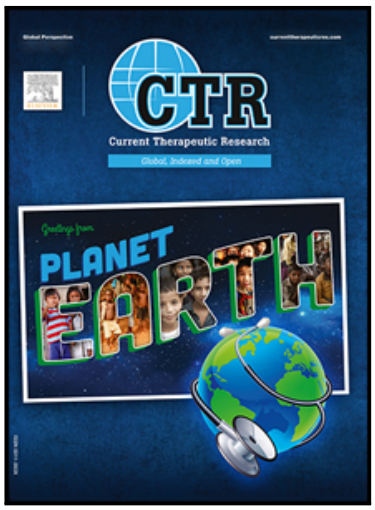

Structural Optimization of the Diarylurea PSNCBAM-1, an Allosteric Modulator of Cannabinoid Receptor 1

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Highlights

- PSNCBAM-1 is an allosteric modulator of the cannabinoid receptor 1
- Derivatives of PSNCBAM-1 were made, to reduce the total rings in the structure
- Several derivatives maintained allosteric activity, as shown by binding experiments
- Some calculated physicochemical properties for these derivatives are provided

Structural Optimization of the Diarylurea PSNCBAM-1, an Allosteric Modulator of Cannabinoid Receptor 1

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Abstract

Background

Structure-activity relationship studies improve the pharmacological and pharmacokinetic properties of a lead compound such as PSNCBAM-1, an allosteric modulator of the cannabinoid receptor 1 (CB_1).

Objectives

Here, several derivatives of PSNCBAM-1 were synthesized with the aim of reducing the number of rings within its structure and enhancing the solubility of the compounds. The derivatives studied contain substituents previously shown to enhance binding of agonists (i.e. a cyano group, and a pyrimidine ring), with a reduced number of rings compared to the parent compound, PSNCBAM-1.

Methods

The synthesized compounds were tested for the enhancement of the binding of orthosteric CB_1 agonist CP55,940 in the presence of varying concentrations of each test compound. Select compounds were also tested for their effects on CB_1 inverse agonist SR141716A binding. The compounds are also subjected to computational analysis of drug-like properties and solubility.

Results

Consistent with a positive allosteric modulator for orthosteric ligand binding, compounds LDK1317 (12a), LDK1320 (12b), LDK1321 (6a), LDK1323 (8a), and LDK1324 (6b) all enhanced the binding of agonist CP55,940 to some degree. Reduction in the number of rings did

not abolish the activity. The new lead compounds LDK1317 (12a) and LDK1321 (6a) showed improved drug-like properties and enhanced solubility in silico.

Conclusions

In contrast to PSNCBAM-1, the synthesized compounds are analogs with fewer rings. The compounds LDK1317 (12a) and LDK1321 (6a) contained only 2 or 3 rings, respectively, and showed the binding parameters (K_B = 110 nM, α = 2.3, and K_B = 85 nM, α = 5.9). Further, the computationally predicted drug-like properties and solubility suggest these compounds are acceptable new lead compounds for further development of CB_1 allosteric modulators.

Keywords

allosteric modulator, cannabinoid, CB_1 receptor, diarylurea

Introduction

The cannabinoid receptor 1 (CB_1) is a rhodopsin-like G protein coupled receptor (GPCR)[1], and is the most abundant GPCR in the brain.[2,3] Orthosteric ligands for CB_1 include the endogenous cannabinoids 2-arachidonyl glycerol and anandamide, as well as the phytocannabinoid, Δ^9-tetrahydrocannabinol, and synthetic compounds such as the agonist CP55,940 and the inverse agonist SR141716A.[4-10] CB_1 is implicated in pathways involving pain, hunger, emotional state, and neurodegenerative disease, thus making it an attractive therapeutic target for maladies impacting these pathways.[11-13]

In addition to orthosteric compounds, which bind the site where the endogenous ligands bind, several allosteric modulators for CB_1 have been identified such as ORG27569[14] and PSNCBAM-1.[15] An allosteric modulator binds to a site which is topographically distinct from the orthosteric site.[14,16] There are several advantages to targeting the allosteric site, such as subtype selectivity, spatiotemporal control, pathway selectivity, and a ceiling effect which may minimize overdose risk.[17-20]

PSNCBAM-1 is an allosteric modulator of CB_1, first characterized by Horswill and colleagues in 2007.[15] It displayed properties characteristic of a compound which promotes an active conformation of CB_1 in that it enhanced binding of the CB_1 agonist CP55,940, while reducing the binding of the inverse agonist SR141716A.[15] However, PSNCBAM-1 had non-competitive, inhibitory effects in GTPγS and cAMP assays, and caused reduced food intake and body weight in rats.[15]

