Supporting Information

Conformational Restriction Leading to a Selective CB_2 Cannabinoid Receptor Agonist Orally Active Against Colitis

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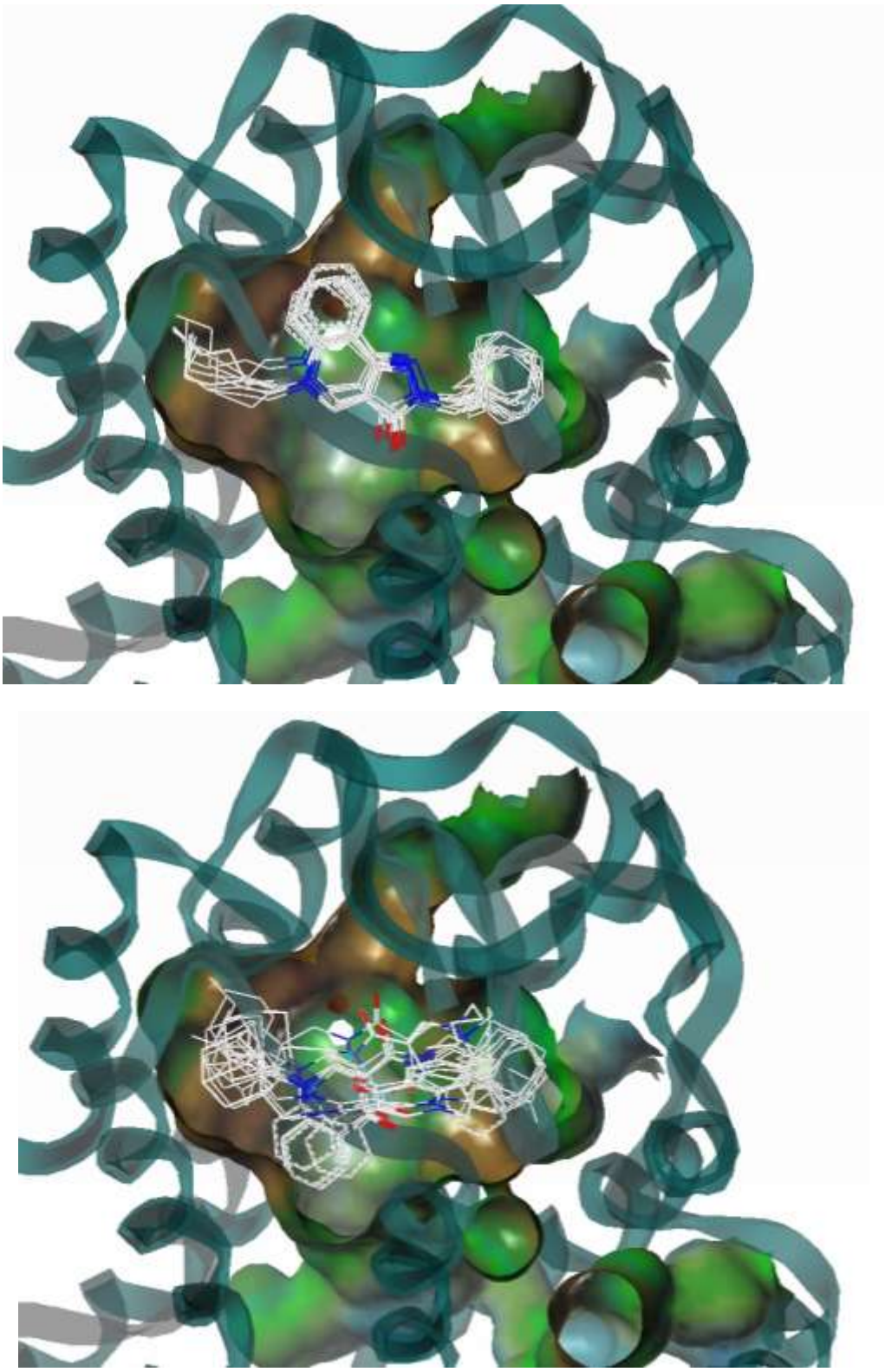
Contents

Figure S1. Docking poses of compound 26 (top) and ALICB353 (bottom) in an agonist-biased state model of the CB_2 receptor.



