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Design and synthesis of novel peptidomimetics for cancer immunotherapy

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Abstract: Tumor cells benefit from some certain signals, which are referred to as "immune checkpoints", to escape immune-mediated destruction. With that in mind, it is believed that the blockade of these points, such as programmed cell death Ligand-1 (PD-L1) and programmed cell death 1 (PD-1), can restore an adaptative immune response against tumoral cells. In this study, we have designed and synthesized some novel peptidomimetics with a 2-aminobenzathiazole scaffold, which targets the PD-1/PDL-1 pathway. In the viability assay, it was found that these compounds decreased the proliferation of peripheral blood mononuclear cells in the concentration of 10 uM. Overall, our results indicate that these novel compounds are potential checkpoint inhibitors for cancer immunotherapy.

Keywords: Immunotherapy; PD-L1; PD-1; peptidomimetics; 2-aminobenzothiazole. ©2020 ACG Publications. All right reserved.

1. Introduction

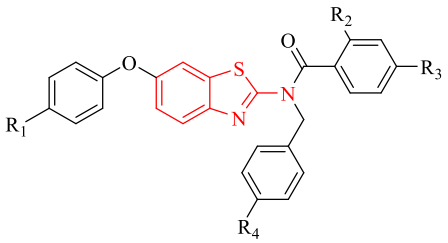
Programmed death-1 (PD-1) is found on the activated T cells as a negative regulator of immune responses. When PD-1 binds to its ligands programmed death ligand-1 (PD-L1) and ligand-2 (PD-L2), T cell responses are inhibited. PD-L1 is expressed on dendritic cells, T cells, B cells, and cancer cells, besides macrophages. In the tumor microenvironment, it has been shown that PD-L1 is upregulated and its expression interferes with anti-tumor T cell responses[1]. Therefore, blocking PD-1 and PD-L1 pathway with antibodies is currently used in cancer therapies[2]. To this end, FDA approved five monoclonal antibodies against the programmed cell death 1 receptor (PD-1) (Nivolumab, Pembrolizumab) or programmed cell death ligand 1 (PD-L1) (Atezolizumab, Avelumab,

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Durvalumab)[3,4]. However, it became evident that resistance against therapeutic antibodies and tumor recurrence are prevalent complications, as a result of which their use is very much hindered[5].
To tackle these limitations, an exquisite approach is the development of small-molecular weight inhibitors. This is because this class of compounds reportedly displays unique therapeutic behaviors (such as, amenability for oral administration, improved tumor penetration, and elimination of immunogenicity issues), which enable them to compete with antibodies[6]. Notwithstanding, the development of such compounds is notably scarce to this date, which stems from a general challenge. That is, the interaction surface of PD-1/PDL-1 is observed to be relatively flat and remarkably hydrophobic, which poses a major constraint against designing inhibitors[7]. Thus, it is no surprise that the repertoire of compounds designed to antagonize the interaction of PD-1/PDL-1 is culminated by only a handful of examples. In one of the rare examples, Bristol-Myers Squibb (BMS) has disclosed a tri-aromatic scaffold, which harbors with a mono-ortho substituted biphenyl substructure[8]. Another one came from Aurigene Ltd., which developed peptidomimetics composed of 3-4 amino acids fused with hydrazine or urea linker[9]. Of note, it would appear that the notion of employing small-molecular weight inhibitors in immunotherapy remained somewhat underexplored, in comparison to antibodies.

Driven by this rationale, we have been intrigued by devising scaffolds to fabricate novel immunotherapeutic agents targeting the PD-1/PDL-1 pathway. To this respect, we screened numerous compounds from literature through computational methods and fortuitously, we came to find out that 2-amino-benzothiazole-based peptidomimetics exhibit high affinity towards the active site of PD-L1. In light of docking studies, we have subsequently constructed a library of peptidomimetics, which are specific to PD-L1. Then, five of these molecules (**1a-e**) with the highest score in docking studies were chosen for synthesis and subsequent studies (Figure 1). Since these small molecules are designed as such that they target cancer-related immunological pathways, the effects of the molecules on the immune system's cells were analyzed in terms of cell proliferation and viability. In this manuscript, we report the chemistry and the synthesis of these compounds, in conjunction with their activity in cell proliferation and viability assays.



1a-e

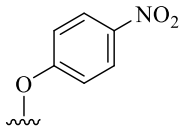
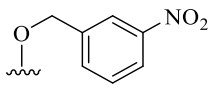
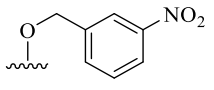
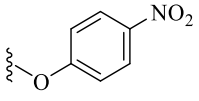
1a	**R₁:** $C(CH_3)_3$	**R₂:**		**R₃:**	H	**R₄:**	H
1b	**R₁:** $C(CH_3)_3$	**R₂:**		**R₃:**	H	**R₄:**	H
1c	**R₁:** OCH_3	**R₂:**		**R₃:**	H	**R₄:**	H
1d	**R₁:** F	**R₂:**	H	**R₃:**		**R₄:**	CH_3
1e	**R₁:** F	**R₂:**	H	**R₃:**	H	**R₄:**	H

Figure 1. The novel peptidomimetics **1a-e** designed in the work (the 2-aminobenzathiazole core is marked in red).

2. Experimental

2.1. Chemical Material and Apparatus

Methyl 4-hydroxybenzoate, 4-tertiary-butylphenol, 1-fluoro-4-nitrobenzene, ethyl 2-hydroxy benzoate, 3-nitrobenzyl chloride, potassium carbonate, benzoyl chloride, and benzyl chloride were purchased from Merck. Ammonium thiocyanate, bromine, sodium hydroxide, and diisopropylethylamine (DIPEA) were obtained from Sigma Aldrich. Diethylaminosulfur trifluoride (DAST) and 4-(4-fluorophenoxy)aniline were purchased from Alfa Aesar, and ChemImpex, respectively. DMSO, DMF, ethyl acetate, hexane, DCM, acetic acid, and formic acid were purchased from Merck. DCM was dried over calcium hydride (Aldrich, Germany) overnight, purified through distillation, and kept under nitrogen atmosphere until use. Other reagents and solvents were directly used without any purification.

^{1}H-NMR and ^{13}C-NMR were acquired on an Agilent VNMRS Spectrometer at 500 MHz, and 125 MHz, respectively. Coupling constant values were given in Hertz and chemical shifts were reported in δ (ppm) with respect to the internal standard TMS. Splitting patterns were described as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad signal).

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy measurements were recorded using a Perkin-Elmer Spectrum BX FT-IR spectrometer over the range of 4000-500 cm^{-1} with a maximum OPD resolution of 1 cm^{-1}.

2.2. Biological Materials and Apparatus

Peripheral blood was obtained from healthy donors who did not have inflammatory diseases or infections for at least ten days (n=3). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Histopaque 1077, Sigma, St. Louis, MO, USA). To evaluate the effects of the molecules on the cell viability, PBMCs were treated with different doses of the molecules (0.01 uM, 0.1 uM, 10 uM). After 24 hours the viability of PBMCs was assessed with propidium iodide (25 ng/mL) staining and analyses were performed with flow cytometry (FACSAria II, Becton Dickinson, San Jose, CA, USA).

