'Design, synthesis, and activity evaluation of small-molecule inhibitors of PD-1/PD-L1 interaction'

Summary

Programmed Death 1 (PD-1) protein has been demonstrated to have a significant impact on the regulation of autoimmunity, including both: induction and maintenance of peripheral tolerance. However, the binding of PD-1 with its ligand PD-L1 results in the suppression of the body's immune responses. The formulation of PD-1/PD-L1 complex is also associated with the inhibition of T cells proliferation, cytokines release, and cytotoxic effects, what leads to the apoptosis of tumor-specific T lymphocytes. Nonetheless, the introduction of the antagonist, which targets PD-1/PD-L1 interaction proved directly, that immunity can be modulated through inhibition of PD-1/PD-L1 pathway. Nowadays immunotherapeutic methods are gradually replacing more traditional ways of cancer treatment such as chemotherapy and radiotherapy. The implementation of immunotherapeutic strategies through the involvement of monoclonal antibodies (mAbs), plainly demonstrated that modulation of the immune responses can restore the function of specific T lymphocytes and normalize anti-tumor responses, thus being an effective strategy in the treatment of cancer. Unfortunately, apart from the mentioned advantages, the use of mAb in therapy is associated with numerous disadvantages, such as a poor pharmacokinetic profile when administered orally, adverse immune-related side effects (irAE), and poor tumor penetration due to the large size (150 kDa) of the antibody. Additionally, monoclonal antibodies based therapies are correlated with high costs, which restricted the adaptability of this immunotherapeutic approach. Moreover, the impressive outcomes of clinical trials received with the use of mAbs, lead that small molecule inhibitors targeting the PD-1/ PD-L1 checkpoint are intensively sought after. The application of small molecule PD-1/PD-L1 antagonists could overcome the disadvantages associated with the use of mAbs while providing an alternate immunotherapeutic approach. Therefore, the search for low-molecular-weight inhibitors of the PD-1/PDL1 immune checkpoint is an intensively developing research topic, and the optimization of the structure of potential antagonists has also become the subject of my interest.

The main objective of my research involved structure optimization, the performance of organic synthesis, and the analyses of the biochemical activity of the obtained compounds in terms of their use as PD-L1 antagonists. In the presented doctoral dissertation ten different groups of small-molecule inhibitors were designed, obtained, and studied.

Initially, as part of the conducted research, PD-1/PD-L1 immunomodulators were synthesized as 1,1'-biphenyl derivatives. Fragments constituting the cores of individual groups of molecules were optimized due to methods such as: in silico screening of designed short fragments with AutoDock Vina integrated into PyRx software, nuclear magnetic resonance spectroscopy (NMR) combined with weak-Antagonist Induced Dissociation Assay (w-AIDA), time-resolved fluorescence measurements (HTRF, Homogenous Time-Resolved Fluorescence) as well as following the known, disclosed so far in the literature structures of PD-L1 protein antagonists. Using convergent and linear synthesis, inhibitors derived from 2H-benzo[b][1,4]oxazin-3(4H)-one, 2-bromo-1,1'-biphenyl, 1,1':2',1''-terphenyl, 2-fluoro-1,1'-biphenyl, 2-chloro-1,1'-biphenyl, 2-iodo-1,1'-biphenyl were successfully synthesized. In total 29 short PD-1/PD-L1 final inhibitors were obtained. Presented herein thesis describes the synthetic routs and the full and precise characterization of obtained inhibitors.

Preliminarily, the affinity of the obtained compounds was determined using the HTRF technique. The compounds exhibiting considerable affinity for disruption of PD-1/PD-L1 complex, were further tested due to hPD-1/hPD-L1 immune checkpoint blockade assay. The observed in the assay increase of luciferase activity proved the TCR-mediated stimulation of Jurkat T-cells for some of the compounds.

Among the obtained compounds, **3.17** inhibitor as a 2-bromo-1,1'-biphenyl derivative provided the most satisfactory results. The molecule was characterized by an half maximal inhibitory concentration value-IC_{50} of 15.0 ± 0.2 nM and half maximal effective concentration-EC_{50} of 6.6 ± 0.8 μM and lack of cytotoxicity up to 100 μM concentration of the compound. Furthermore, binding profile of **3.17** with dimeric PD-L1 was studied due to Protein-Ligand Interaction Profiler. Analyses of the obtained results enabled to determine biphenyl core as being responsible for anchoring of the inhibitor within the hydrophobic cleft of dimeric PD-L1, hence providing π-π stacking with $Tyr56_A$. Other important non-bond interactions were found between the central aromatic ring of **3.17** and $Tyr56_B$. Among detected interactions hydrogen bonding between OH-group from $Tyr56_B$ and N atom from solubilizer and between N atom from $Asn63_B$ and O atom from -COOH group of L-pipecolinic acid were found to have a stabilizing effect on molecule's binding mode. Also observed salt bridge formed between tertiary aminic solubilizer and -COOH group from $Asp122_A$ proves the importance of incorporation of solubilizing agent into molecules structure.

Moreover, the presented herein work comprises the description of obtained high-quality crystal structure of 2-fluoro-1,1'-bifenyl derived compound – **3.54** with dimeric h-PD-L1. The

obtained data allowed to understand the molecular interactions of **3.54** with the target and shows that 1,4-benzodioxane moiety maintaining its interaction with $Tyr56_A$ and providing further contacts with $Ile54_A$ and $Ala121_B$ is crucial in the molecule's binding mode. Furthermore except for numerous detected hydrophobic interactions of the molecule, hydrogen bonding between tris(hydroxymethyl)aminomethane solubilizing group and $Asp122_A$ can be observed. The latest one proves directly, that solubilizing agent is not only responsible for the compound's solubility but also essential in the molecule's binding mode.

As a continuation of the research, an attempt to synthesize a group of elongated, small-molecule PD-1/PD-L1 immune checkpoint antagonists was undertaken. Consequently, 30 final structures derived from groups such as 2,3-dihydro-1H-indene, 2,2'-dimethyl-1,1'-biphenyl, 2-fluoro-2'-methyl-1,1'-biphenyl, and 3,4-dihydro-2H-benzo[b][1,4]oxazine were obtained. Among the indicated, pseudo-C2-symmetric derivatives of 2-fluoro-2'-methyl-1,1'-biphenyl and 2,2'-dimethyl-1,1'-biphenyl were found to exhibit the best properties. From obtained derivatives, eight (**7.29**, **7.31**-**7.37**) were characterized by means of IC_{50} up to 15 nM. Nine of obtained antagonists (**5.6-5.8**, **7.31**-**7.36**) were indicated by EC_{50} up to 500 nM. The applied modification of small molecules related to their elongation and enlargement of their structure contributed to strengthening the binding of inhibitors by increasing the number of its interactions with the PD-L1 protein. To conclude, the results of my research, related to both Structure-Activity Relationship (SAR) analysis and the preparation of compounds due to organic synthesis manners, may contribute to the formulation of a new class of potential inhibitors of the PD-1/ PD-L1 immune checkpoint.

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