Several SAR studies have been performed on derivatives of PSNCBAM-1.[21-23] A finding from these studies is that a non-cyclic substitution in the 2-pyrrolidinylpyridine position, such as a dimethylamino, is favored.[21] In addition, it was suggested that the electron-withdrawing cyano

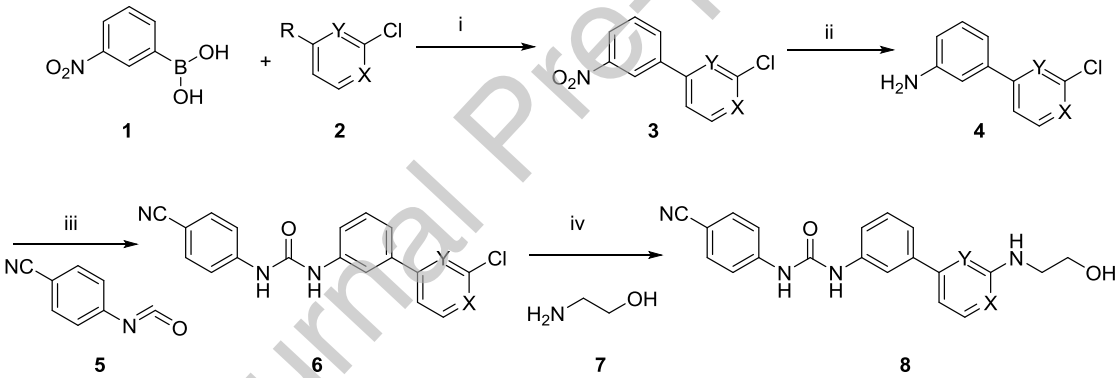
group may play additional roles such as replacing the water molecule in the receptor-ligand complex, which in turn improved the potency of the modulator compared to the original chloro group of PSNCBAM-1.[21, 22] In binding experiments of CB_1 using agonist CP55,940 as the tracer, the EC_{50} of the derivative of PSNCBAM-1 which was cyano-substituted was 55 nM, compared to the EC_{50} of PSNCBAM-1, which was 167 nM,[21] suggesting it is important for the cyano group to maintain its position. By shifting the substituent to the *meta* position, the affinity for CB_1 decreases.[22] The NH group of the urea also appears to be essential for the compound to influence CP55,940 binding.[23] Khurana and colleagues[22] replaced the pyridine ring of PSNCBAM-1 with a pyrimidine ring. Two scaffolds were made where the nitrogens of the pyrimidine ring were in different positions. For both of these newly synthesized PSNCBAM-1 derivatives, they maintained the ability to positively modulate the binding of the orthosteric agonist CP55,940. Although the compounds had a lowered binding affinity, they showed a greater degree of positive cooperativity than the parent compound, indicated by a higher α value.[22]In the lead selection and optimization for central nervous system (CNS) drug discovery, the preferred number of rings within a structure is up to three.[24] The lead compound PSNCBAM-1 possesses four rings. In this study, several derivatives of PSNCBAM-1 with reduced numbers of rings (two or three) were synthesized and tested for their ability to potentiate orthosteric agonist CP55,940 binding. All compounds in this series have a cyano group which replaces the chloro group of the lead compound PSNCBAM-1, while some compounds in this series feature a pyrimidine ring as opposed to the pyridine ring of the lead compound (see Figure 1). Our work in reducing the rings of PSNCBAM-1 to optimize the scaffold met the endpoints of this study that aim at enhancing the drug-like properties[25] and improving aqueous solubility.

Materials and Methods

Compound Synthesis

The target compounds were synthesized according to the methods illustrated in Scheme 1-3. Generally, the target compounds 6, 10 and 12 were prepared from coupling a commercially available (i.e., 9 -) 1or synthesized arylamine (i.e. 4) with 4-Cyano isocyanate (5). To synthesize the target compound 8, the diaryl urea 6 was further reacted with ethanolamine in heated anhydrous *N,N*-dimethyl formaldehyde (DMF) in the presence of potassium carbonate.

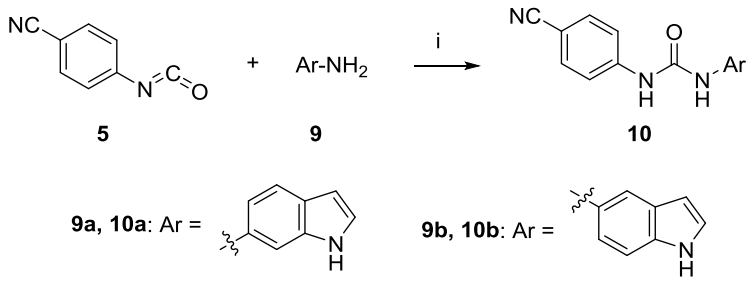
Scheme 1: Synthesis Route for Pyridinyl and Pyrimidinyl Biphenyl Ureas **6** and **8**[a]