PBMCs ($10x10^5$ cells/well) labelled with carboxyfluorescein succinimidyl ester (CFSE, 5 μM; CellTraceTM, Invitrogen, Eugene, OR, USA) were treated with 0.01 uM, 0.1 uM, 10 uM of the molecules under anti-CD3 stimulation (HIT3a; 25 ng/mL). After 96 hours, the effects of the molecules on the proliferative capacity of PBMCs were analyzed by dilution of CFSE and the viability of PBMCs was assessed by propidium iodide with flow cytometry.

2.3. Chemistry

2.3.1. Molecular Modeling Studies

The crystal structures of the programmed cell death receptor ligand 1 (PD-L1) (PDB: 5J8O, 2.3 Å, in complex with low molecular mass inhibitor 6GZ) were obtained from the RSCB Protein Data Bank[10]. Ligand (6GZ) was retained and all other non-protein atoms were removed from the crystal structure. Hydrogen atoms were added using the "Protonate3D" tool (MOE software package, version 2019.0102, chemical computing group, Inc., Montreal, Canada)[11]. The molecular structures of the ligands were built using the MOE software package. The most prevalent protomer at physiological pH value was assigned to the ligands and the ligand structures were energy minimized (MMFF94x force field)[12-17]. Docking calculations were performed with the FlexX (version 4.1; BioSolveIT GmbH, Sankt Augustin, Germany, 2019). The best scoring three poses for each ligand were subjected to refinement calculations. To this end, the docked ligand and the binding pocket (defined as all residues within 6.5 Å of the docked ligand) were energy minimized and rescored using the GBVI/WSA force field.

*2.3.2. The Synthesis of Compounds **1a-1e***

*2.3.2.1. General Procedure for the Synthesis of **3a-b***

In a solution of **2a-b** (7.09 mmol) and 1-fluoro-4-nitrobenzene (1 g, 7.09 mmol) dissolved in DMF (10 mL), K_2CO_3 (3.33 g, 24.15 mmol) was suspended under an inert atmosphere. Then, the reaction was stirred overnight at 70 °C. The mixture was cooled down to room temperature and diluted with ethyl acetate (30 mL). Insoluble material was filtered and the remaining solution was washed with distilled water (3×15 mL), and brine (15 mL), respectively. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure.

*1-t-butyl-4-(4-nitrophenoxy)benzene (**3a**):* Yield=79%. ^{1}H-NMR ($CDCl_3$): 8.39 (2H, d, *J*=7.3); 7.42 (2H, d, *J*=7.5); 7.12 (4H, d, *J*=8.3); 1.33 (9H, s). ^{13}C-NMR (DMSO-d_6): δ 163.5; 152.5; 148.2; 142.7; 127.5; 126.4; 120.3; 117.4; 30.5, 31.5. IR (KBr tablet) ν_{max}/cm^{-1}: 2963-2867, 1586. The compound was obtained as yellow solid.

*1-methoxy-4-(4-nitrophenoxy)benzene (**3b**)*: Yield=77%. ^{1}H-NMR (DMSO-d_6): δ 8.21 (2H, d, *J*= 9.0 Hz); 7.12 (2H, d, *J*=8.8 Hz); 7.07-7.00 (4H, m); 3.77 (3H, s). ^{13}C-NMR (DMSO-d_6): δ 164.2; 157.2; 147.7; 142.3; 126.6; 122.4; 116.9; 115.9; 55.9. IR (KBr tablet) ν_{max}/cm^{-1}: 1502; 1233; 1584. The compound was obtained as yellow solid.

*2.3.2.2. General procedure for the synthesis of **4a-b***

To a solution of **3a-b** (7.37 mmol) in methanol (30 mL), $SnCl_2$ (5.5 g, 29.5 mmol) dissolved in HCl (5 mL, %37) was added dropwise and then, the reaction was stirred at 80 °C for 5 hours. The solvent was evaporated under reduced pressure and the reaction mixture was basified to pH=11 with a 1% NaOH solution. The resulting suspension was centrifuged and the precipitate was extracted with DCM (3×30 mL) and distilled water (3×30 mL). The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure.

*4-(4-(t-butyl)phenoxy)aniline (**4a**)*: Yield=47%. ^{1}H-NMR (DMSO-d_6): δ 7.32 (4H, d, *J*=7.3); 6.82 (4H, d, *J*=7.3); 4.82 (2H, s); 1.33 (9H, s). ^{13}C-NMR (DMSO-d_6): δ 156.2; 149.2; 144.6; 140.4; 126.4; 120.7; 118.3; 116.4; 122.0; 34.0; 31.7. IR (KBr tablet) ν_{max}/cm^{-1}: 3443-3369, 2960-2904, 1620. The compound was obtained as yellow solid.

*4-(4-methoxyphenoxy)aniline (**4b**)*: Yield=62%. ^{1}H-NMR (DMSO-d_6): δ 6.84 (2H, d, *J*=9.1 Hz); 6.80 (2H, d, *J*=9.1 Hz); 6.69 (2H, d, *J*=8.7 Hz); 6.55 (2H, d, *J*=8.7 Hz); 4.86 (2H, s); 3.67 (3H, s). ^{13}C-NMR (DMSO-d_6): δ 159.3; 156.1; 155.2; 150.2; 132.3; 127.8; 120.1; 115.6; 55.9. IR (KBr tablet) ν_{max}/cm^{-1}: 1493; 1206; 3379. The compound was obtained as yellow solid.

*2.3.2.3. General Procedure for the Synthesis of **5a-c***

4a-c (2.51 mmol) and NH_4SCN (0.69 g, 9.1 mmol) were dissolved in a mixture of formic acid/acetic acid (20%, 75 mL) under an inert atmosphere. While the reaction mixture was protected from light and kept at a temperature of -3 to 0 °C with ice and salt, a solution of bromine (0.182 mL) in glacial acetic acid (18.2 mL) was added dropwise in one hour. Then, the light shield was removed, the reaction was warmed to room temperature and was stirred overnight. The reaction mixture was basified to pH=11 with NaOH pellets in the presence of ice/water and the product was extracted with EtOAc (4×60 mL). The combined organic phase was filtered through celite and washed with water (2×60 mL), saturated $NaHCO_3$ (2×40 mL), respectively. The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure.

*6-(4-t-butylphenoxy)benzo[d]thiazol-2-amine (**5a**)*: Yield=80%. ^{1}H-NMR (DMSO-d_6): δ 7.39 (2H, s); 7.37 (1H, d, *J*=8.7 Hz); 7.35 (2H, d, *J*=8.7 Hz); 6.90 (2H, d, *J*=7.7 Hz); 6.87 (2H, d, *J*=8.7 Hz); 1.25

(9H, s). ^{13}C-NMR (DMSO-d_6): δ 166.6; 156.0; 151.1; 149.4; 145.3; 132.3; 126.9; 118.6; 117.8; 117.4; 112.4; 34.2; 31.5. IR (KBr tablet) ν_{max}/cm^{-1}: 3440-3126; 2960-2904; 1680; 1627-1586. The compound was obtained as brown solid.

*6-(4-methoxyphenoxy)benzo[d]thiazole-2-amine (**5b**)*: Yield=81%. ^{1}H-NMR (DMSO-d_6): δ 7.35 (2H, s); 7.29 (1H, s); 7.27 (1H, d, *J*=8.6 Hz); 6.93 (4H, s); 6.83 (1H, d, *J*=8.6 Hz); 3.72 (3H, s). ^{13}C-NMR (DMSO-d_6): δ 166.4; 155.5; 152.3; 151.4; 149.2; 132.5; 119.6; 116.9; 120.0; 115.4; 111.4; 55.8. The compound was obtained as brown solid.

