2f solubilizer tag (Fig. 2B). Therefore, the presented spatial orientation of the solubilizer tag was based on the possible protein-ligand interactions.

2.5. Crystal structure of compound 2a and Cambridge Structural Database analysis

Compound 2a crystallizes in the centrosymmetric space group I2/a (Table S2). The asymmetric unit consists of one molecule in the zwitterionic form (Fig. 3A, Figures S1-3). Additionally, there are 4 water molecules, from which two (namely O3W and O4W) are located at a special position and represent two alternative molecules’ locations. Water molecule O2W is disordered and refined in two positions with site occupancies of 55% and 45%. All water molecules form a net of hydrogen bonds propagating in a channel along [100]. The presence of the water channels in the proximity of the solubilizing tag confirms the hydrophilic properties of this molecular fragment. The fluctuating water molecules’ positions lead to disorder within the solubilizing tag (β-proline and its carboxylic substituent) with refined site occupancies of 54% and 46%. The two alternative positions are shown in Fig. 3B (the less abundant conformation is shown in green).

Apart from hydrogen bonds involving water molecules, the strongest observed intermolecular interaction is a charge-assisted hydrogen bond (salt bridge), formed between protonated amine of the β-proline and the carboxylate anion of the neighboring molecule. This interaction propagates parallel to the water channels. The corresponding salt-bridge interaction is also observed in the protein-ligand crystal structure presented here, where the protonated amine of 2f can interact with the anionic form of B_Asp122. In the crystal of 2a several C-H…O interactions are observed (Table S3), which additionally stabilize the crystal structure.

The molecular conformation of the main aromatic, m-terphenyl core is well conserved for the small molecule and protein-ligand crystal structures. The superposition of the m-terphenyl fragment for compound 2a in its crystal form (Fig. 3C, the molecule with carbon atoms in gray) on the one observed for compound 2f in the binding cavity of the PD-L1 dimer (Fig. 3C, the molecule with cyan carbon atoms) is almost identical with the RMSD for aromatic rings carbon atoms of ~0.11 Å). For the structure 2a the torsion angles C1-C2-C7-C16 (TOR1) and C1-C6-C17-C22 (TOR2) are 56.25° and 46,64°, respectively. The mutual aromatic fragments' orientation may be defined also by angles between planes of phenyl rings 1–3 (marked as blue numbers in Fig. 3A) with angles 1/2 (ANG1), 1/3 (ANG2), and 2/3 (ANG3) being 53.85°, 46.06°, and 87.44°, respectively. Such a spatial orientation of π-electron-rich fragments may be the main characteristic, responsible for binding to the PD-L1 dimer as it matches the corresponding m-terphenyl angles in a co-crystal structure. Therefore, we postulate that a correct preorientation of the core m-terphenyl scaffold in our inhibitors is primarily responsible for its strength in dissociating PD-1/PD-L1 complex as it avoids a thermodynamic penalty as no
“torsion adjustments” are required for the inhibitor. Interestingly, such mutual aromatic rings’ arrangement is not very strictly defined and conserved for different m-terphenyl-containing structures and strongly depends on substituents. The Cambridge Structural Database (CSD, Ver. 5.43, November 2021) (Groom et al., 2016) search revealed a wide range of values for all the analyzed geometrical parameters (the histograms showing the statistical distribution of TOR1-2 and ANG1-3 are presented in Figures S4-8) with maximum counts for TOR1-2 in range ±(80–100)° ANG1-2 in the range 75–90° and ANG3 45–60°. From the statistical point of view, the m-terphenyl derivatives presented here adopt peculiar geometry not strongly represented in CSD results which can be defined as a strong pharmacophore feature for the PD-1/PD-L1 inhibitors, perhaps justifying why such scaffolds were not reported previously.

The solubilizing tag of 2a and 2f in crystal structures presented here is oriented differently. When a ligand is bound to PD-L1, the geometry of this molecular fragment is the most sensitive to the environment as it is exposed to the solvent and, therefore, may display a high disorder level, which can be confirmed by the low coverage of the $2F_o-F_c$ electron density map (at contour level 3σ) from Fig. 3, panel B. The mobility of this fragment may suggest the formation of interactions both with protein and solvent, in a competitive and even interchanging manner.