Experimental Section

Chemistry.

General Information. All commercial reagents and solvents were used without further purification. Analytical thin-layer chromatography was performed on precoated Kieselgel $60F_{254}$ plates (Merck); the spots were located by UV (254 and 366 nm) and/or with iodine. Silica gel 60 230-400 mesh purchased from Merck was used for column chromatography. Preparative thick-layer chromatography (TLC) was performed using silica gel from Merck and the compounds were extracted from the silica using $CHCl_3$/MeOH (8:2, v/v). All melting points were determined with a Büchi 535 capillary apparatus and remain uncorrected. ^{1}H NMR spectra were obtained using a Brücker 300 MHz spectrometer, chemical shift (δ) were expressed in ppm relative to tetramethylsilane used as an internal standard, *J* values are in hertz, and the splitting patterns were designated as follow: s singlet, d doublet, t triplet, m multiplet. All compounds were analyzed by HPLC-MS on a HPLC combined with a Surveyor MSQ (Thermo Electron) equipped with an APCI-source. All tested compounds showed a purity of > 96% in $APCI^+$ mode.

Diethyl 2-((phenylamino)methylene)malonate **1**, ethyl 4-oxo-1,4-dihydroquinoline-3-carboxylate **2** and ethyl 4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxylate **4** were already described in a previous report.[1] Thionation reactions leading to compound **6** and **7** were accomplished as described previously.[2]

1-Pentyl-1*H*-benzo[*d*][1,3]oxazine-2,4-dione (3). Under nitrogen atmosphere, to a stirred solution of NaH (1.5 g, 61.3 mmol, 60% in mineral oil) in dry DMF (200 mL), isatoic anhydride (10 g; 61.3 mmol) was added followed by 1-bromopentane (15.2 mL, 123 mmol). The mixture was refluxed for 14 h. DMF was removed under reduced pressure and the residue

taken up in water and extracted with EtOAc. The organic layer was then dried over $MgSO_4$ and the solvent removed under reduced pressure to give a yellow oil which was purified by silica gel chromatography (dichloromethane). Compound **3** was isolated as colorless oil (9.3 g, 65%). IR (cm^{-1}) 1777, 1721. ^{1}H NMR ($CDCl_3$) δ 8.16 (d, 1H, J = 8.1 Hz), 7,77 (t, 1H, J = 9.0 Hz), 7,30 (t, 1H, J = 8.1 Hz), 7,17 (d, 1H, J = 8.0 Hz), 4,05 (t, 2H, J = 8.3 Hz), 1,80-1,75 (m, 2H), 1,43-1,39 (m, 4H), 0,92 (t, 3H, J = 7.1 Hz). LC-MS ($APCI^+$) *m/z* 234.2 (MH^+).

Ethyl 4-oxo-1-pentyl-2-phenyl-1,4-dihydroquinoline-3-carboxylate (5). NaH (0.4 g, 15.4 mmol, 60% in mineral oil) was added portionwise, at 0 °C, to a solution of ethyl benzoylacetate (2.7 mL, 15.4 mmol) in 80 mL of dry DMF. After 30 minutes, compound **3** in 20 mL of dry DMF was added and the mixture was heated at 120 °C for 1 h. After cooling to room temperature, the solvent was distilled off to give a brown oil. Water was added and subsequent extraction with CH_2Cl_2 afforded an oil which was triturated in diethyl ether to give a white powder (3.6 g, 64%). mp 118 °C. IR (cm^{-1}) 1725, 1619. ^{1}H NMR (DMSO-d_6) δ 8.26 (d, 1H, J = 8.9 Hz), 7.93-7.79 (m, 2H), 7.59-7.42 (m, 6H), 3.95 (t, 2H, J = 8.0 Hz), 3,80 (q, 2H, J = 7.3 Hz), 1.68-1.52 (m, 2H), 1.12-0.95 (m, 4H), 0,77 (t, 3H, J = 7.1 Hz), 0,70 (t, 3H, J = 7.1 Hz). LC-MS ($APCI^+$) *m/z* 364.5 (MH^+).