*6-(4-fluorophenoxy)benzo[d]thiazol-2-amine (**5c**)*: The compound **5c** was synthesized from commercially available 4-(4-fluorophenoxy) aniline **4c**, according to the same procedure. Yield=85%. ^{1}H-NMR ($CDCl_3$): δ 7.50 (1H, d, *J*=8.7 Hz); 7.20 (1H, d, *J*=2.2 Hz); 7.08-6.94 (5H, m); 6.06 (2H, bs). ^{13}C-NMR ($CDCl_3$): δ 165.7; 159.6; 157.7; 153.5; 153.2; 145.6; 131.3; 119.8; 119.2; 118; 116.3; 116.2; 111.1. IR (KBr tablet) ν_{max}/cm^{-1}: 3351; 3071; 2732; 2176; 1865; 1638; 1535; 1500; 1114; 1092. The compound was obtained as white solid.

*2.3.2.4. General Procedure for the Synthesis of **7a-c***

6a-b (6.02 mmol), 2-fluoro-4-nitrobenzene or 3-nitrobenzyl chloride (5.96 mmol), and K_2CO_3 (3.33 g, 24.08 mmol) were stirred in DMF (30 mL) at 50 °C temperature for 24 h. The reaction was cooled to room temperature, the insoluble material was filtered and the solvent is removed under reduced temperature. The crude material was dissolved in ethyl acetate (25 mL) and washed with water (3x25 mL). The organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The compound was purified with flash column chromatography using 1:3 EtOAc/Hexanes, as eluent.

*Ethyl 2-(4-nitrophenoxy)benzoate (**7a**)*: Yield=82%. ^{1}H-NMR ($CDCl_3$): δ 8.21 (2H, d, *J*=9.3 Hz); 8.05 (1H, dd, J_1=7.6 Hz, J_2=1.6 Hz); 7.62 (1H, td, J_1=8.2 Hz, J_2=1.6 Hz); 7.39 (1H, td, J_1=7.6 Hz, J_2=1.65 Hz); 7.16 (1H, dd, J_1=8.2 Hz, J_2=1.1 Hz); 6.93 (2H, d, *J*=9.3 Hz); 4.22 (2H, q, *J*=9.3 Hz); 1.16 (3H, t, *J*=9.3 Hz). The compound was obtained as white solid.

*Methyl 4-(4-nitrophenoxy)benzoate (**7b**)*: Yield=87%.^{1}H-NMR ($CDCl_3$): δ 8.24 (2H, d, *J*=9.3 Hz); 8.12 (2H, d, *J*=8.8 Hz); 7.11 (4H, d, *J*=9.8 Hz); 3.93 (3H, s). ^{13}C-NMR ($CDCl_3$): δ 166.2; 161.9; 159.0; 143.5; 132.1; 126.8; 126.1; 119.5; 118.3; 52.3. IR (KBr tablet) ν_{max}/cm^{-1}: 3109; 3081; 2957; 1711; 1606; 1583; 1525; 1513; 1235. The compound was obtained as white solid.

*Ethyl 2-((3-nitrobenzyl)oxy)benzoate (**7c**)*: Yield=62%. ^{1}H-NMR ($CDCl_3$): δ 8.45 (1H, s); 8.20 (1H, dd, J_1=7.6 Hz, J_2=1.1 Hz); 7.91 (1H, d, *J*=7.6 Hz); 7.88 (1H, dd, J_1=7.6 Hz, J_2=1.6 Hz); 7.59 (1H, t, *J*=7.6 Hz) 7.48 (1H, td, J_1=7.6 Hz, J_2=2.2 Hz); 7.07 (1H, t, *J*=8.2 Hz); 7.03 (1H, t, *J*=8.2 Hz); 5.26 (2H, s); 4.43 (2H, q, *J*=7.1 Hz); 1.38 (3H, t, *J*=7.1 Hz). The compound was obtained as white solid.

*2.3.2.5. General Procedure for the Synthesis of **8a-c***

To a solution of **7a-c** (3.32 mmol) in dioxane (22.5 mL), 1.4 M sodium hydroxide solution (10 mL) was added and the reaction mixture was heated to 50 °C for 2 h. Upon the completion of the reaction that is monitored with TLC, dioxane was removed under reduced pressure. After the acidification of the remaining solution to pH=2 with 1 N HCl, the product precipitated. Then, it was isolated with suction and dried under vacuum overnight.

*2-(4-nitrophenoxy)benzoic acid (**8a**)*: Yield = 70%. ^{1}H-NMR (DMSO-d_6): 8.21 (2H, dt, J_1=9.0 Hz, J_2=2.2 Hz); 8.12 (1H, dd, J_1=7.6 Hz, J_2=1.3 Hz); 7.66 (1H, td, J_1=8.2 Hz, J_2=1.3 Hz); 7.39 (1H, td, J_1=7.6 Hz, J_2=1.3 Hz); 7.14 (1H, d, *J*=8.2 Hz); 6.97 (2H, dd, J_1=9.0 Hz, J_2=2.2 Hz). IR (KBr tablet) ν_{max}/cm^{-1}: 3107-3075; 2955-2869; 1728; 1669. The compound was obtained as white solid.

*4-(4-nitrophenoxy)benzoic acid (**8b**)*: Yield=72%. ^{1}H-NMR (DMSO-d_6): δ 13.02 (1H, bs); 8.28 (2H, d, *J*=9.3 Hz); 8.03 (2H, d, *J*=8.0 Hz); 7.25 (4H, d, *J*=8.8 Hz). ^{13}C-NMR (DMSO-d_6): δ 166.9; 162.0; 158.9; 143.4; 132.3; 127.7; 126.7; 120.1; 119.2. IR (KBr tablet) ν_{max}/cm^{-1}: 3538; 3084-2957; 1676. The compound was obtained as white solid.

*2-((3-nitrobenzyl)oxy)benzoic acid (**8c**)*: Yield=79%. ^{1}H-NMR (DMSO-d_6): δ 8.39 (1H, s); 8.26 (1H, d, *J*=8.2 Hz); 8.19 (1H, d, *J*=7.6 Hz); 7.85 (1H, d, *J*=7.6 Hz); 7.65 (1H, t, *J*=8.2 Hz); 7.58 (1H, t, *J*=7.2 Hz); 7.18 (1H, t, *J*=7.2 Hz); 7.10 (1H, d, *J*=8.2 Hz); 5.40 (2H, s). ^{13}C-NMR (DMSO-d_6): δ 167.6; 157.2; 148.3; 140.0; 133.8; 133.5; 131.3; 130.3; 122.9; 122.2; 121.9; 121.2; 114.4; 68.8. IR (KBr tablet) ν_{max}/cm^{-1}: 3091; 2922-2859; 1662; 1580. The compound was obtained as white solid.

*2.3.2.6. General Procedure for the Synthesis of **9a-9c***

To a solution of **8a-c** (1.83 mmol) in dry DCM (25 mL) chilled in an ice-bath, DAST (725 µL, 5.49 mmol) was added under an inert atmosphere. Then, the ice-bath was removed and the reaction was stirred at room temperature. Upon the completion of the reaction, which was monitored with TLC, the reaction mixture was washed with a mixture of ice/water (3x30 mL), and brine (1x20 mL), respectively. The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure.

