PNAS
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Supplementary Information for

Adjuvant effect of the novel TLR1/2 agonist Diprovocim synergizes with anti-PD-L1 to eliminate melanoma in mice

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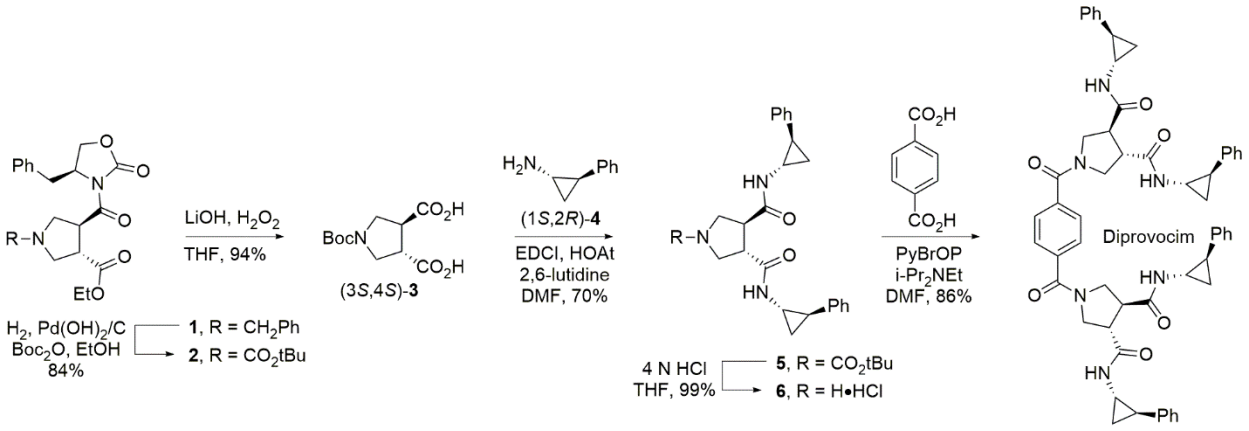
This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1809232115

Supplementary Information Text

Methods

Synthesis of Diprovocim



(3*S*,4*S*)-1-*tert*-Butyl 3-Ethyl 4-((*S*)-4-Benzyl-2-oxooxazolidine-3-carbonyl)pyrrolidine-1,3-dicarboxylate (**2**). (3*S*,4*S*)-Ethyl 1-benzyl-4-((*S*)-4-benzyl-2-oxooxazolidine-3-carbonyl)pyrrolidine-3-carboxylate[1] (**1**, 3.43 g, 7.86 mmol) and Boc_2O (1.80 g, 8.25 mmol, 1.05 equiv) were dissolved in ethanol (EtOH, 50 mL) at room temperature. $Pd(OH)_2/C$ (500 mg) was added and the reaction mixture was sparged with nitrogen (N_2) for 15 min. A 3-way flushing adapter, equipped with a hydrogen (H_2) filled balloon and vacuum source, was attached. The headspace above the reaction mixture was evacuated until the solvent began to boil, then backfilled with H_2. This vacuum/fill process was repeated 10-15 times to maximize H_2 in the headspace. After stirring for 18 h, the reaction mixture was filtered through a 6 cm Celite plug, rinsing with EtOH aliquots (3 × 15 mL) thoroughly, and concentrated. Flash column chromatography (SiO_2, 25% EtOAc/hexanes) provided 2.93 g (84%) of **2** as a clear, viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.19 (m, 5H), 4.69 (dd, *J* = 9.0, 4.5 Hz, 1H), 4.52 (q, *J* = 7.7 Hz, 1H), 4.29 – 4.14 (m, 4H), 3.95 – 3.75 (m, 2H), 3.60 (m, 2H), 3.52 – 3.27 (m, 2H), 2.86 – 2.71 (m, 1H), 1.46 (s, 9H), 1.28 (t, *J* = 7.5 Hz, 3H). HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{31}N_2O_7$ $[M+H]^+$ 447.2126, found 447.2126.