2a. X= N, Y= N, R= Cl **3a, 4a, 6a, 8a**: X= N, Y= N
2b. X=CH, Y= N, R= Br **3b, 4b, 6b, 8b**: X=CH, Y= N

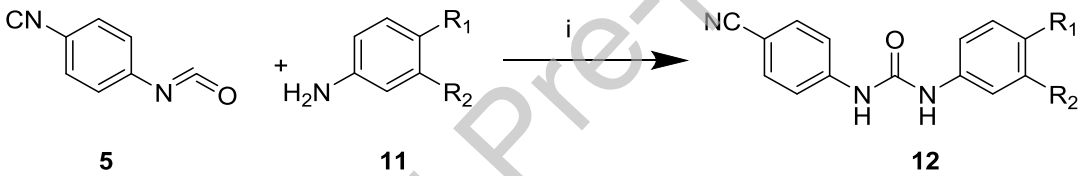
[a]Reagents and conditions: (i) Na_2CO_3, $Pd(PPh_3)_4$, DME, 80 ^oC, 8-12h; (ii) $SnCl_2{\cdot}2H_2O$, DCM:MeOH=1:1, 0^oC-rt, 8-12h for **4a**; or $SnCl_2{\cdot}2H_2O$, EtOAc: EtOH=1:1, reflux, 6-8h for **4b**; (iii) DCM, 0 ^oC -rt, 3h; (iv) DMF, K_2CO_3, 120 ^oC

Scheme 2: Synthesis Route for Diaryl Urea **10**



Reagents and conditions: (i) DCM, 0 ^{o}C- rt., 3h.

Scheme 3: Synthesis Route for Diaryl Urea **12**



11a, 12a: R_1 = H, R_2 = $NCOCH_3$; 11**b, 12b**: R_1 = $N(CH_3)_2$, R_2 = H

Reagents and conditions. (i). Dichloromethane, 0 ^{o}C- RT, 2h

General Procedure A for the synthesis of diarylurea compounds (**6a-6b, 10a-10b, 12a-12b**). To the solution of 1.5 mmol of amine in anhydrous DCM (5 − 8 mL) was added the selected isocyanate **5** (1.8 mmol, 1.2 equiv.) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min and then at room temperature for 2 h-4 h. The reaction was monitored by TLC (30% acetone in hexane or 50 − 70% ethyl acetate in hexane). After completion of the reaction, the suspension was filtered. The filtered solid was further washed with dichloromethane (2 mL) and diethyl ether (5 mL) successively and then dried in a vacuum oven to provide the desired compounds.

2-Chloro-4-(3-nitrophenyl)pyrimidine (3a). In a 3 neck round bottomed flask, argon gas was bubbled through a mixture of 3-nitrophenylboronic acid (**1**, 2.68 g, 16.10 mmol), 2,4-dichloropyrimidine (**2a**, 2 g, 13.42 mmol), Na_2CO_3 (4.26 g, 40.26 mmol), dimethoxyethane (80 mL), and H_2O (7 mL) for 20-25 min. Then the palladium catalyst $Pd(PPh_3)_4$ (1.54 g, 1.34 mmol) was added, and the reaction mixture was refluxed for 12 hours and monitored by TLC. Upon completion of the reaction, the mixture was allowed to cool to room temperature and was filtered through a small Celite pad. The filtrate was washed with water (2 X 20 mL), and the organic compound was extracted with ethyl acetate (3 × 30 mL). The combined organic phase was washed with water, brine and dried over Na_2SO_4. Filtration and removal of solvent *in vacuo* provided the crude product, which was purified by silica gel Combiflash chromatography (0-70% dichloromethane in hexane) to afford the compound **3a** (1.88 g, 59.8%) as a white solid; mp 147−149 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.95 (t, *J*= 1.8 Hz, 1H), 8.78 (d, *J*= 5.4 Hz, 1H), 8.52 (dt, *J*= 7.8, 0.9 Hz, 1H), 8.42 (ddd, *J*= 8.1, 2.1, 0.9 Hz, 1H), 7.74-7.79 (m, 2H). MS (ESI): m/z = 236.015$[M+H]^+$.