*2-(4-nitrophenoxy)benzoyl fluoride (**9a**)*: Yield=80%. ^{1}H-NMR (DMSO-d_6): δ 8.24 (2H, d, *J*=8.2 Hz); 8.10 (1H, d, *J*=8.1 Hz); 7.89 (1H, t, *J*=7.8 Hz); 7.53 (1H, t, *J*=7.5 Hz); 7.36 (1H, d, *J*=7.3 Hz); 7.14 (2H, d, *J*=7.1). IR (KBr tablet) ν_{max}/cm^{-1}: 3080-2965; 1800; 1590. The compound was obtained as yellow solid.

*4-(4-nitrophenoxy)benzoyl fluoride (**9b**)*: Yield=80%. ^{1}H-NMR (DMSO-d_6): δ 8.30 (2H, d, *J*=8.2 Hz); 8.10 (2H, d, *J*=8.0 Hz); 7.33 (2H, d, *J*=7.3 Hz); 7.30 (2H, d, *J*=7.3 Hz). IR (KBr tablet) ν_{max}/cm^{-1}: 3068-2873; 1736; 1676. The compound was obtained as yellow solid.

*2-((3-nitrobenzyl)oxy)benzoyl fluoride (**9c**)*: Yield=80%. ^{1}H-NMR (DMSO-d_6): δ 8.41 (1H, s); 8.18 (1H, d, *J*=8.1 Hz); 7.94 (2H, t, *J*=7.9 Hz); 7.78 (1H, t, *J*=7.8 Hz); 7.70 (1H, t, *J*=7.7 Hz); 7.37 (1H, d, *J*=7.4 Hz); 7.15 (1H, t, *J*=7.2); 5.43 (2H, s). IR (KBr tablet) ν_{max}/cm^{-1}: 3091-2853; 1803. The compound was obtained as yellow solid.

*2.3.2.7. General Procedure for the Synthesis of **10a-e***

Acyl fluoride (**9a-c**, 1.45 mmol) and the corresponding 2-amino-benzothiazole derivative (**5a-c**, 0.73 mmol) were dissolved in dry DCM (30 mL). DIPEA (503 µL, 2.90 mmol) was added and the reaction was stirred at 45 °C overnight. The reaction was allowed to cool down to room temperature and then, it was washed with 10% citric acid (3×20 mL), distilled water (2×20 mL), and saturated $NaHCO_3$ (2×20 mL), respectively. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The product was washed with cold methanol and dried under vacuum.

*N-(6-(4-(t-butyl)phenoxy)benzo[d]thiazol-2-yl)-2-(4-nitrophenoxy)benzamide (**10a**)*: The compound **10a** was synthesized from 2-(4-nitrophenoxy)benzoyl fluoride **9a** and 6-(4-t-butylphenoxy)benzo[d]thiazol-2-amine **5a**. Yield=70%. ^{1}H-NMR (DMSO-d_6) δ ppm: 10.09 (1H, s); 8.14 (1H, d, *J*=8.9 Hz); 7.69 (1H, s); 7.69 (1H, d, *J*=8.7 Hz); 7.61 (2H, d, *J*=8.7 Hz); 7.37 (2H, d, *J*=8.7 Hz); 7.30 (1H, d, *J*=7.6 Hz); 7.07 (1H, t, *J*=2.5 Hz); 7.03 (1H, dd, *J*=8.7 Hz, *J*=7.9 Hz); 6.92 (2H, d, *J*=8.7 Hz); 6.68 (1H, t, *J*=7.4 Hz); 6.62 (1H, d, *J*=8.2 Hz); 1.20 (9H, s). IR (KBr tablet) ν_{max}/cm^{-1}: 3068; 2959-2866; 1747; 1700; 1674. The compound was obtained as yellow solid.

N-(6-(4-(t-butyl)phenoxy)benzo[d]thiazol-2-yl)-2-((3-nitrobenzyl)oxy)benzamide (**10b**): The compound **10b** was synthesized from 2-(3-nitrophenoxy)benzoyl fluoride **9c** and 6-(4-t-butylphenoxy)benzo[d]thiazol-2-amine **5a**. Yield=70%. ^{1}H-NMR (DMSO-d_6): δ 12.22 (1H, s); 8.41 (1H, s); 8.15 (1H, d, *J*=9.2 Hz); 7.99 (1H, d, *J*=6.3 Hz); 7.73 (1H, d, *J*=7.5 Hz); 7.65 (1H, t, *J*=8.7 Hz); 7.65 (1H, d, *J*=7.9 Hz); 7.65 (1H, s); 7.35 (1H, t, *J*=7.5 Hz); 7.36 (2H, d, *J*=8.6 Hz); 7.27 (1H, d, *J*=8.3

Hz); 7.11 (1H, t, *J*=8.0 Hz); 7.11 (1H, d, *J*=9.5 Hz); 6.91 (2H, d, *J*=8.6 Hz); 1.25 (9H, s). IR (KBr tablet) ν_{max}/cm^{-1}: 3313; 2956-2849; 1735; 1665. The compound was obtained as yellow solid.

N-(6-(4-methoxyphenoxy)benzo[d]thiazole-2-yl)-2-((3-nitrobenzyl)oxy)benzamide ***(10c)***: The compound **10c** was synthesized from 2-(3-nitrobenzyl)oxy) benzoyl fluoride **9c** and 6-(4-methoxyphenoxy)benzo[d]thiazole-2-amine **5b**. Yield=77%. ^{1}H-NMR (DMSO-d_6): δ 10.23 (1H, s); 8.10 (1H, d, *J*=7.5 Hz); 7.92 (1H, t, *J*=15.6 Hz); 7.73 (1H, s); 7.72 (1H, d, *J*=8.7 Hz); 7.63 (1H, d, *J*=7.4 Hz); 7.56 (1H, s); 7.54 (1H, t, *J*=15.5 Hz); 7.49 (1H, d, *J*=7.7 Hz); 7.47 (1H, d, *J*=6.0 Hz); 7.08 (1H, d, *J*=8.7 Hz); 6.99 (1H, t, *J*=9.0 Hz); 6.95 (2H, d, *J*=5.8 Hz); 6.93 (2H, d, *J*=5.7 Hz); 5.72 (2H, s); 3.71 (3H, s). ^{13}C-NMR (DMSO-d_6): δ 174.0; 1667.9; 157.4; 148.3; 139.7; 136.6; 134.3; 134.0; 131.6; 130.4; 129.5; 129.3; 128.3; 127.8; 123.0; 122.2; 121.3; 120.9; 120.7; 115.6; 114.5; 112.5; 66.6; 55.9. IR (KBr tablet) ν_{max}/cm^{-1}: 3061; 1728; 1668; 1600; 1210. The compound was obtained as yellow solid.

N-(6-(4-fluorophenoxy)benzo[d]thiazol-2-yl)-4-(4-nitrophenoxy)benzamide ***(10d)***: The compound **10d** was synthesized from 4-(4-nitrophenoxy) benzoyl fluoride **9b** and 6-(4-fluorophenoxy)benzo[d]thiazol-2-amine **5c** and was purified through crystallization from cold methanol. Yield=60%. ^{1}H-NMR (DMSO-d_6): δ 8.31 (2H, d, *J*=7.1 Hz); 8.27 (2H, d, *J*=7.1 Hz); 7.71 (2H, d, *J*=7.1 Hz); 7.64 (2H, d, *J*=7.1 Hz); 7.30 (6H, m); 7.12 (3H, m). ^{13}C-NMR (DMSO-d_6): δ 166; 162; 160; 159; 158; 157; 154; 153; 145; 143; 133; 132; 131; 130; 126; 125; 124; 122; 121; 120; 119; 118; 117; 116; 115; 114; 111. The compound was obtained as yellow solid.