(3*S*,4*S*)-1-(*tert*-Butoxycarbonyl)pyrrolidine-3,4-dicarboxylic Acid (**3**).[2] (3*S*,4*S*)-1-*tert*-Butyl 3-ethyl 4-((*S*)-4-benzyl-2-oxooxazolidine-3-carbonyl)pyrrolidine-1,3-dicarboxylate ((3*S*,4*S*)-**2**, 2.06 g, 4.63 mmol) was dissolved in anhydrous tetrahydrofuran (THF, 20 mL) and cooled to 0 °C. Hydrogen peroxide (2.10 mL, ca. 18.5 mmol, 4.0 equiv, 30% w/v) was added dropwise to the stirred reaction solution. After 3-5 min, $LiOH•H_2O$ (500 mg, 11.9 mmol) was added. After 2 h, additional LiOH (470 mg, 11.2 mmol) was added, along with H_2O (10 mL) and THF (15 mL). The reaction mixture was stirred 3 h, warming to room temperature. Saturated aqueous Na_2SO_3 (10 mL) was added, and the THF was removed under a N_2 stream. The resulting mixture was poured into H_2O (200 mL) and extracted with methylene chloride (CH_2Cl_2, 2 × 100 mL) to remove the oxazolidinone. The aqueous phase was acidified with the addition of aqueous

[1] Prepared as a single stereoisomer according to: Bao J, Baker RK, Parsons WH, Rupprecht K. U.S. Patent 6,489,354 B1, pg 158.

[2] Modified procedure from: Ma Z, Wang S, Cooper CS, Fung AKL, Lynch JK, Plagge F, Chu DTW. Asymmetric dipolar cycloaddition reactions: a practical, convergent synthesis of chiral pyrrolidines. *Tetrahedron Asymm.* **8**, 883–887 (1997).

1 N HCl to pH 2 (ca. 75 mL). The aqueous phase was extracted with ethyl acetate (EtOAc, 3 × 125 mL), and the organic extracts were dried over Na_2SO_4, filtered and concentrated to provide 1.13 g (94%) of (*S*,*S*)-**3** as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.59 – 3.48 (m, 2H), 3.41 – 3.31 (m, 2H), 3.30 – 3.18 (m, 2H), 1.39 (s, 9H).

(3*S*,4*S*)-*tert*-Butyl 3,4-Bis(((1*S*,2*R*)-2-phenylcyclopropyl)carbamoyl)pyrrolidine-1-carboxylate (**5**). (3*S*,4*S*)-1-(*tert*-Butoxycarbonyl)pyrrolidine-3,4-dicarboxylic acid ((*S*,*S*)-**3**, 775 mg, 2.99 mmol), (1*S*,2*R*)-*trans*-2-phenylcyclopropylamine ((1*S*,2*R*)-**4**, 816 mg, 6.13 mmol, 2.05 equiv, commercially available from D-L Chiral Chemicals), and 1-hydroxy-7-azabenzotriazole (HOAt, 895 mg, 6.58 mmol, 2.20 equiv) were dissolved in anhydrous dimethylformamide (DMF, (15 mL) under a N_2 atmosphere. 2,6-Lutidine (1.75 mL, 14.9 mmol, 5.00 equiv) was added slowly. Upon dissolution of the reagents (ca. 15 min), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI•HCl, 1.43 g, 7.47 mmol, 2.50 equiv) was added in one portion, and the reaction mixture was stirred for 18 h, after which it was poured into aqueous 1 N HCl (150 mL) and EtOAc (100 mL). The aqueous phase was extracted with EtOAc (2 × 75 mL), and the combined organic phases were washed with aqueous 1 N HCl (75 mL), saturated aqueous $NaHCO_3$ (75 mL), and saturated aqueous NaCl (50 mL) sequentially. The organic phase was dried over Na_2SO_4, filtered and concentrated. Flash column chromatography (SiO_2, 50% EtOAc/hexanes) provided 1.02 g (70%) of **5**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.24 (m, 5H), 7.23 – 7.09 (m, 5H), 6.61 (s, 1H), 6.43 (s, 1H), 3.85 (t, *J* = 9.7 Hz, 1H), 3.68 (m, 1H), 3.60 (t, *J* = 10.5 Hz, 1H), 3.42 (t, *J* = 10.4 Hz, 1H), 3.27 (q, *J* = 10.0, 9.3 Hz, 1H), 3.12 (t, *J* = 9.7 Hz, 1H), 2.88 (m, 2H), 2.05 (ddt, *J* = 9.8, 6.4, 3.4 Hz, 2H), 1.46 (s, 9H), 1.24 (q, *J* = 6.6 Hz, 2H), 1.13 (dt, *J* = 10.1, 5.3 Hz, 2H). HRMS (ESI-TOF) *m/z* calcd for $C_{29}H_{36}N_3O_4$ $[M+H]^+$ 490.2700, found 490.2705.