2-Chloro-6-(3-nitrophenyl)pyridine (3b). The compound **3b** was synthesized from 3-nitrophenylboronic acid (952 mg, 5.75 mmol), 2-bromo-6-chloropyridine **2b** (850 mg, 4.42 mmol), Na_2CO_3 (1.39 g, 13.26 mmol), $Pd(PPh_3)_4$ (508 mg, 0.44 mmol), dimethoxyethane (25 mL), and H_2O (2.3 mL) according to the procedure described for compound **3a**. The crude compound was purified by Combiflash chromatography (0- 30 % ethyl acetate in hexane) to afford the compound **3b** (540 mg, 52.2%) as white solid; mp 128- 129 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.86 (t, *J* = 1.9 Hz, 1H, CH), 8.40 (dt, *J* = 7.8, 1.1 Hz, 1H, CH), 8.31 (ddd, *J* = 8.2, 2.1, 0.8 Hz, 1H, CH), 7.66-7.84 (m, 3H, CH), 7.38 (dd, *J* = 7.5, 1.0 Hz, 1H, CH). MS (ESI): m/z = 235.020$[M+H]^+$

3-(2-Chloropyrimidin-4-yl)aniline (4a). The 3-nitrophenyl)pyrimidine **3a** (815mg, 3.46 mmol) in the mixture of DCM and methanol (1:1) was added $SnCl_2{\cdot}2H_2O$ (4.68g, 20.76 mmol) and stirred at 0 ^{o}C for 1 h and then at room temperature. The reaction was monitored by TLC (30% ethyl acetate in hexane). Upon completion of the reaction, it was cooled to room temperature and condensed *in vacuo*. It was then

treated with saturated $NaHCO_3$ solution (60 mL) and the solid precipitated out. The solid was filtered under vacuum and the filtrate was extracted with ethyl acetate (2 X 40 mL). The organic layer was washed with water, brine and dried over anhydrous Na_2SO_4. Filtration and removal of solvent provided the crude solid, which was purified using silica gel Combiflash chromatography (0-30 % ethyl acetate in hexane) to provide **4a** (348 mg, 48 %) as a light yellow solid; mp 101-105 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.63 (d, J = 6.0 Hz, 1H, CH), 7.63 (d, J = 5.3 Hz, 1H, CH), 7.50 (t, J = 3.0 Hz, 1H, CH), 7.40 (dt, J = 9.0, 3.0 Hz, 1H, CH), 7.31 (d, J = 9.0 Hz, 1H, CH), 6.86 (ddd, J = 7.8, 2.4, 0.9 Hz, 1H, CH), 3.9 (s, 2H, NH). MS (ESI): m/z = 206.041$[M+H]^+$.

3-(6-Chloropyridin-2-yl)aniline (4b). The compound 3-nitrophenyl)pyridine **3b** (100 mg, 0.43 mmol) in the mixture of EtOAc and EtOH (1:1) was added with $SnCl_2.2H_2O$ (298.3, 2.98 mmol) at room temperature. The reaction mixture was then refluxed for 6-8 h and monitored by TLC (30% ethyl acetate in hexane). Upon completion of the reaction, the reaction mixture was cooled to room temperature and condensed *in vacuo* and treated with saturated $NaHCO_3$ solution (60 mL) and filtered. The filtrate was then extracted with ethyl acetate (2 x 40 mL). The combined organic layer was washed with water, brine and dried over anhydrous Na_2SO_4. Filtration and removal of solvent provided the crude solid, which was purified using silica gel Combiflash chromatography (0-30% ethyl acetate in hexane) to provide **4b** (68 mg, 77 %) as a light yellow solid; mp 76–80 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62-7.73 (m, 2H, CH), 7.42 (t, J = 1.8 Hz, 1H, CH), 7.23-7.34 (m, 3H, CH), 6.77(dd, J = 7.8, 1.2 Hz, 1H, CH), 3.84 (brs, 2H, NH). MS (ESI): m/z = 205.045$[M+H]^+$.

1-(3-(2-Chloropyrimidin-4-yl)phenyl)-3-(4-cyanophenyl)urea (6a, LDK1321). The compound **6a** was synthesized from amine **4a** (308.46 mg, 1.5 mmol), and the 4-cyanophenyl isocyanate **5** (259.43 mg, 1.8 mmol) in anhydrous DCM according to general procedure A. The crude compound was purified using Combiflash chromatography (0-50% ethyl acetate in hexane) to provide product **6a** (302 mg, 57%) as a white solid; mp 241-243 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.79 (d, J= 5.4 Hz, 1H), 8.73 (s, 1H),

8.63 (s, 1H), 8.41 (t, *J* = 1.9 Hz, 1H), 8.01(d, *J* = 5.4 Hz, 1H), 7.68-7.88 (m, 6H), 7.52 (t, *J* = 7.9 Hz, 1H). MS (ESI): m/z = 350.073$[M+H]^+$.

1-(3-(6-Chloropyridin-2-yl)phenyl)-3-(4-cyanophenyl)urea (6b, LDK1324). The compound **6b** was synthesized from amine **4b** (145 mg, 0.71 mmol), and 4-cyanophenyl isocyanate **5** (122.5 mg, 0.85 mmol) in anhydrous DCM according to general procedure A. The crude compound was purified using flash chromatography (0-50% ethyl acetate in hexane) to provide product **6b** (180 mg, 72%) as a white solid; mp 180-184 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.20 (s, 1H, NH), 9.13 (s, 1H, NH), 8.19 (t, *J* = 1.8 Hz, 1H, CH), 7.96 (d, *J* = 3.3 Hz, 1H, CH), 7.95 (s, 1H, CH), 7.57 - 7.76 (m, 6H, CH), 7.45 (dd, *J* = 5.9, 2.4 Hz, 1H, CH), 7.42-7.47 (m, 1H, CH). MS (ESI): m/z = 349.078$[M+H]^+$.