General Procedure for the Preparation of 2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-ones (8-17) from 4-Thioxoquinolines 6 and 7.

A mixture of compound **6** or **7** (1 equiv.) and hydrazine monohydrate or monosubstituted (3 equiv.) in absolute EtOH was refluxed for 14 h. DIPEA (3.2 equiv.) was also added in the case when the monosubstituted hydrazine was in its hydrochloride salt form. After cooling to room temperature, solvent was evaporated and the residue partitioned in H_2O-CH_2Cl_2. The organic phase was washed both with water and brine, dried and evaporated to give a

yellow oil. The latter was chromatographied on silica gel (CH_2Cl_2/MeOH 95:5, v/v) and the subsequent yellow solid was crystallized in acetonitrile to give the title compound as yellow crystals.

5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (8). Yield**:** 90%. Mp > 250 °C. IR (cm^{-1}) 1610. ^{1}H NMR (DMSO-d_6) δ 11.42 (s, 1H), 8.67 (s, 1H), 8.14 (d, 1H, J = 7.9 Hz), 7,82 (d, 1H, J = 8.4 Hz), 7,66 (t, 1H, J = 7.3 Hz), 7.52 (t, 1H, J = 7.6 Hz), 4,38 (t, 2H, J = 7.3 Hz), 1.82-1.68 (m, 2H), 1.38-1.25 (m, 4H), 0.84 (t, 3H, J = 6.4 Hz). LC-MS (APCI$^+$) *m/z* 256.2 (MH^+).

5-pentyl-4-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (9). Yield**:** 94%. mp 250 °C. IR (cm^{-1}) 1654. ^{1}H NMR (DMSO-d_6) δ 9.06 (s, 1H), 8.39 (d, 1H, J = 6.4 Hz), 7.74 (d, 1H, J = 8.7 Hz), 7.67 (t, 1H, J = 7.3 Hz), 7.62-7.44 (m, 6H), 4,00 (t, 2H, J = 7.6 Hz), 1.68-1.54 (m, 2H), 1.14-0.95 (m, 4H), 0.71 (t, 3H, J = 6.7 Hz). LC-MS (APCI$^+$) *m/z* 332.3 (MH^+).

5-Pentyl-2,4-diphenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (10). Yield**:** 61%. mp > 250 °C. IR (cm^{-1}) 1672. ^{1}H NMR ($CDCl_3$) δ 8.58 (d, 1H, J = 7.6 Hz), 8.20 (d, 1H, J = 8.7 Hz), 7.70-7.11 (m, 12H), 4.08 (t, 2H, J = 8.4 Hz), 1.78-1.69 (m, 2H), 1.27-1.17 (m, 4H), 0.84 (t, 3H, J = 6.7 Hz). LC-MS (APCI$^+$) *m/z* 408.2 (MH^+).

2-(4-Methoxyphenyl)-5-pentyl-4-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (11). Yield**:** 41%. mp > 250 °C. IR (cm^{-1}) 1280, 1661. ^{1}H NMR ($CDCl_3$) δ 8.56 (d, 1H, J = 7.6 Hz), 8. 10 (d, 2H, J = 9.1 Hz), 7.66-7.45 (m, 8H), 6,92 (d, 2H, J = 7.3 Hz), 4.11 (t, 2H, J = 8.1 Hz), 3.81 (s, 3H), 1.78-1.69 (m, 2H), 1.27-1.19 (m, 4H), 0.84 (t, 3H, J = 6.7 Hz). LC-MS (APCI$^+$) *m/z* 438.1 (MH^+).