2.6. Molecular modeling of m-terphenyl ligands’ interactions with dimer PD-L1

Initially, the docking procedures were conducted and optimized with PD-L1 homodimer coordinates with m-terphenyl analog stabilizing the dimer (PDB ID 7NLD (Muszak et al., 2021a)) taken from Protein Data Bank (Berman et al., 2000). Meanwhile, the crystal structure of the PD-L1 dimer with compound 2f has been obtained. The docking study performed for the protein-ligand co-crystal structure presented in this paper gave similar results to those obtained in the early stage of docking experiments. Thus, we describe here only the last set of results, shown in the summarized graphical form in Fig. 4A (results in the numerical form with ChemPLP scoring function values are included in the Supplemental file 2).

The best PLP scoring function result was obtained for Compound A, which is one of the most potent PD-L1 ligand amongst all investigated molecules described so far. Nonetheless, the correlation between the studied ligand’s ranking and HTRF results is insignificant with p-value of 0.178 according to the Pearson correlation test and an overall correlation value of 0.7 (Figure S9). The general observation based on the docking results is that the positive charge generated on the protonated amine in the solubilizer tag corresponds to a higher-scoring function result compared to the neutral form of the ligand. This is related to the $N^+\text{H}^+\text{B}^-$Asp122 salt bridge formation that has a strong positive influence on the protein-ligand complex stabilization due to its strength (Riccio et al., 2021).

The re-docking procedure resulted in good compatibility of the 2f conformation observed in the crystal structure with the generated pose, with well-reconstructed protein-ligand interactions (Fig. 4B and C). The calculated RMSD is 0.84 Å (based on all non-hydrogen atoms of the 2f ligand), with very well-conserved m-terphenyl geometry. The highest discrepancy between the structure and predicted model was observed for 2,3-dihydro-1,4-benzodioxine moiety and the solubilizer tag region, further confirming that the solubilizer tag is in fact highly mobile in the protein/ligand complex and we only approximate its conformation (Fig. 4D). The first
mentioned difference in ligand’s orientation within the binding cavity is related to the slightly different conformation of the dioxane ring in the docked vs. native poses. That can be explained by the presence of a water molecule in the deep region of the binding site for the crystal data (Fig. 2B) which was removed for the docking procedure, leading to a more spacious binding site. Additionally, a small translational shift of the docked compound lacking it by ca. 0.5 Å, however, still preserving all key interactions (Fig. 4D).

The solubilizer tag orientation is a less trivial issue. The comparison of all the best-scored poses for each of the investigated ligands revealed a highly conserved location and orientation of the m-terphenyl fragment with a strong diversity in the solubilizer tag geometry (Fig. 4E). This result hints that indeed the role of this terminal fragment may be related to increasing the ligand’s solubility rather than the additional stabilization of the compound in the PD-L1 homodimer binding site (however, we do not see a clear correlation of HTRF/scoring results to the in silico predicted LogS – Figure S9). More likely, this observation can imply high mobility of the solubilizer tag, and the competition between polar amino acids in the cavity’s entrance and environmental water molecules to form optimal interactions with this molecular fragment.

The log S prediction results show that all studied compounds in their neutral form are only moderately soluble (-6 < log S<-4) (Figure S9). However, the majority of the presented compounds are in their cationic forms, due to the protonation of the amine group within the solubilizer tag at physiological pH, which affects the resulting compounds’ final solubility. Additionally, the predicted logP seems to be optimal only for Compound A, whereas for most of the tested compounds, this important pharmacological parameter is in the range of 4–5, implying high lipophilicity of the m-terphenyl derivatives and hindering its accessibility in a polar water environment.