N-(6-(4-fluorophenoxy)benzo[d]thiazol-2-yl)benzamide ***(10e)***: The compound **10e** was synthesized from benzoyl chloride and 6-(4-fluorophenoxy)benzo[d]thiazol-2-amine **5c** and the crude product was purified by column chromatography (silica gel, 1:3 EtOAc/Hexanes). Yield=53%. ^{1}H-NMR ($CDCl_3$): δ 8.06 (2H, d, *J*=7.1 Hz); 7.63 (1H, t, *J*=7.1 Hz); 7.54 (2H, t, *J*=8.2 Hz); 7.50 (1H, d, *J*=2.7 Hz); 7.40 (1H, d, *J*=8.8 Hz); 7.27 (2H, d, *J*=8.8 Hz); 7.07 (1H, d, *J*=8.8 Hz); 7.02 (2H, d, *J*=8.8 Hz). The compound was obtained as yellow solid.

2.3.2.8. General Procedure for the Synthesis of ***1a-1e***

In a solution of benzyl halide (1.5 mmol) and the corresponding intermediate **10a-10e** (0.78 mmol) in DMF (10 mL), K_2CO_3 (0.32 g, 2.34 mmol) was suspended under inert atmosphere and the reaction was stirred 90 °C overnight. Then, the reaction was cooled down to room temperature and insoluble material was filtered. The solvent was evaporated under reduced pressure. The crude material was dissolved in DCM (20 mL) and washed with cold distilled water (3×20 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The solid was washed with cold methanol and the product is dried under a vacuum.

N-benzyl-N-(6-(4-(t-butyl)phenoxy)benzo[d]tiazol-2-yl)-2-(4-nitrophenoxy)benzamide ***(1a)***: The compound **1a** was synthesized from benzyl chloride and *N*-(6-(4-(tertiary-butyl)phenoxy)benzo[d]-thiazol-2-yl)-2-(4-nitrophenoxy)benzamide **10a**. Yield=70%. ^{1}H-NMR (DMSO-*d6*) δ ppm: 8.23 (2H, d, *J*=9.0 Hz); 8.14 (1H, d, *J*=7.8 Hz); 8.10 (1H, d, *J*=9.2 Hz); 7.98 (2H, d, *J*=9.2 Hz); 7.85 (1H, t, *J*=5.4 Hz); 7.81 (2H, d, *J*=9.1 Hz); 7.63 (1H, t, *J*=8.7 Hz); 7.45 (1H, t, *J*=2.6 Hz); 7.31 (1H, s); 7.07 (2H, t, *J*=7.6 Hz); 7.01 (1H, dd, *J*=8.7 Hz, *J*=7.9 Hz); 6.95 (1H, d, *J*=9.0 Hz); 6.90 (2H, d, *J*=8.7 Hz); 6.84 (1H, d, *J*=8.7 Hz); 5.72 (2H, s); 1.24 (9H, s). IR (KBr tablet) ν_{max}/cm^{-1}: 3064-2850; 1729; 1674. The compound was obtained as yellow solid.

N-benzyl-N-(6-(4-(t-butyl)phenoxy)benzo[d]thiazol-2-yl)-2-((3-nitrobenzyl)oxy)benzamide ***(1b)***: The compound **1b** was synthesized from benzyl chloride and *N*-(6-(4-(t-butyl)phenoxy)benzo[d]thiazol-2-yl)-2-((3-nitrobenzyl)oxy)benzamide **10b**. Yield=70%. ^{1}H-NMR (DMSO-d_6): δ 8.40 (1H, s); 8.38 (2H, s); 8.16 (1H, d, *J*=7.2 Hz); 8.10 (1H, d, *J*=8.0 Hz); 7.93 (1H, d, *J*=7.6 Hz); 7.90 (2H, d, *J*=7.3 Hz); 7.71 (1H, t, *J*=6.1 Hz); 7.69 (1H, t, *J*=8.1 Hz); 7.63 (1H, t, *J*=11.1 Hz); 7.61 (1H, t, *J*=7.7 Hz); 7.37 (2H, d, *J*=8.8 Hz); 7.15 (1H, d, *J*=8.4 Hz); 7.09 (1H, d, *J*=7.0 Hz); 7.04 (1H, t, *J*=7.4 Hz); 6.92 (2H, d, *J*=7.8 Hz); 6.83 (1H, d, *J*=7.9 Hz); 5.77 (2H, s); 5.34 (2H, s); 1.23 (9H,s). ^{13}C-NMR (DMSO-d_6): δ 174.9; 166.9; 157.4; 155.2; 153.8; 148.2; 146.3; 140.3; 136.3; 133.7; 132.6; 131.7; 130; 129.2; 128.3; 127.7;

127.6; 127.5; 127.2; 122.9; 121.9; 121.2; 199.2; 118.2; 114.6; 114.3; 113.7; 68.9; 48.8; 34.4; 31.7. IR (KBr tablet) ν_{max}/cm^{-1}: 3039-2866; 1619; 1527. The compound was obtained as yellow solid.

*N-benzyl-N-(6-(4-methoxyphenoxy)benzo[d]thiazole-2-yl)-2-((3-nitrobenzyl)oxy)benzamide (**1c**)*: The compound **1c** was synthesized from benzyl chloride and *N*-(6-(4-methoxyphenoxy)benzo[d]-thiazole-2-yl)-2-((3-nitrobenzyl)oxy)benzamide **10c**. Yield=69%. ^{1}H-NMR (DMSO-d_6): δ 8.35 (1H, s); 8.23 (1H, d, *J*=7.5 Hz); 8.14 (1H, d, *J*=8.1 Hz); 7.86 (1H, d, *J*=7.6 Hz); 7.74 (1H, d, *J*=7.6 Hz); 7.47 (1H, t, *J*=15.1 Hz); 7.31-7.24 (8H, m); 7.05 (1H, t, *J*=8.9 Hz); 7.04 (1H, d, *J*=7.4 Hz); 6.98 (2H, d, *J*=8.9 Hz); 6.93 (2H, d, *J*=9.0 Hz); 5.80 (2H, s); 5.72 (2H, s); 3.72 (3H, s). IR (KBr tablet) ν_{max}/cm^{-1}: 1720; 1599; 1234. The compound was obtained as yellow solid.

*N-(4-methylbenzyl)-(6-(4-fluorophenoxy)benzo[d]thiazol-2-yl)-4-(4-nitropheoxy)benzamide (**1d**)*: The compound **1d** was synthesized from 4-methylbenzyl chloride and *N*-(6-(4-fluorophenoxy)benzo[d]-thiazol-2-yl)-4-(4-nitrophenoxy)benzamide **10d**. Yield=97%. ^{1}H-NMR (DMSO-d_6): δ 8.36 (2H, d, *J*=7.1 Hz); 8.28 (2H, d, *J*=7.1 Hz); 7.66 (2H, t, *J*=8.2 Hz); 7.32-7.07 (13H, m); 5.78 (2H, s); 2.23 (3H, s). ^{13}C-NMR (DMSO-d_6): δ 173; 167; 162; 159; 158; 157; 154; 153; 143; 137; 133; 132; 131; 130; 129; 128; 127; 126; 121; 120; 119; 118; 117; 116; 112; 113; 49; 21. The compound was obtained as yellow solid.