5-Pentyl-4-phenyl-2-(3-(trifluoromethyl)phenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (12). Yield**:** 35%. mp > 250 °C. IR (cm^{-1}) 1099, 1666. ^{1}H NMR ($CDCl_3$) δ 8.58 (d, 1H, J = 8.1 Hz),

8.49 (d, 1H, *J* = 8.1 Hz), 7.72-7.36 (m, 11H), 4.30 (t, 2H, *J* = 7.3 Hz), 1.78-1.62 (m, 2H), 1.27-1.20 (m, 4H), 0.90 (t, 3H, *J* = 6.7 Hz). LC-MS ($APCI^+$) *m/z* 472.2 (MH^+).

5-Pentyl-2-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (13). Yield**:** 64%. mp > 250 °C. IR (cm^{-1}) 1658. ^1H NMR ($CDCl_3$) δ 8.51 (d, 1H, *J* = 8.1 Hz), 8.31 (s, 1H), 8.26 (d, 1H, *J* = 8.1 Hz), 7.82 (d, 1H, *J* = 8.4 Hz), 7.57-7.47 (m, 6H), 4.27 (t, 2H, *J* = 7.3 Hz), 1.81-1.62 (m, 2H), 1.47-1.27 (m, 4H), 0.94 (t, 3H, *J* = 6.4 Hz). LC-MS ($APCI^+$) *m/z* 332.3 (MH^+).

2-(4-Methoxyphenyl)-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (14). Yield**:** 44%. mp > 250 °C. IR (cm^{-1}) 1294, 1645. ^1H NMR ($CDCl_3$) δ 8.62 (d, 1H, *J* = 7.6 Hz), 8.38 (s, 1H), 8.11 (d, 2H, *J* = 7.9 Hz), 7,83 (d, 1H, *J* = 8.1 Hz), 7.67 (t, 1H, *J* = 7.4 Hz), 7.64 (t, 1H, *J* = 7.3 Hz), 7.02 (d, 2H, *J* = 7.9 Hz,), 4.27 (t, 2H, *J* = 7.3 Hz), 3.86 (s, 3H), 1.96-1.88 (m, 2H), 1.59-1.56 (m, 4H), 0.93 (t, 3H, *J* = 6.4 Hz). LC-MS ($APCI^+$) *m/z* 362.1 (MH^+).

5-Pentyl-2-(3-(trifluoromethyl)phenyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (15). Yield**:** 38%. mp > 250 °C. IR (cm^{-1}) 1117, 1660. ^1H NMR ($CDCl_3$) δ 8.60-8.50 (m, 2H), 7.71 (s, 1H), 7.61-7.40 (m, 6H), 4.29 (t, 2H, *J* = 7.3 Hz), 2.06-1.94 (m, 2H), 1.59-1.52 (m, 4H), 0.94 (t, 3H, *J* = 7.0 Hz). LC-MS ($APCI^+$) *m/z* 400.1 (MH^+).

2-Cyclohexyl-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (16). Yield**:** 77%. mp > 250 °C. IR (cm^{-1}) 1634. ^1H NMR ($CDCl_3$) δ 8.43 (d, 1H, *J* = 7.6 Hz), 8.39 (s, 1H), 7.63-7.48 (m, 3H), 4.24 (t, 2H, *J* = 7.2 Hz), 1.86-1.19 (m, 17H), 1.00 (t, 3H, *J* = 6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.5, 141.5, 141,2, 135.0, 129.4, 126.1, 123.8, 121.2, 116.3, 107.6, 54.8, 53.1, 32.0, 28.8, 28.7, 25.8, 25.5, 22.2, 13.9. LC-MS ($APCI^+$) *m/z* 338.2 (MH^+). HRMS calcd for $C_{21}H_{28}N_3O$ $[M + H]^+$ 338.2227, found 338.2217.