1-(4-Cyanophenyl)-3-(3-(2-((2-hydroxyethyl)amino)pyrimidin-4-yl)phenyl)urea (8a, LDK1323). The solution of **6a** (50mg, 0.14 mmol) in anhydrous DMF (2.5 mL) was added to ethanolamine **7** (17.71 mg, 0.29 mmol) and K_2CO_3 (40 mg, 0.29 mmol). The reaction mixture was stirred and heated at 110 °C for 8 hours. The reaction was monitored by TLC (10% methanol in dichloromethane). Upon completion of the reaction, it was cooled to room temperature and quenched by adding 30 mL of water and extracted with ethyl acetate (3 x 12mL). The organic layer was washed with water, brine and dried over anhydrous Na_2SO_4. Filtration and removal of the solvent *in vacuo* provided the crude compound. The crude compound was purified using Combiflash chromatography (0-10% MeOH in dichloromethane) to provide the product **8a** (37 mg, 70%) as a light yellow solid; mp 189-193 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.27 (s, 1H), 9.04 (s, 1H), 8.35 (d, *J* = 5.1 Hz, 1H), 8.18 (s, 1H), 7.76 - 7.64 (m, 6H), 7.43 (t, *J* = 6.0 Hz, 1H), 7.07 (d, *J* = 4.5 Hz, 1H), 4.71 (t, *J*= 5.4 Hz, 1H), 3.59 (brs, 2H), 3.42 (brs, 2H). MS (ESI): m/z = 375.149 $[M+H]^+$.

1-(4-Cyanophenyl)-3-(3-(6-((2-hydroxyethyl)amino)pyridin-2-yl)phenyl)urea (8b, LDK1325). The compound **8b** was synthesized from **6b** (100 mg, 0.29 mmol) and ethanolamine **7** (35 mg, 0.58 mmol) in anhydrous DMF (5 mL) in the presence of K_2CO_3 (80 mg, 0.58 mmol) according to the procedure for **8a**.

The crude compound was purified by Combiflash chromatography (0-10% MeOH in DCM) to provide product **8b** (25 mg, 23%) as a light yellow solid; mp 225-230 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.46 (s, 1H, NH), 9.14 (s, 1H, NH), 8.04 (s, 1H, CH), 7.32-7.75 (m, 8H), 6.97 (d, J = 6.0 Hz, 1H), 6.56 (t, J = 5.1 Hz, 1H), 6.46 (d, J = 8.3 Hz, 1H), 4.76 (t, J = 5.2 Hz, 1H, OH), 3.59 (brs, 2H), 3.42 (brs, 2H). MS (ESI): m/z = 374.154 $[M+H]^+$.

1-(4-Cyanophenyl)-3-(1*H*-indol-6-yl)urea (10a, LDK1319). The compound **10a** was synthesized from amine **9a** (50 mg, 0.38 mmol), and 4-cyanophenyl isocyanate **5** (65 mg, 0.45 mmol) in anhydrous dichloromethane according to the general procedure A. The pure product was obtained as a white solid (97 mg, 92%); mp 235-238 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.97 (s, 1H), 9.73 (s, 1H), 8.74 (s, 1H), 7.62-7.78 (m, 5H), 7.42 (d, J = 8.4 Hz, 1H), 7.24 (s, 1H), 6.86 (dd, J = 8.5, 1.7 Hz, 1H), 6.35 (s, 1H). MS (ESI): m/z = 277.101$[M+H]^+$.

1-(4-Cyanophenyl)-3-(1H-indol-5-yl)urea (10b, LDK1318). The compound **10b** was synthesized from amine **9b** (50 mg, 0.38 mmol), and 4-cyanophenyl isocyanate **5** (66 mg, 0.46 mmol) in anhydrous dichloromethane according to the general procedure A. The pure product was obtained as a white solid (96 mg, 91.4%); mp 238-240 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ10.99 (s, 1H, NH), 9.11 (s, 1H, NH), 8.60 (s, 1H, NH), 7.62-7.73 (m, 5H, CH), 7.30-7.33 (m, 2H, CH), 7.08 (dd, J = 8.7, 1.6 Hz, 1H, CH), 6.36 (s, 1H, CH). MS (ESI): m/z = 277.101$[M+H]^+$.