The weak correlation between the docking results and the biological tests can be related to the still not fully understood mechanism of action of PD-L1 ligands, which equally may be based on the enhancement of cell-surface PD-L1 dimerization as well as influencing some intracellular processes (J. J. Park et al., 2021). The synergistic mechanism involving intracellular actions can be highly reduced due to a limited cell membrane penetration ability by the preferential ionic form (at pH 7.4) for most of the PD-L1 ligands presented here. On the contrary, the positive charge on the protonated amine of the solubilizer tag strengthens the protein-ligand interaction and complex stabilization as discussed above. For this reason, all considered physicochemical parameters such as ionization of the compound, low solubility and high lipophilicity can be treated as limiting factors, which cooperatively influence the biological activity of PD-L1 ligands.

3. Conclusions

The success of cancer therapy by inhibition of negative immune regulation awarded the 2018 Nobel Prize in Physiology or Medicine jointly to James P. Allison and Tasuku Honjo. It fueled the development of small molecular inhibitors (SMIs) disrupting the PD-1/PD-L1 immune checkpoint. Despite the great interest resulting in numerous patents and publications on the PD-L1-targetted SMIs, the understanding of the mode of action of small inhibitors on PD-L1 at a molecular level is still not well established. Classically, anti-PD-L1 SMIs’ scaffolds are divided into the biaryl core responsible for the PD-L1 dimerization, followed by the aryl with an ether-linked group, to increase the number of “binding anchors”, and terminated by the solubilizer tag accountable primarily for the enhancement of the compound solubility index (nowadays, many deviations
from this classical outline are reported, such as mirrored compounds, etc. (Guzik et al., 2019b). The question also arises, whether the solubilizing fragment influences ligand-protein binding and may be rationally designed to increase the potency of SMIs to stabilize the ligand-induced PL-L1 homodimerization. This led us to the formulation of guidelines for anti-PD-L1 SMIs. Continuing the work on the meta-chloro-terphenyl scaffold, we found that its characteristic pre-orientation of the aromatic rings in the inhibitor's scaffold to engage PD-L1's $\text{A}_{\text{Tyr56}}, \text{A}_{\text{Met115}}, \text{B}_{\text{Tyr123}}$ in strong hydrophobic/π-interactions favors sub-nanomolar inhibitory constants. Therefore, the correct terphenyl substitution (with e.g., a halogen or a methyl in ortho position) leading to steric hindrance and lowering the resonance effect as it was shown in the ligand's crystal structure and the following CSD search presented in this manuscript is a valid and straightforward strategy for the development of strong conformational scaffolds and pharmacophore model.

Solubilizer tags are often considered to increase the solubility of anti-PD-L1 compounds which are usually quite hydrophobic. However, based on the performed in-silico modeling routine and the obtained experimental results we did not find a correlation. Clearly, poor inhibitor's solubility can lead to its aggregation in the polar environment (such as buffers) and lower its effective concentration. However, the connection between anti-PD-L1 SMIs and their lipophilicity is a convoluted process we do not understand fully yet. Also, the second most postulated argument that solubilizers provide additional, stabilizing contacts with PD-L1, such as the hydrogen bond between the $\text{B}_{\text{Arg125}}$ and the terminal hydroxyl group of the 2f compound reported here, seems not very obvious as the poor electron density around this terminal part of the inhibitor suggests a high degree of flexibility of this fragment. A more likely explanation of the "solubilizer tag" role is that due to its high degree of conformational changes in the PD-L1 dimer-formed binding cavity, it prevents water molecules from penetrating the hydrophobic core of the ligand-protein complex. This is illustrated in our docking routine where resulting poses with similar predicted binding scores represent various "solubilizer tag" orientations. In our work, we decorated the m-chloro-terphenyl scaffold with polar amino acid derivatives, such as proline, pipeliconic acid, or isonipecotic acid conjugated with various terminal groups including hydroxyl, amides, acyl hydrazides, or ethanolamine groups. Nearly half of the reported compounds were more potent in the disruption of PD-1/PD-L1 complex than a well-known BMS 1166 compound at 5 nM final concentration (BMS 1166 disrupts 42% of the preformed PD-1/PD-L1 complex). Especially, β-proline series (2a-h) proved to be potent as all terminal fragments of this group gave better results than the positive control BMS 1166, suggesting sub-nanomolar IC$_{50}$s.