General procedure for the preparation of 2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-ones (18-27) from compounds 8 and 9.

NaH (1.5 equiv., 60% in mineral oil) was added portionwise at 0 °C, to a solution of compound **8** or **9** (1 equiv.) in dry DMF (20 mL for 1.2 mmol of **8** or **9**). After 30 minutes, the appropriate alkyl halide (1.5 equiv.) was added and the mixture was heated at 90 °C for 14 h. After cooling to room temperature, the reaction medium was concentrated to dryness and the residue taken up in water and extracted with CH_2Cl_2. The resulting oil was then purified by silica gel chromatography (CH_2Cl_2/MeOH 95:5, v/v) followed by crystallization in acetonitrile to give yellow crystals.

2-Benzyl-5-pentyl-4-phenyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (17). Yield**:** 88%. mp > 250 °C. IR (cm^{-1}) 1651. ^{1}H NMR ($CDCl_3$) δ 8.28 (d, 1H, *J* = 7.6 Hz), 7.96 (d, 1H, *J* = 8.7 Hz), 7.80-7.05 (m, 12H), 4.95 (s, 2H), 4.08 (t, 2H, *J* = 8.4 Hz), 1.63-1.48 (m, 2H), 1.16-1.04 (m, 4H), 0.82 (t, 3H, *J* = 6.7 Hz). LC-MS ($APCI^+$) *m/z* 422.2 (MH^+).

5-Pentyl-4-phenyl-2-(3-phenylpropyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (18). Yield**:** 30%. mp 250 °C. IR (cm^{-1}) 1651. ^{1}H NMR ($CDCl_3$) δ 8.45 (d, 1H, *J* = 7.3 Hz), 7.91 (d, 1H, *J* = 8.1 Hz), 7.87-6.92 (m, 12H), 4.30 (t, 2H, *J* = 8.3 Hz), 4.03 (t, 2H, *J* = 7.6 Hz), 2.72 (t, 2H, *J* = 7.6 Hz), 1,95 (m, 2H), 1.64-1.49 (m, 2H), 1.27-1.16 (m, 4H), 0.85 (t, 3H, *J* = 6.5 Hz). LC-MS ($APCI^+$) *m/z* 450.2 (MH^+).

2-Benzyl-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (19). Yield**:** 74%. mp > 250 °C. IR (cm^{-1}) 1644. ^{1}H NMR ($CDCl_3$) δ 8.39 (s, 1H), 8.36 (d, 1H, *J* = 7.6 Hz), 7.72 (t, 1H, *J* = 8.1 Hz), 7.64 (d, 1H, *J* = 8.4 Hz), 7.58-6.99 (m, 6H), 5.26 (s, 2H), 4.26 (t, 2H, *J* = 7.3 Hz), 1.91-1.62 (m, 2H), 1.49-1.27 (m, 4H), 1,00 (t, 3H, *J* = 6.4 Hz). LC-MS ($APCI^+$) *m/z* 346.2 (MH^+).

5-Pentyl-2-(3-phenylpropyl)-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (20). Yield**:** 35%. mp > 250 °C. IR (cm^{-1}) 1634. ^{1}H NMR ($CDCl_3$) δ 8.43 (d, 1H, *J* = 7.6 Hz), 7.72 (m, 3H), 7.25-7.17 (m, 5H), 4.34-4.24 (m,, 4H), 2.73 (t, 2H, *J* = 7.0 Hz), 2.23 (m, 2H), 1.77 (m, 2H), 1,39 (m, 4H), 1.07 (t, 3H, *J* = 6.4 Hz). LC-MS ($APCI^+$) *m/z* 374.2 (MH^+).