***N*-(3-(3-(4-Cyanophenyl)ureido)phenyl)acetamide (12a, LDK1317).** The biphenyl urea **12a** was synthesized from *N*-(3-aminophenyl)acetamide **11a** (0.050 g, 0.33 mmol) and 4-isocyanatobenzonitrile **5** (0.056 g, 0.39 mmol) in 8 mL of – anhydrous dichloromethane according to the general procedure A. The crude product was purified by trituration with diethyl ether to provide **12a** (85 mg, 87.5%) as a yellow solid; mp 240-243 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.94 (s, 1H), 9.10 (s, 1H), 8.90 (s, 1H), 7.77 (s, 1H), 7.72 (d, J = 8.55 Hz, 2H), 7.62(d, J = 8.55 Hz, 2H), 7.20-7.16 (m, 3H), 2.02 (s, 3H). MS (APCI): m/z = 295.11 ($M+H^+$).

1-(4-Cyanophenyl)-3-(4-(dimethylamino)phenyl)urea (12b, LDK1320). The biphenyl urea **12b** was synthesized from dimethyl aniline **11b** (0.1 g, 0.73 mmol) and 4-isocyanatobenzonitrile **5** (0.115 g, 0.80 mmol) in 10 mL of dichloromethane according to the general procedure A. The crude product was purified by multiple titrations with diethyl ether to provide **12b** (85 mg, 87.5%) as a white solid; mp 219-221 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.06 (s, 1H), 8.49 (s, 1H), 7.59-7.77 (m, 4H), 7.25 (d, J = 8.94 Hz, 2H), 6.70 (d, J = 9.03 Hz, 2H), 2.83 (S, 6H). MS (APCI): m/z = 281.13 ($M+H^+$).

Receptor Expression and Membrane Preparation

Human embryonic kidney 293T (HEK293T) cells were seeded at 1,000,000 cells/ 100-mm plate, and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and 3.5 mg/mL glucose. They were incubated at 37 ˚C with 5% carbon dioxide. The next day, cells were transfected with 20 µg of human CB_1 receptor cloned into pcDNA3.1, using the calcium phosphate method.[26] Transfected cells were harvested, and the membranes prepared as previously described,[27] 21 hours after transfection.

Equilibrium Binding Assays

CB_1-expressing membrane preparations (5 µg) were incubated with nine concentrations of the allosteric modulator (1 nM-10 µM). In each reaction, [^{3}H]CP55,940 (150.2 Ci/mmol; Perkin Elmer), a radiolabeled tracer which is an orthosteric agonist of CB_1, was also added at 0.5 nM. Or, for select allosteric compounds (LDK1321, LDK1323, or LDK 1324), [^{3}H]SR141716A (56 Ci/mmol; Perkin Elmer), a radiolabeled tracer which is an orthosteric inverse agonist of CB_1, was added at a concentration of 1 nM instead of the orthosteric agonist. Nonspecific binding was determined by treatment of the membranes with 10 µM of either untritiated CP55,940 (Tocris) or untritiated SR141716A (Tocris). Membranes were incubated for 60 minutes, at 30^{o}C. The reaction was terminated by the addition of 300 µL of TME (Tris-Mg2+-EDTA) buffer with 5% bovine serum albumin (BSA). Harvesting of the mixture was performed with a Brandel cell

harvester with Whatman GF/C filter paper. Measurement of bound radioactivity was performed using liquid scintillation counting.

Data Analysis

The binding data collected were subjected to nonlinear regression, fitted to a log (dose) versus response curve to determine the EC_{50} using Prism 7.02 (Graphpad Software, LaJolla, CA). Binding analysis was performed in the graphs as the mean ± S.E. (error bars) and summarized in Table 1 as the corresponding 95% confidence limits. The physicochemical properties including solubility were obtained from computational prediction using the ChemAxon program (Chemicalize, SanDiego, CA).

Results and Discussion

The compounds reported in this study (Figure 1) were generated to explore whether reducing the number of rings within the scaffold of PSNCBAM-1 was possible and to identify lead compounds with improved drug-like properties. Eight analogs of PSNCBAM-1 and the parent compound were tested using equilibrium binding for their ability to enhance binding of the CB_1 agonist CP55,940. For comparison, PSNCBAM-1 was tested and consistent with the literature value,[22] K_B= 55 nM. The cooperativity factor, α = 3.6, indicates positive cooperativity with agonist CP55,940 (Figure 2A, Table 1).