Ionization/protonation state of anti-PD-L1 inhibitors is often neglected and/or not considered in the SMIs in silico design. Nevertheless, this parameter is crucial as it can affect the binding energy and complex stabilization by highly favorable salt bridge formation, which leads to an enhancement of the biological activity towards PD-L1 for both macrocyclic peptides and SMIs (Riccio et al., 2021). Application of this information in the in silico approach can increase the predictive power of the molecular docking-based method for the studied protein-ligand system. Additionally, the potential ionization of the putative drug molecule may be a critical factor influencing bioavailability and altering the properties of cell penetration. The latter would be especially important in the case of the dual surface-internal/cytoplasmic mode of action of anti-PD-L1 small inhibitors.
Guidelines formulated here for PD-L1 SMIs shed more light on the often-neglected subject of the importance of the solubilization tag. Through the extensive biological, biochemical, and structural analysis exemplified by the *meta*-chloro-terphenyl scaffold, we aimed to structure the current knowledge about the importance and complex function of the solubilizing tag in the design of PD-L1 SMIs.

4. Experimental section

4.1. Synthesis

All the reagents and solvents which were used for synthesis and characterization of the compounds were received from commercial suppliers such as Sigma Aldrich, Alfa-Aesar, Acros, Ambeed, and others, they were used without further purification. The NMR spectral data were recorded with Bruker Avance NMR spectrometer, $^1$H NMR at 600, and $^{13}$C NMR at 151 MHz, reporting chemical shifts ($\delta$) in ppm and coupling constants (J) in Hz. All chemical shifts were analyzed in correspondence to the solvent peaks (DMSO-$d_6$, MeOD-$d_4$, CDCl$_3$). Thin-layer chromatography (TLC) was performed on aluminum sheets precoated with Silica Gel 60 F$_{254}$ (Merck). Techniques of visualization of TLC plates included radiation with a UV lamp at 254 nm wavelength. Purification of compounds was conducted by flash chromatography on the Grace Reveleis X2 Flash Chromatography System with the Grace Resolv Silica Cartridges. The LCMS measurements were recorded on the UPLC-MS/MS system consisting of a Waters ACQUITY UPLC coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were performed with the use of the Acquity UPLC BEH C$_{18}$ column; 2.1 $\times$ 100 mm, and 1.7 $\mu$m particle size, equipped with Acquity UPLC BEH C$_{18}$ VanGuard pre-column; 2.1 $\times$ 5 mm, and 1.7 $\mu$m particle size. The column was maintained at 40°C, and eluted under gradient conditions using from 95–0% of eluent A over 10 min, at a flow rate of 0.3 mL min$^{-1}$. Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v). All final compounds were determined to have at least 92% of purity. High-resolution mass spectrometry (HRMS) analyses were carried out with the microTOF-QII spectrometer using the ESI ionization technique.

4.2. Homogenous Time-Resolved Fluorescence

The certified Cis-Bio assay kit was used to perform the HTRF assay. Following the standard protocol, the measurement was carried out at a 20 $\mu$L final volume with a 5 nM concentration of hPD-L1 and 50 nM concentration of hPD-1 in the final formulation. Separate dilution series were performed to calculate the half-maximal inhibitory concentration (IC$_{50}$) of the most potent compounds. Analyte and detection reagents (anti-analyte conjugated donor and acceptor conjugated antibody) were placed in the proper wells on the microplate as suggested in the Cis-Bio protocol. The plate was left for 1 h of incubation and it was read on an HTRF certified microplate reader Tecan Spark 20M. Output data were subjected to background subtraction on negative control, and normalization in correlation to the positive control, and averaged.