2,5-Dipentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (21). Yield**:** 77%. mp 154 °C. IR (cm^{-1}) 1630. ^{1}H NMR ($CDCl_3$) δ 8.41 (dd, 1H, *J* = 7.6 Hz, *J* = 1.5 Hz), 8.25 (s, 1H), 7.59-7.51 (m, 3H), 4.24 (t, 2H, *J* = 7.3 Hz), 4.06 (t, 2H, *J* = 7.3 Hz), 1.91 (m, 4H), 1.38 (m, 8H), 0.90 (m, 6H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 142.5, 141.9, 134.9, 129.7, 126.6, 123.7, 121.0, 116.6, 55.1, 45.3, 29.2, 29.0, 28.8, 22.4, 22.3, 14.0, 13.9. LC-MS ($APCI^+$) *m/z* 326.2 (MH^+). HRMS calcd for $C_{20}H_{28}N_3O$ $[M + H]^+$ 326.2227, found 326.2218.

2-Hexyl-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (22). Yield**:** 73%. mp 157 °C. IR (cm^{-1}) 1632. ^{1}H NMR ($CDCl_3$) δ 8.40 (dd, 1H, *J* = 7.9 Hz, *J* = 1.2 Hz), 8.25 (s, 1H), 7.58-7.51 (m, 3H), 4.24 (t, 2H, *J* = 7.3 Hz), 4.06 (t, 2H, *J* = 7.3 Hz), 1.91 (m, 4H), 1.38 (m, 10H), 0.90 (m, 6H). NMR ($CDCl_3$, 75 MHz) δ 161.6, 144.6, 142.3, 134.7, 130.1, 127.2, 124.0, 120.8, 116.9, 105.5, 55.6, 46.1, 31.5, 29.2, 28.8, 28.7, 26.4, 22.5, 22.2, 14.0, 13.8. LC-MS ($APCI^+$) *m/z* 340.3 (MH^+). HRMS calcd for $C_{21}H_{30}N_3O$ $[M + H]^+$ 340.2383 found 340.2374.

2-(Cyclopropylmethyl)-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (23). Yield**:** 47%. mp 187 °C. IR (cm^{-1}) 1632. ^{1}H NMR ($CDCl_3$) δ 8.41 (dd, 1H, *J* = 7.6 Hz), 8.25 (s, 1H), 7.58-7.48 (m, 3H), 4.24 (t, 2H, *J* = 7.3 Hz), 3.89 (d, 2H, *J* = 7.3 Hz), 1.91 (m, 2H), 1.39 (m, 5H), 0.92 (t, 3H, *J* = 7.0 Hz), 0.47 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 162.2, 141.8, 141.3, 135.1, 129.6, 126.3, 123.7, 121.1, 116.4, 107.2, 54.9, 49.2, 28.8, 28.7, 22.3, 13.8, 11.1, 3.63. LC-MS ($APCI^+$) *m/z* 310.1 (MH^+). HRMS calcd for $C_{19}H_{24}N_3O$ $[M + H]^+$ 310.1914, found 310.1907.

2-(Cyclohexylmethyl)-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (24). Yield**:** 37%. mp 200 °C. IR (cm^{-1}) 1631. ^{1}H NMR ($CDCl_3$) δ 8.41 (d, 1H, *J* = 7.6 Hz), 8.38 (s, 1H), 7.63-7.48 (m, 3H), 4.24 (t, 2H, *J* = 7.3 Hz), 3.88 (d, 2H, *J* = 7.3 Hz), 1.91-1.19 (m, 17H), 0.92 (t, 3H, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 162.5, 141.6, 141.2, 135.1, 129.5, 126.2, 123.6, 121.0, 116.4, 107.2, 54.9, 51.0, 37.9, 30.7, 29.7, 28.8, 28.7, 26.5, 25.8, 22.3, 13.9. LC-MS ($APCI^+$) *m/z* 352.2 (MH^+). HRMS calcd for $C_{22}H_{30}N_3O$ $[M + H]^+$ 352.2383, found 352.2372.