All eight analogs also featured a cyano group which was previously shown to enhance the allosteric properties of PSNCBAM-1-derived compounds.[22] Of these derivatives, three compounds displayed no binding: LDK1318 (10b), LDK1319 (10a), and LDK1325 (8b). The compounds LDK1318 (10b) and LDK1319 (10a) were structurally similar in that both featured an indole ring (Figure 1). LDK 1323 (8a) and LDK1325 (8b) featured an ethanolamine attached to the pyrimidine or pyridine ring, respectively (Figure 1). All of these compounds were optimized from PSNCBAM-1 with reduced number of rings (two or three rings), which is preferred for therapeutic agents used in the CNS.[24]

Compounds LDK1321 (6a) and LDK1324 (6b) were designed to retain the three aromatic rings of PSNCBAM-1 while its pyrrolidinyl ring was removed. LDK1321 (6a) showed a K_B = 85 nM and α = 5.9 suggesting that this compound retained key features of receptor modulation of PSNCBAM-1 (Table 1). LDK1324 (6b) maintained the pyridine ring PSNCBAM-1 and also featured a chlorine substituent (Figure 1), and enhanced binding of CP55,940 (Figure 2B). In the CP55,940 binding experiments, the K_B = 300 nM and the cooperativity factor, α = 5.6, indicative of positive cooperativity (Table 1). While the K_B is higher than that of the parent compound,

PSNCBAM-1 (55 nM), the cooperativity factor for LDK1324 (6b) is greater than one, indicating that this compound maintains the ability to positively enhance the binding of CP55,940 (Table 1).

LDK1320 (12b) featured a dimethylamino group which replaced the two rings (i.e. the pyridine ring and the pyrrolidine ring) (Figure 1). LDK1320 (12b) enhanced binding of agonist CP55,940 (Figure 2C), and showed positive cooperativity, as indicated by its $\alpha = 5.4$ (Table 1). However, the $K_B = 830$ nM, thus the binding affinity is weaker than PSNCBAM-1 (Table 1). Replacement of the pyridine ring (Figure 1) with an acetamide group maintains some enhancement of binding of CP55,940 as with LDK1317 (12a), which exhibited with a $K_B = 110$ nM and an $\alpha = 2.3$ (Figure 2D, Table 1). Results from testing these two compounds indicate that diarylureas which possess only two aromatic rings may still be capable of binding the allosteric site and cause positive binding cooperativity.

Compound LDK1323 (8a) featured a pyrimidine ring in place of the pyridine ring of the parent compound, and also had an ethanolamine group attached to the pyrimidine ring (Figure 1). This compound has a modest, but positive impact on the binding of agonist CP55,940 (Figure 2E). The compound in this series with both the lowest K_B (85 nM) and the highest cooperativity factor (5.9) was LDK1321 (6a) (Table 1). This compound, like LDK1323 (8a), has a pyrimidine ring instead of the pyridine ring of PSNCBAM-1, but with a chlorine replacing the pyrrolidine group (Figure 1), and was successful in enhancing the binding of agonist CP55,940 (Figure 2F).

Select compounds were also tested for their ability to decrease the binding of the inverse agonist SR141716A. LDK1323 (8a) displayed a modest decrease in SR141716A binding, while LDK1321 (6a) and LDK1324 (6b) demonstrated a robust, dose-dependent decrease of SR141716A binding (Figure 3A-C). All compounds with this orthosteric inverse agonist had K_B

values in the micromolar range (1.4, 6.8. and 1.8 μM for LDK1321 (6a), LDK1323 (8a), and LDK1324 (6b), respectively), and cooperativity factors of less than 1, which is indicative of negative binding cooperativity (Figure 3). This negative cooperativity of binding with an inverse agonist is a characteristic of other positive allosteric modulators of CB_1, including PSNCBAM-1.[14,15] Since the positive allosteric modulator stabilizes CB_1 in an activated form that enhances CP55,940 binding, one would expect the modulator to have a lower affinity for an inverse agonist (e.g. SR141716A) in its presence relative to its absence and a negative cooperativity factor. As one would expect, SR141716A binding is decreased as one employs a higher concentration of allosteric modulator (Figure 3A-C).

For those where there is binding, it is established by the binding assay to CP55,940 with allosteric modulator that there is positive cooperativity. That is expected for an activated receptor. That the CP55,940 (agonist) binding has an alpha factor greater than 1.0 argues that the allosteric modulator activates the receptor and therefore is positive allosteric modulator (PAM)-like. That SR141716A (inverse agonist) binds an activated receptor less well and the alpha factor is less than one agrees with that.[28]