4.3. PD-1/PD-L1 immune checkpoint blockade (ICB) assay

For the *in vitro* analysis of the bioactivity of the molecules an Immune Checkpoint Blockade assay was performed (Cheng et al., 2015). CHO/TCRAct/PD-L1 cells (Promega), overexpressing an artificial TCR-activator construct and PD-L1, were seeded on 96-well white plates at the density of 10 000 cells/well. The
next day, the cells were overlaid with the effector Jurkat T cells (Jurkat-ECs, Promega, 20 000 cells/well), overexpressing PD-1 and containing construct assuring an NFAT-induced expression of luciferase, either in the presence of the indicated concentrations of the compounds with DMSO-only as a control (the concentration of DMSO was kept constant at 0.1%) or durvalumab (Selleckchem), as a positive control, with untreated cells as controls. Activation of the Jurkat-ECs, reflected by luciferase activity, was monitored by luminescence measurements after 6 h of incubation (37°C, 5%CO₂), and 20 min of additional incubation with the Bio-Glo™ Assay reagent (Promega) at room temperature. The luminescence was read on the Spark microplate reader (Tecan). The data is presented as fold induction of the luminescence signal relative to either untreated (for durvalumab) or DMSO-treated (for compounds) cells. Data points represent mean ± SD values from duplicates.

4.4. Protein expression and crystallization

The plasmid encoding of the PD-L1 protein (amino acids 18–134) was used to transform E. coli BL21 strain. The bacteria carrying the plasmid were then grown at 37 °C until reaching an OD600nm of 0.6–0.8. The protein expression was then induced by adding a final concentration of 1 mM IPTG and the expression was then carried overnight at the same temperature. The PD-L1 protein was then isolated in the form of inclusion bodies, which were subsequently washed using a protocol previously described (Magiera-Mularz et al., 2017b). After washing the inclusion bodies, the protein was refolded by slowly dropping around 50 mg of inclusion bodies into a solution containing 0.1 M Tris-HCl pH 8.0, 1 M L-arginine-HCl, 0.25 mM oxidized glutathione, and 0.25 mM reduced glutathione. After the refolding step, the protein was dialyzed 3 times against buffer containing 10 mM Tris-HCl pH 8.0 and 20 mM NaCl. The final step of purification consisted of size exclusion chromatography using a Superdex 75 column and 10 mM Tris- HCl, 20 mM NaCl based buffer for crystallization or PBS buffer for NMR based experiments. The correctly folded state and purity of the PD-L1 protein were checked by NMR and SDS-PAGE respectively.

The freshly purified protein was concentrated to 5 mg/mL and then mixed in a 1:3 molar ratio with an excess of the 2f inhibitor, finally the solution was centrifuged for 1 minute at max speed. The clarified solution was then used for screening using multiple available commercial screens. The diffraction quality crystals were obtained from a sitting drop set up at room temperature with a buffer containing 27% w/v PEG 3350, 0.1 M Bis-Tris propane, pH 7.0 and 0.2 M Lithium sulfate. Crystals were cryo-protected using glycerol and flash frozen with liquid nitrogen.

The X-ray diffraction data were collected at the BL13 - XALOC beamline at ALBA (Barcelona, Spain) (Juanhuix et al., 2014). The data were indexed, integrated, and scaled using XDS, SCALE, and Aimless (Evans & Murshudov, 2013; Kabsch, 2010; Krug et al., 2012). The initial phase estimates was obtained by molecular replacement calculated in Phaser using PDB:5C3T as a model (McCoy et al., 2007). The refinement was performed using CCP4cloud (Winn et al., 2011) and verified using Coot (Adams et al., 2010; Emsley & Cowtan, 2004; Joosten et al., 2014). The structure was deposited in the Protein Data Bank with accession number 8P1O. The protein-ligand interactions were assessed using PLIP server (Adasme et al., 2021) and visualized using PyMol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

4.5. Crystal structure determination for compound 2a
X-ray diffraction data for single crystals of compound 2a was collected using XtalLAB Synergy-S four-circle diffractometer with a mirror monochromator and a microfocus CuKα radiation source (λ = 1.5418 Å). The CryoStream cryostat system was used to allow low-temperature experiments, performed at 100(2) K. The obtained data sets were processed with CrysAlisPro software (Rigaku-Oxford Diffraction; CrysAlisPro Oxford Diffraction Ltd, Abingdon, England V 1. 171. 36. 2.; release 27-06-2012 CN, 2006). The phase problem was solved with direct methods using SIR2014. (Burla et al., 2015). Parameters of the obtained model were refined by full-matrix least-squares on F2 using SHELXL-2014/6 (Sheldrick, 2008). Calculations were performed using WinGX integrated system (ver. 2014.1) (Farrugia, 1999). Figures were prepared with Mercury 4.0 software (MacRae et al., 2020).