(3*S*,4*S*)-N^3,N^4-Bis((1*S*,2*R*)-2-phenylcyclopropyl)pyrrolidine-3,4-dicarboxamide Hydrochloride (**6**). (3*S*,4*S*)-*tert*-Butyl 3,4-bis(((1*S*,2*R*)-2-phenylcyclopropyl)carbamoyl)-pyrrolidine-1-carboxylate (**5**, 998 mg, 2.04 mmol) was suspended in anhydrous THF (2 mL) at room temperature. 4 N HCl (8 mL, 4.0 M solution in dioxane) was added dropwise to the vigorously stirred reaction solution. After stirring 3 h at room temperature, during which some product had precipitated from the reaction mixture, the solvents were removed by N_2 stream over 16 h. The residual solids were suspended in anhydrous THF and reconcentrated in vacuo (3 × 5 mL) to ensure complete removal of the dioxane and excess HCl. This process was repeated with anhydrous Et_2O (3 × 5 mL) to provide 870 mg (99%) of **6** as an amorphous white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.35 (s, 2H), 8.76 (d, *J* = 4.4 Hz, 2H), 7.26 (t, *J* = 7.6 Hz, 4H), 7.20 – 7.06 (m, 6H), 3.76 – 3.62 (m, 1H), 3.55 – 3.42 (m, 1H), 3.26 (t, *J* = 8.2 Hz, 2H), 3.21 – 3.11 (m, 2H), 2.90 – 2.78 (m, 2H), 1.99 (ddd, *J* = 9.6, 6.3, 3.4 Hz, 2H), 1.26 – 1.13 (m, 4H). HRMS (ESI-TOF) *m/z* calcd for $C_{24}H_{28}N_3O_2$ $[M+H]^+$ 390.2176, found 390.2178.

Diprovocim: (3*S*,3'*S*,4*S*,4'*S*)-1,1'-Terephthaloylbis(N^3,N^4-bis((1*S*,2*R*)-2-phenylcyclo-propyl)pyrrolidine-3,4-dicarboxamide). (3*S*,4*S*)-N^3,N^4-Bis((1*S*,2*R*)-2-phenylcyclopropyl)-pyrrolidine-3,4-dicarboxamide hydrochloride (**6**, 500 mg, 1.17 mmol, 2.20 equiv) and terephthalic acid (benzene-1,4-dicarboxylic acid, 89 mg, 0.53 mmol, 1.00 equiv) were dissolved in anhydrous DMF (6 mL) at room temperature. *i*-Pr_2NEt (0.280 mL, 1.60 mmol, 3.00 equiv) was added, followed by bromo-*tris*-pyrrolidinophosphonium hexafluorophosphate (PyBrOP, 497 mg, 1.07 mmol, 2.00 equiv) after 5 min and the mixture was stirred at 23 °C for 18 h. After 18 h, the reaction mixture was diluted with EtOAc (300 mL) and washed with aqueous 0.5 N HCl (2 × 150 mL). The aqueous phase was extracted with EtOAc (1 × 50 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (100 mL) and saturated aqueous NaCl (75