2-(2-Cyclohexylethyl)-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (25). Yield**:** 57%. mp 160 °C. IR (cm^{-1}) 1621. ^{1}H NMR ($CDCl_3$) δ 8.41 (d, 1H, *J* = 7.9 Hz), 8.24 (s, 1H), 7.60-7.48 (m, 3H), 4.24 (t, 2H, *J* = 7.3 Hz), 4.06 (t, 2H, *J* = 7.3 Hz), 1.91-1.19 (m, 17H), 0.92 (m, 5H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 156.5, 148.5, 143.1, 134.2, 130.8, 128.3, 124.4, 120.5, 117.5, 103.2, 56.5, 45.4, 36.3, 35.2, 33.0, 29.0, 28.6, 26.4, 26.1, 22.2, 13.8. LC-MS ($APCI^+$) *m/z* 366.2 (MH^+). HRMS calcd for $C_{23}H_{32}N_3O$ $[M + H]^+$ 366.2540, found 366.2530.

***N*2-(Adamantylmethyl)-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (26)**. Yield: 32%. mp 225 °C. IR (cm^{-1}) 1629. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.39 (d, 1H, *J* = 7.6 Hz), 8.36 (s, 1H), 7.63-7.47 (m, 3H), 4.23 (t, 2H, *J* = 7.3 Hz), 3.73 (s, 2H), 1.97-1.39 (m, 21H), 0.93 (t, 3H, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.9, 142.7, 141.3, 135.0, 129.7, 126.6, 123.8, 121.0, 116.6, 106.2, 57.2, 55.1, 40.6, 36.9, 35.7, 28.8, 28.4, 22.3, 13.8. LC-MS ($APCI^+$) m/z 404.2 $[M + H]^+$. HRMS calcd for $C_{26}H_{34}N_3O$ $[M + H]^+$ 404.2689, found 404.2696.

2-(1-Adamantylethyl)-5-pentyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (27). Yield : 35%. mp 237 °C. IR (cm^{-1}) 1625. ^{1}H NMR ($CDCl_3$) δ 8.40 (d, 1H, *J* = 7.9 Hz), 8.26 (s, 1H), 7.63-7.47 (m, 3H), 4.24 (t, 2H, *J* = 7.2 Hz), 4.06 (m, 2H), 3.73 (s, 2H), 2.01-1.33 (m, 23H), 0.93 (t, 3H, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.9, 141.1, 135.1, 129.5, 126.2, 123.7, 121.0, 116.4,

107.4, 54.9, 43.1, 42.3, 40.0, 37.2, 32.0, 28.8, 28.7, 22.3, 13.9. LC-MS (APCI+) m/z 418.2 (MH+). HRMS calcd for $C_{27}H_{36}N_3O$ $[M + H]^+$ 418.2853, found 418.2843.

In vitro Pharmacology

Competition Binding Assay. Stock solutions of the compounds were prepared in DMSO and further diluted with the binding buffer to the desired concentration. Final DMSO concentrations in the assay were less than 0.1%. The competitive binding experiments were performed with little modifications of a protocol described earlier.[3] Briefly [^{3}H]-CP-55,940 (0.5 nM) as radioligand for the human CB_1 and the human CB_2 cannabinoid receptor, respectively, were added to 6 µg of membranes resuspended in 550 µL (final volume) binding buffer (20 mM Hepes, 5 mM $MgCl_2$, 1 mM EDTA, 0.3% bovine serum albumine, pH 7.4). After 1 h at 30 °C, the incubation was stopped and the solutions were rapidly filtered over Unifilter-96 GF/C glass fiber pre-soaked in binding buffer on a Filtermate Unifilter 96-Harvester (PerkinElmer) and washed 20 times with ice-cold binding buffer without serum albumin. The radioactivity on the filters was measured using a TopCount NXT Microplate Scintillation Counter (PerkinElmer) using 60 µL of MicroScint 40 (PerkinElmer) after 30 min resting. Assays were performed at least in triplicate. The nonspecific binding was determined in the presence of 5 µM (*R*)-(+)-WIN 55,212-2 (Sigma).