All non-hydrogen atoms were refined anisotropically. All hydrogen atoms attached to carbon atoms were positioned with the idealized geometry and refined using the riding model with the isotropic displacement parameter $U_{iso}[H] = 1.2 \times U_{eq}[C]$ for all but the methyl group, for which $U_{iso}[H] = 1.5 \times U_{eq}[C]$ was applied. The hydrogen atom at N24, being a result of the tertiary amine protonation, was located on the Fourier difference map and refined with no restraints on $U_{iso}$ parameter. In the structure water channels are observed, propagating along [100] axis. The four water molecules in the asymmetric unit are highly disordered, with two of them located in the proximity of the crystallographic 2-fold axis, leading to two alternative positions of O3w and O4w water molecules. Due to the observed disorder of water molecules, their hydrogen atoms’ coordinates were obtained by the Fourier difference map inspection, supported by CALC-OH predictive algorithm (Nardelli, 1999) available in the WinGX suite (Farrugia, 1999). Additionally, those hydrogen atoms were refined using the riding model with the isotropic displacement parameter $U_{iso}[H] = 1.5 \times U_{eq}[O]$, with restraints on distances and angles (DFIX and DANG commands, respectively) to maintain relatively good water molecule geometry. The disordered water channels lead to the partial, positional disorder within the β-proline fragment which is involved in interactions with the mentioned water molecules. This disorder was modeled based on the Fourier difference map inspection and the site occupancies were determined during the refinement procedure. Figures presenting asymmetric unit and packing scheme (Figs. S1 and S2) as well as crystal data and refinement results (Table S1) are shown in the Supplementary Information.

Crystal structure data of 2a has been deposited with the Cambridge Crystallographic Data Centre - accession no. CCDC 2231594

4.6. Molecular modeling

4.6.1 Protein preparation procedure

Prior to the molecular docking simulations, the PD-L1 dimer was prepared using Maestro (Version 12.5.139) (Schrödinger Release 2020-3: Maestro, Schrödinger, LLC, New York). The hydrogen atoms were added in the idealized positions. The missing side chains were added using the conformers’ library. The ligand originally bound to the protein as well as water molecules were removed for the next step of the experiment.

4.6.2 Ligands preparation procedure

The 3D geometries of the investigated *m*-terphenyl derivatives were obtained from SMILES with OpenBabel (version 2.4.1). (O’Boyle et al., 2011). Additionally, the possible protonation at pH = 7.4 was predicted with
Calculator Plugins in Marvin 19.12.0, 2019, ChemAxon (http://www.chemaxon.com). The ionic form was applied if 60% or more molecules were ionized at the set pH. For the range of 40–60% both forms (neutral & ionized) were considered. Otherwise, the compound was kept in the neutral form. The ionization was introduced in Maestro 3D Builder. The initial geometries were minimized with the OPLS3 force field (Harder et al., 2016).

4.6.3 Molecular Docking Procedure

Molecular docking experiments were performed using GOLD 2021.3.0 (Genetic Optimisation for Ligand Docking) software (Verdonk et al., 2003). The binding region was defined based on the location of the native ligand, including all atoms within 8 Å, to prevent binding to the corresponding, mirror-like site formed in the homodimer. During the semi-flexible docking process, ligands were allowed flexibility to find the most probable binding pose, using a genetic algorithm (GA) implemented in GOLD [M4]. The empirical ChemPLP scoring function was applied to evaluate the obtained results. Three top-scored results have been recorded for each docked molecule. Compound A, (J.-J. Park et al., 2021), BMS-1166 (L. Chupak et al., 2015; L. S. Chupak & Zheng, 2015), m-terphenyl analog from 7NLD crystal structure (Muszak et al., 2021b) and 2f compound have been used as reference ligands in the docking procedure, after their geometry randomization (conversion of SMILES to .mol2 followed by energy minimization), and ionization state prediction as described in “Ligand preparation procedure” section. A full table of the docking results is attached to the Supplementary Materials file 2.