mL). The organic phase was dried over Na_2SO_4, decanted and concentrated. Flash column chromatography (SiO_2, 5–8% MeOH/CH_2Cl_2) provided diprovocim. Diprovocim could be further purified by trituration with cold (0 °C) 1:1 Et_2O/EtOAc (3 ×5 mL), decanting off the liquid phase to provide 421 mg (86%) of pure diprovocim. $[\alpha]_D^{26}$ +57 (*c* 0.33, EtOH). IR (neat) ν_{max} 3259, 1633, 1539, 1426, 1386, 1073, 695 cm^{-1}. ^1H NMR (600 MHz, DMSO-d_6) δ 8.42 (d, *J* = 4.3 Hz, 2H), 8.29 (d, *J* = 4.3 Hz, 2H), 7.56 (s, 4H), 7.27 – 7.21 (m, 8H), 7.19 – 7.09 (m, 8H), 7.09 – 7.03 (m, 4H), 3.80 (dd, *J* = 12.0, 8.6 Hz, 2H), 3.71 – 3.58 (m, 2H), 3.51 (ddd, *J* = 15.6, 11.2, 8.2 Hz, 4H), 3.19 (q, *J* = 8.4 Hz, 2H), 3.10 (q, *J* = 8.1 Hz, 2H), 2.90 – 2.80 (m, 2H), 2.80 – 2.73 (m, 2H), 1.97 (ddd, *J* = 9.6, 6.4, 3.4 Hz, 2H), 1.86 (ddd, *J* = 9.5, 6.3, 3.4 Hz, 2H), 1.21 – 1.13 (m, 4H), 1.13 – 1.05 (m, 4H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 171.65, 170.93, 167.46, 141.28, 141.19, 137.71, 128.17, 128.14, 127.09, 125.83, 125.79, 125.60, 51.48, 48.74, 46.95, 45.83, 45.07, 32.54, 32.45, 25.87, 23.90, 23.81, 15.33, 15.24. HRMS (ESI-TOF) *m/z* calcd for $C_{56}H_{57}N_6O_6$ $[M+H]^+$ 909.4334, found 909.4334.

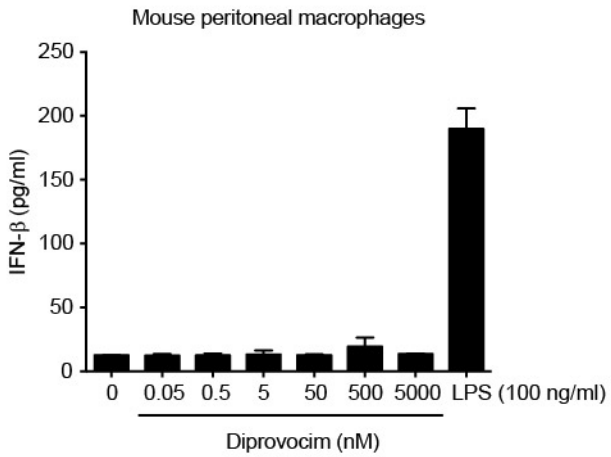


Fig. S1. Diprovocim does not stimulate IFN-β secretion by mouse peritoneal macrophages. IFN-β in the supernatants of mouse peritoneal macrophages after treatment with Diprovocim or LPS for 4 h. The means of three independent samples are plotted. P values were determined by Student's t test; no significant differences were found between responses of unstimulated cells (0 nM) and Diprovocim-stimulated cells. Results are representative of two independent experiments.