[^{35}S]-GTPγS Assays. The binding experiments were performed at 30 °C in tubes containing 10 µg of protein in 0.5 mL (final volume) binding buffer (20 mM Hepes, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% bovine serum albumin, pH 7.4) supplemented with 30 µM GDP. The assay was initiated by the addition of [^{35}S]-GTPγS (0.1 nM, final concentration). After 1 h at 30 °C, the incubation was stopped and the solutions were rapidly filtered over Unifilter-96 GF/B glass fiber and washed 20x times with the ice-cold binding buffer. The radioactivity on the filters

was counted as mentioned above. Assays were performed in triplicate. The nonspecific binding was measured in the presence of 100 µM Gpp(NH)p. Results were expressed as EC_{50} (nM) and E_{max} (%).

Data Analysis. Ki and EC_{50} values were determined by nonlinear regression analysis performed using the GraphPad prism 5.0 program (GraphPad Software, San Diego). Statistical signification of [^{35}S]-GTPγS assay results was assessed using a Student's t test.

In vivo Pharmacology

Experiments were performed after approval by the Ethics Committee for Animal Experimentation from Lille (Authorization number 00448.01). Males C57Bl6 mice (n = 10 per group) had free access to standard mouse chow and tap water. For colitis induction, mice were anesthetized by subcutaneous administration of xylazine–ketamine (50 mg/kg) in saline for 90–120 min and received an intrarectal administration of TNBS (40 µL, 150 mg/Kg) dissolved in a 1:1 mixture of 0.9% NaCl with 100% ethanol. Compound **26** (0.1, 1 and 10 mg/Kg in carboxymethyl cellulose, *per os*) and JWH133 (0.1 mg/Kg, i.p injection) were administered once daily over 7 days, starting 2 days before TNBS administration (colitis induction). Animals were euthanized 5 days after TNBS administration. Macroscopic and histological indications of inflammation in each colon were evaluated blindly by two investigators. Macroscopic score rates lesions on a scale from 0 to 10 based on features reflecting inflammation, such as hyperemia, thickening of the bowel, and extent of ulceration. A colon specimen located precisely 2 cm above the anal canal was used for histological evaluation following May–Grunwald Giemsa staining. Histologic score grading on a scale from 0 to 6 takes into account the degree of inflammation infiltrate, the presence of erosion, ulceration, or necrosis, and the depth and surface extension of lesions. The other

parts of the colon were frozen and used to measure cytokines mRNA levels. For quantification of colonic cytokines levels by real-time PCR, total RNA from colon was extracted using Nucleospin RNAII (Macherey Nagel, Hoerdt, France) and then reverse transcribed using the high-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Real time PCR was performed using SYBR Green (Applied Biosystems, Foster City, USA). Specific primers for TNFα (TNFα F: CCACCACGCTCTTCTGTCTA and TNFα R; GAGGCCATTTGGGAACTTCT), IL-1β (IL1β F; AGCTCTCCACCTCAATGGAC and IL1β R; AGGCCACAGGTATTTTGTCG) and POLR2A (POLR2A F: CCCACAACCAGCTATCCTCAA and POLR2A R: GGTGCTGTGGGTACGGATACA) acting as internal control were designed using the Primer Express Program (Applied Biosystems, Foster City, USA). For graphical representation of quantitative PCR data, raw cycle threshold values (Ct values) obtained for target samples were deducted from the Ct value obtained for internal control transcript levels, using the ΔΔCt method as follows: ΔΔCt = (Ct,target-Ct,control)treatment-(Ct,target-Ct,control)nontreatment, and the final data were derived from 2-ΔΔCt.

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