Declarations

Accession code

Crystallographic data for compound 2a has been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication no. CCDC 2231594. Copies of the data can be obtained, free of charge, on application to CCDC (e-mail: deposit@ccdc.cam.ac.uk).

The structure factors and final models of PD-L1 complexes with inhibitor 2f was deposited into the Protein Data Bank with the accession numbers 8P1O/

Authors contributions


Declaration of competing interest

The authors declare no competing interests.
Data availability

Data will be made available on request to ewa.surmiak@uj.edu.pl.

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References


**Schemes**

Scheme 1 is available in the Supplementary Files section

**Figures**
Figure 1

The bioactivity of the molecules in the *in vitro* PD-1/PD-L1 Immune Checkpoint Blockade (ICB) assay. (A) A schematic representation of the assay, in which the effector Jurkat T cells (Jurkat-ECs) were incubated with the stimulator CHO/TCRAct/PD-L1 cells in the presence of the tested molecules. (B) The graph presents the fold induction of the activation of Jurkat-ECs cells in the presence of either the indicated compounds (1 µM or 6.4 µM) or therapeutic anti-PD-L1 antibody (1 µg/ml), relative to controls. Untreated cells (ctrl.) served as controls for the durvalumab treatment and DMSO-treated cells as controls for the compound treatments. Data points represent mean ± SD values from duplicates.
Figure 2

Crystal co-structure of PD-L1 and 2f inhibitor. A) A surface/cartoon representation of PD-L1 dimer with A subunit in red and B subunit in blue. The 2f inhibitor is presented in a cyan stick style with atoms color-coded. B) The 2f molecule and its protein surrounding, showing interactions with PD-L1 amino acids, superimposed on the difference 2F_o - F_c electron density Fourier map leveled at 3σ (gray isomesh). The inhibitor 2f is colored cyan, A PD-L1 amino acids are colored red, while B PD-L1’s are colored blue. Hydrogen bond is indicated with blue continuous line, hydrophobic interactions in gray dashed line, π-stacking in gray dashed line with gray spheres, salt bridge as yellow dashed lines with yellow spheres, waters are indicated as red crosses.
Figure 3

A: The asymmetric unit of 2a crystal structure - here the more abundant conformation of the organic compound with marked aromatic rings with a numerical tag. B: Four disordered water molecules are located in the proximity of the solubilizing tag, leading to disorder within β-proline and its carboxylic substituent - the less abundant conformation shown as green small shares. C: Superposed molecules 2a(gray) in geometry observed in the small-molecule crystal structure and 2f (cyan) in the conformation observed in protein-ligand complex crystal show good agreement within the main terphenyl core, with RMSD ~0.11 Å for aromatic C atoms. Displacement ellipsoids of non-hydrogen atoms are drawn at the 30% probability level. H-atoms are presented as small spheres with an arbitrary radius. The superposed molecules are shown in stick representation with H-atoms removed for figure clarity.
Figure 4

A: The summarised representation of docking results for all the described *meta*-terphenyl derivatives, including reference PD-L1 ligands: compound A, (J.-J. Park et al., 2021), BMS-1166 (L. Chupak et al., 2015; L. S. Chupak & Zheng, 2015), *meta*-terphenyl analog from 7NLD crystal structure (Muszak et al., 2021b); B and C: The 2D-ligand interactions plots (*Schrödinger Release 2020-3: Maestro, Schrödinger, LLC, New York*) for 2f conformer observed in the protein-ligand crystal structure and 2f re-docking result, respectively; D: The superposition of native 2f conformer and its re-docking result; and E: The superposition of all docked *meta*-terphenyl derivatives (the best-scored poses)

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.png
- Supplementarymaterial.docx