*N-benzyl-N-(6-(4-fluorophenoxy)benzo[d]thiazol-2-yl)benzamide(**1e**)*: The compound **1e** was synthesized from benzyl bromide and *N*-(6-(4-fluorophenoxy)benzo[d]thiazol-2-yl)benzamide **10e**. Yield=98%. ^{1}H-NMR ($CDCl_3$): δ 5.78 (2H, s); 7.03 (5H, m); 7.24 (1H, t, *J*=7.1 Hz); 7.27 (2H, d, *J*=8.8 Hz); 7.41 (6H, m); 7.53 (2H, t, *J*=8.2 Hz); 7.67 (1H, d, *J*=8.8 Hz); 8.38 (2H, d, *J*=7.1 Hz). ^{13}C-NMR ($CDCl_3$): δ 175; 167; 159; 158; 154; 152; 135; 129; 128; 127; 120; 118; 116; 112; 49. The compound was obtained as yellow solid.

3. Results and Discussion

To design benzothiazole-based peptidomimetics **1a-e**, we examined the crystal structure of human PD-L1 with Bristol-Myers Squibb (BMS) compounds (PDB: 5J8O). In this co-crystal structure, the biphenyl group of the ligand 6GZ forms hydrophobic interaction with Tyr56B, Met115B, Ile54B, Ala121A (Figure 2, left). The 2-Bromo 1,4-disubstituted phenyl group of the ligand forms hydrophobic interactions with Tyr56A. A hydrogen bond was formed between the ligand's piperidine ring and the side chain of Gln66A. Furthermore, this co-crystal reveals that the ligand binds PD-L1, rather than PD-1, and more crucially, it effectuates the dissociation of preformed PD-L1/PD-1 complex. Intriguingly, this observation comes to mean that those with analogous binding modes to BMS compounds may indeed behave as PD-1/PD-L1 immune checkpoint modulators. Inspired by this notion, we have designed novel compounds, which consist of benzothiazole scaffold and observed that five of these compounds (**1a-e**) exhibit a high affinity towards PD-L1 (range of GBVI/WSA ΔG scores: -8 to -6 kcal/mol). To illustrate, Figure 2 (right) displays the binding mode of **1a**, wherein the tert-butyl group forms a hydrophobic interaction with Tyr56B, Met115B, Ile54B, Ala121A. The benzothiazole group of the ligand forms a hydrophobic interaction with Tyr56A. A hydrogen bond was formed between the ligand's nitro group and the backbone of Asn63A. The *N*-benzyl group of the ligand form hydrophobic interaction with Val68A.

As for the synthesis of these compounds, we have employed the methodology, which is previously reported by Baell (Scheme 1, A)[18]. Therein, the authors developed a convergent strategy, which relied on the successive assemble of three fragments. Their strategy commences with the synthesis of benzothiazole scaffold (the fragment **1**), to which the fragment **2** is coupled via reductive amination reaction with aldehydes. Afterward, fragment **3** is appended through an acylation reaction. However, we have observed that throughout this research, we failed to reproduce the reductive amination reaction of 2-aminobenzothiazoles with aldehydes, presumably because of the low nucleophilic propensity of the former compound. As even prolonged reaction times or the use of catalysts, such as p-toluene sulfonic acid, did not considerably improve the yield of this reaction, we felt compelled to revise this synthetic approach, whereupon the sequence of the reactions is altered (Scheme 1, B). That is to say, benzothiazoles derivatives are coupled with fragment **3** at first, through acylation reaction. This is

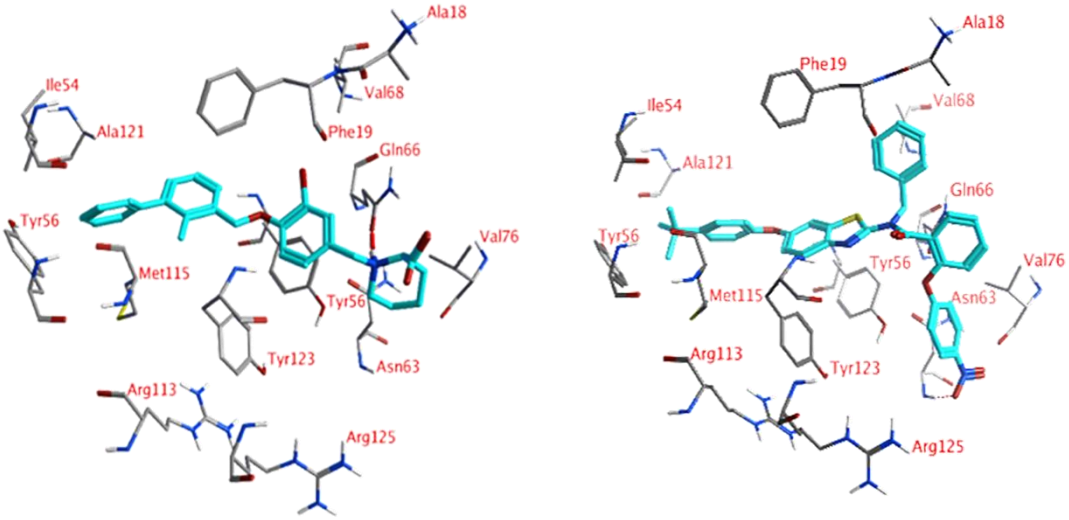
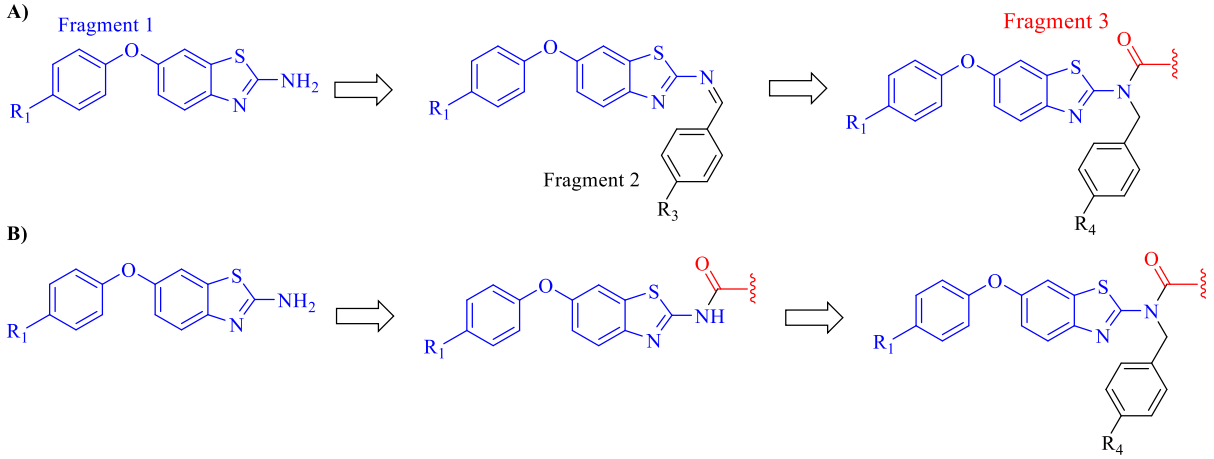