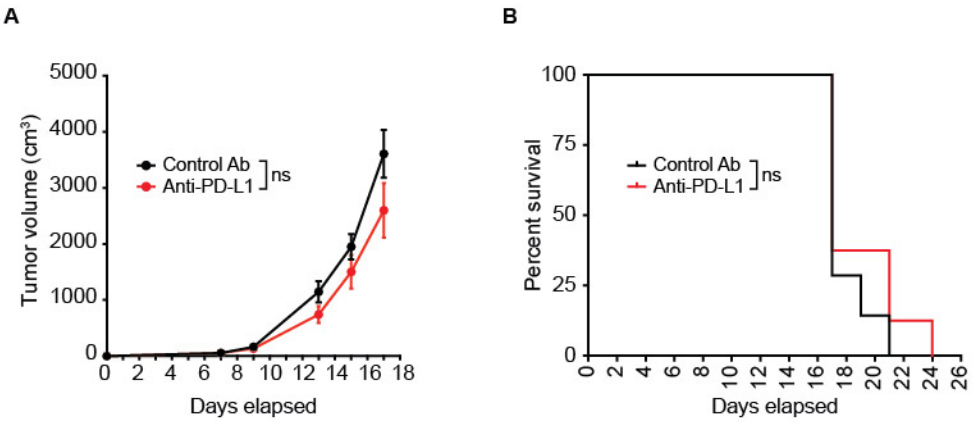


Fig. S2. Anti-PD-L1 does not inhibit B16 tumor growth in mice. C57BL/6J mice (n=8) were injected s.c. with $2x10^5$ B16-OVA melanoma cells on day 0. Anti-PD-L1 (200 μg) or mouse IgG2a isotype control antibody was administered on day 3, 6 and 9 after tumor inoculation by i.p. injection. (A) Tumor volume and (B) percent mouse survival (survivors/total mice) were monitored. P value for tumor volume analysis applies to the final timepoint and was calculated by Student's t test; no significant difference was found between treatments. P values for survival analysis were calculated by Kaplan–Meier analysis; no significant difference was found between treatments. Results are representative of two independent experiments.

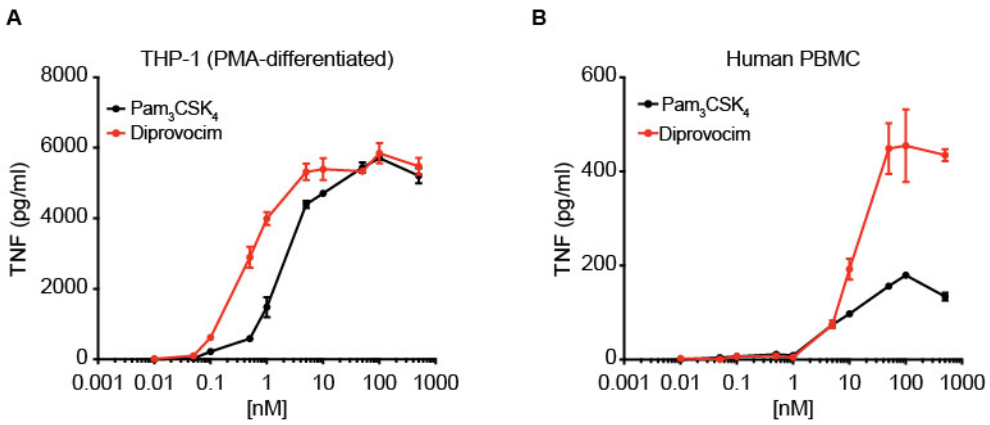


Fig. S3. Diprovocim is more potent than Pam_3CSK_4 in activation of TNF production in human cells. (A and B) TNF in the supernatants of human THP-1 cells (A) and human PBMC (B) after treatment with Diprovocim or Pam_3CSK_4 for 4 h (A) or 24 h (B). The means of three independent samples are plotted. Results are representative of two independent experiments.