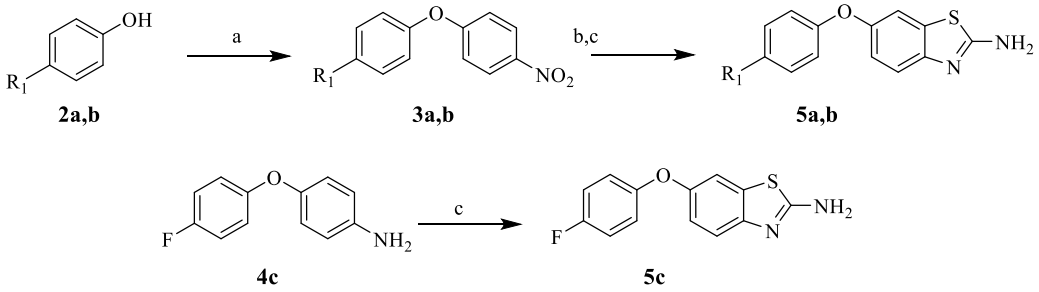
Figure 2. (Left)The crystal structure of 6GZ in the active site of PD-L1 (PDB: 5J8O) and (Right) The docked pose of **1a** (turquoise) in the active site of PD-L1 (PDB: 5J8O) (hydrogen bonds are indicated in red dashed lines).

followed by grafting fragment **2** through the alkylation reaction of the amide group with the derivatives of benzyl halides in the presence of K_2CO_3. Overall, not only did this strategy permit the synthesis of these compounds in moderate-to-good yield but also it was fully reproducible.



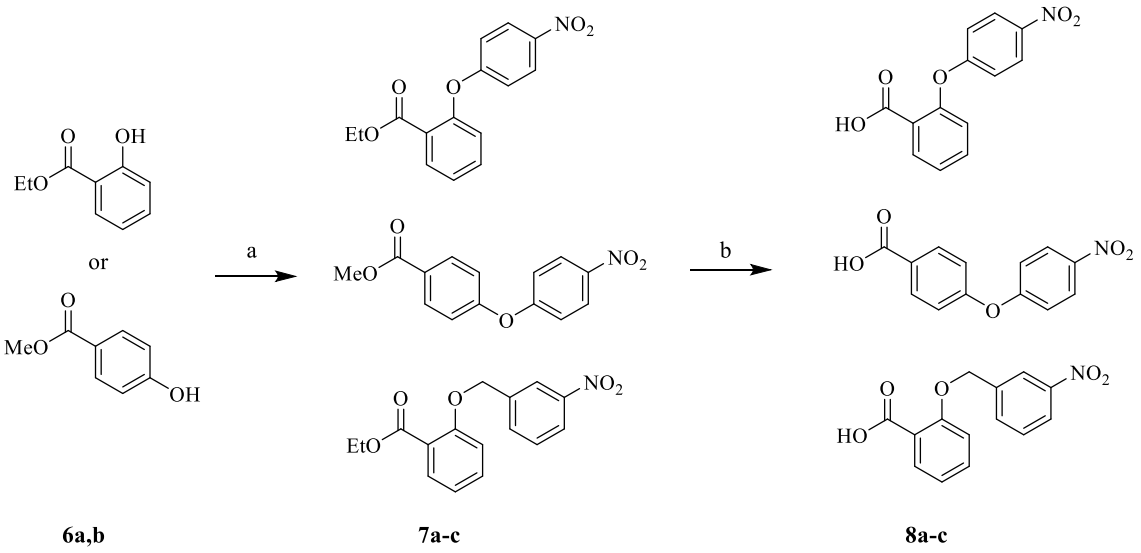
Scheme 1. (**A**) The synthetic route previously reported by Baell *et al*[18] and (**B**) Our alternative approach to prepare 2-aminobenzothiazole-based peptidomimetics.

In concert with this strategy, we have firstly synthesized 2-aminobenzothiazole derivatives **5a-c** (the fragment **1**), as tabulated in Scheme 2. Therein, 4-methoxy phenol and 4-tert-butyl phenol were reacted with 1-fluoro-4-nitrobenzene under basic conditions to afford **3a** and **3b**, in good yields. In the subsequent step, the reduction of nitro groups on **3a** and **3b** with $SnCl_2$ gave the key intermediates **4a** and **4b**.[19] Herein, we should note that 4-(4-fluorophenoxy)aniline was commercially available and was directly utilized in the synthesis of **4c**. Afterward, the benzothiazole scaffolds **5a-c** were prepared through *in situ* bromination and thiocyanation reaction of **4a-c** under acidic condition, as reported in the literature[20]. We should remark that these reactions afforded also some noticeable impurities, in addition to the targeted products **5a**, and **5b**. Naturally, this circumstance demanded purification, however; we have directly used the crude material in the subsequent reaction, after which these impurities are readily removed through washing with cold methanol during the work-up.



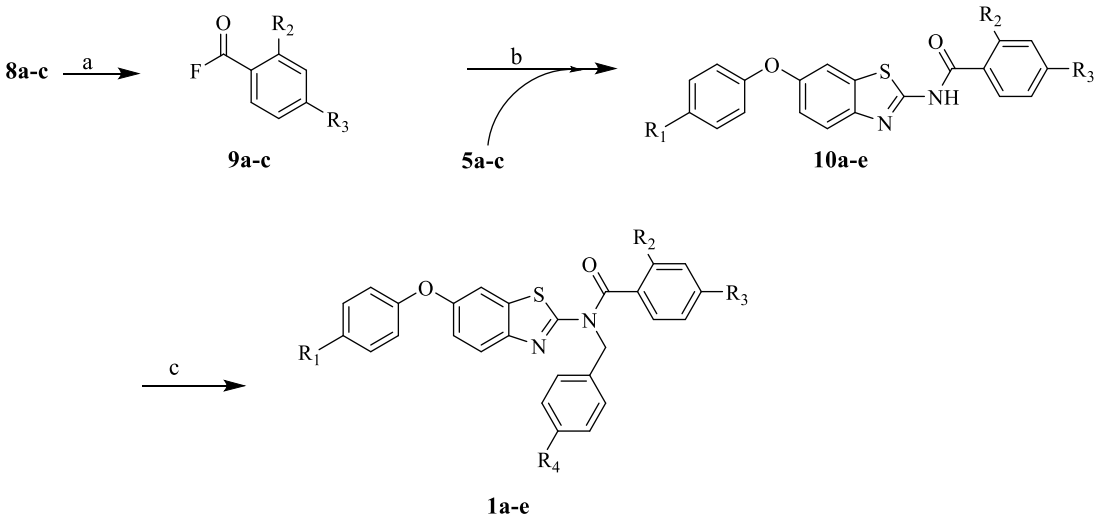
Scheme 2. The synthesis of compounds **5a-c**. Reagents and conditions: (a) 1-fluoro-4-nitrobenzene, K_2CO_3, DMF, 70 °C, overnight; (b) $SnCl_2$, HCl, MeOH, 80 °C, 5 hours; (c) NH_4SCN, Br_2, HCO_2H, CH_3CO_2H, 3-0 °C to RT, overnight.

Next is the synthesis of four intermediates **8a-d**, which constitutes the fragments **2** (Scheme 2). In this synthetic route, ethyl salicylate **6a** or methyl 4-hydroxybenzoate **6b** was alkylated with as 1-fluoro-4-nitrobenzene to give **7a** and **7b** or 3-nitrobenzyl chloride to give **7c**, respectively, in good yields.[21] After the removal of ester groups on **7a-c** with basic hydrolysis, the fragments **8a-c** are obtained in almost quantitative yield. In constructing a library for fragments **2**, our concurrent consideration for ethyl salicylate and methyl 4-hydroxybenzoate as the scaffold is founded on the fact that with their hydroxyl groups on different positions, these compounds can indeed direct the position of aromatic substituents, whereby the hydrophobic interactions between the ligand and amino acids are altered.



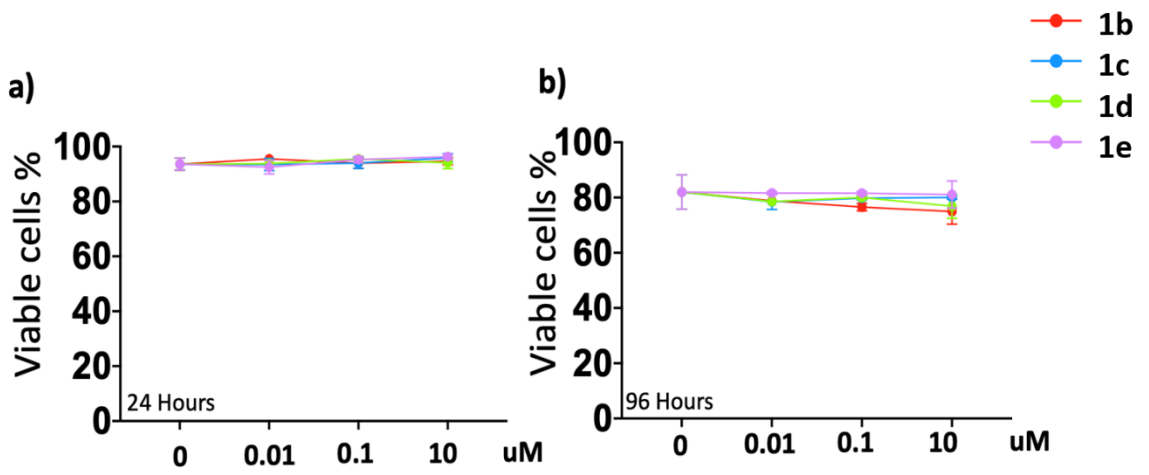
Scheme 3. The synthesis of **8a-c** from ethyl salicylate **6a** or methyl 4-hydroxybenzoate **6b**. Reagents and conditions: (a) 2-fluoro-4-nitrobenzene or 3-nitrobenzyl chloride, K_2CO_3, DMF, 90 °C, overnight; (b) NaOH, H_2O, acetonitrile, 70 °C, 2 h.

In the subsequent step, the carboxylic acid derivatives **8a-c** are converted to acyl fluorides **9a-c** through DAST, which was confirmed with the FT-IR spectra of the products.[22] That is, the stretching of OH on carboxylic acid has disappeared whilst a distinct shift in the frequency of carbonyl stretching is observed. Then, the intermediates **9a-c** are reacted with the corresponding benzothiazoles **5a-c** under reflux condition. In the final step, the fragments **3** are introduced through the alkylation of amide groups on **9a-c** with benzyl halides in the presence of K_2CO_3 to give the final compounds **1a-e** (Scheme 4).[23]

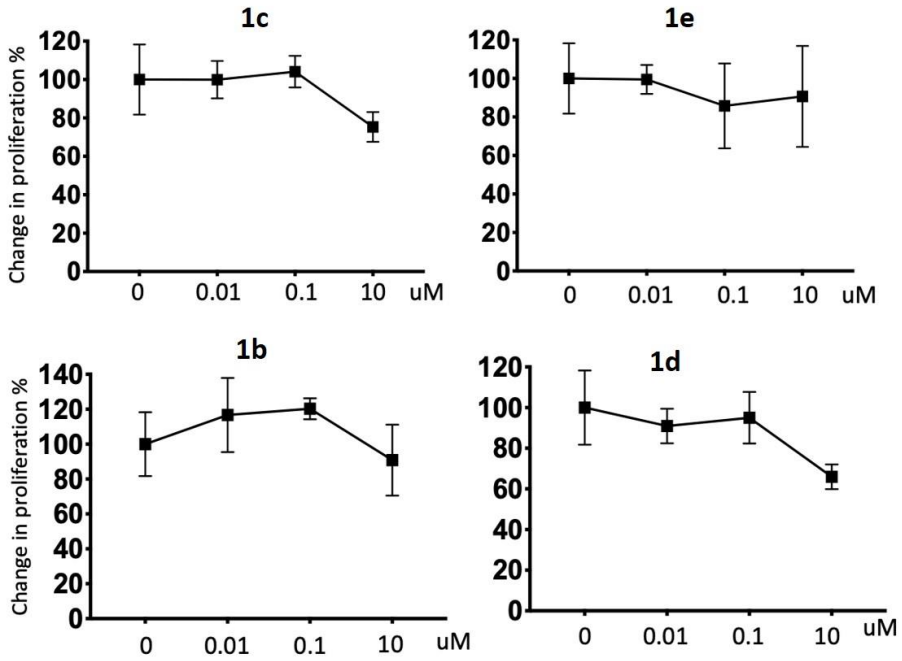


Scheme 4. The synthesis of the compounds **1a-e**. Reagents and conditions: (a) DAST, DCM, 0 °C-RT, 2 hours; (b) **5a-c**, DIPEA, DCM, 45 °C, overnight; (c) benzyl halide, K_2CO_3, DMF, 90 °C, overnight.

Following the design and the synthesis of **1a-e** to target PD-L1 and inhibit the PD-1/PD-L1 axis, their immune regulatory effects were examined on peripheral blood mononuclear cells (PBMCs) with viability and proliferation assays. For viability assays, the cells have abnormal cell membrane function, uptake viability reagent, and stained. These stained cells were measured via flow cytometry and the percentage of viable cells was calculated. According to the viability assay, when the concentration of these molecules was 0.01, 0.1, 10 uM, they did not hamper the viability of PBMCs for 24 and 96 hours of incubation (Scheme 5). For proliferation assay, the amount of diluted proliferation reagent was measured with flow cytometry, and the percentage of the proliferated cells was calculated. In proliferation assays, 0.01 and 0.1 uM of the molecules did not interfere with the proliferation of PBMCs but 10 uM of the molecules decreased the rate of proliferation (Scheme 6). Of these compounds, **1a** exhibited poor solubility in the aqueous milieu, thus, we have been unable to assess its activity in proliferation assays.



Scheme 5. Peripheral blood mononuclear cells (PBMCs) were treated with the different concentrations of the molecules for 24 hours (a) and 96 hours (b) Viability assays were performed with propidium iodide on FACSAriaII Cell Sorter.



Scheme 6. Peripheral blood mononuclear cells (PBMCs) were treated with the different concentrations of the molecules under anti-CD3 stimulation for 72 hours. Analyses were performed with FACSAria II Cell Sorter.

4. Conclusion

In summary, we have demonstrated that 2-amino-benzothiazole composes a novel scaffold targeting the PD-1/PDL-1 pathway. In this regard, we constructed a library of peptidomimetic through docking studies, and then, five of these compounds, which showed the highest score in docking studies, were synthesized. These compounds were tested by the viability and proliferation of PBMCs and it was found that the compounds **1b-e** reduce the viability of PBMCs in the concentration of 10 uM. Overall, our data indicate that these peptidomimetics will lead up to the development of novel immune-checkpoint inhibitors against the PD-1/PD-L1 axis. The pharmacokinetic profile of these novel compounds will be carried out in the subsequent studies, which constitute our future aim.

Acknowledgments

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/organic-communications

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