In order to investigate whether the structural optimization improves the drug-like properties, the compounds shown in Table 1 were assessed with a computational program for their drug-like properties and solubility. The results are shown in Table 2. Compound LDK1317 (**12a)** (K_B= 110 nM, α= 2.3) and LDK 1321 (**6a)** (K_B= 85 nM, α= 5.9) can serve as lead compounds for further development of allosteric modulators from the diarylurea scaffold. Based on the computationally calculated drug-like properties of these molecules in Table 2, reducing the number of rings within the structures of the diarylurea analogs makes these satisfy the Lipinski rule of five and leads to improvement of the distribution constant (LogD) and intrinsic

solubility of the compounds (Table 2). The calculated LogD values obtained in two different pH conditions (pH 7.4 and pH 1.7) indicates that the synthesized compounds could be ionized in acidic media (e.g. the stomach) except compounds LDK1319 (**10a)** and LDK1318 (**10b)**. The calculated LogD and LogP values obtained at pH=7.4 are identical. This indicated that the compounds are in neutral unionized forms in aqueous media at pH 7.4 (e.g. the blood). Introducing ethanolamine into compounds LDK1321 (**6a)** and LDK1324 (**6b)** enhanced the calculated intrinsic solubility (i.e. LDK1323 (**8a)** and LDK1325 (**8b)**) by approximately 20-fold with the cost that LDK1325 (**8b)** lost its binding affinity for the allosteric site. It is noteworthy that reducing the number of rings of diarylurea analogs to 2 significantly enhanced intrinsic solubility by about 400-fold (ie. PSNCBAM-1, 0.000203 mg/mL vs LDK1320 (**12b)**, 0.0873 mg/mL, Table 2).

Conclusions

In this preliminary study, it was found that that reducing the number of rings within the scaffold of PSNCBAM-1 is a viable approach to generate novel lead compounds for developing allosteric modulators of the CB_1 receptor. A fewer number of rings likely holds the key for improving the drug-like properties and solubility. By continuing structure-activity relationship studies based on the allosteric modulator scaffold of PSNCBAM-1, we can develop new lead compounds with improved drug-like properties that target the pharmacologically important CB_1 receptor.

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Conflict of Interest

The authors have no conflicts of interest regarding the content of this article.

References

1. Fredriksson R, Lagerström MC, Lundin L-G, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol.* 2003;63(6):1256-1272.
2. Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon, P, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* 1995;232(1):54-61.
3. Pertwee RG. The therapeutic potential of drugs that target cannabinoid receptors or modulate the tissue levels or actions of endocannabinoids. *AAPS J.* 2005;7(3):E625-E654.
4. Rinaldi-Carmona M, Barth F, Héaulme M, Shire D, Calandra B, Congy C et al. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.* 1994;350:240-244.
5. Galve-Roperh I, Aguado T, Palazuelos J, Guzmán M. The endocannabinoid system and neurogenesis in health and disease. *Neuroscientist.* 2007;13(2):109-114.
6. Rinaldi-Carmona M, Pialot F, Congy C, Redon E, Barth F, Bachy A, et al. Characterization and distribution of binding sites for [3H]-SR141716A, a selective brain (CB1) cannabinoid receptor antagonist, in rodent brain. *Life Sci.* 1996;58(15):1239-1247.
7. MacLennan S, Reynen P, Kwan J, Bonhaus D. Evidence for inverse agonism of SR141716A at human recombinant cannabinoid CB1 and CB2 receptors. *Br J Pharmacol.* 1998;124(4):619-622.

8. Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol.* 1988;34(5):605-613.

9. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science.* 1992;258(5090):1946-1949.

10. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, et al. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol.* 1995;50(1):83-90.

11. Pacher P, Bátkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev.* 2006;58(3):389-462.

12. Walker JM, Huang SM, Strangman NM, Tsou K, Sañudo-Peña MC. Pain modulation by release of the endogenous cannabinoid anandamide. *Proc Natl Acad Sci U.S.A.* 1999;96(21):12198-12203.

13. Porter AC, Felder CC. The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacol Ther.* 2001;90(1):45-60.

14. Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, et al. Allosteric modulation of the cannabinoid CB_1 receptor. *Mol Pharmacol.* 2005;68(5):1484-1495.

15. Horswill J, Bali U, Shaaban S, Keily JF, Jeevaratnam P, Babbs AJ, et al. PSNCBAM- 1, a novel allosteric antagonist at cannabinoid CB1 receptors with hypophagic effects in rats. *Br J Pharmacol.* 2007;152(5):805-814.

16. Christopoulos A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov.* 2002;1(3):198-210.

17. Conn PJ, Christopoulos A, Lindsley CW. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov.* 2009;8(1):41-54.

18. Burford N, Traynor J, Alt A. Positive allosteric modulators of the μ- opioid receptor: a novel approach for future pain medications. *Br J Pharmacol.* 2015;172(2):277-286.

19. Gao Z-G, Jacobson KA. Allosteric modulation and functional selectivity of G protein-coupled receptors. *Drug Discov Today Technol.* 2013;10(2):237-243.

20. Gregory KJ, Noetzel MJ, Niswender CM. Chapter Two - Pharmacology of Metabotropic Glutamate Receptor Allosteric Modulators: Structural Basis and Therapeutic Potential for CNS Disorders. In: Kenakin T, ed. *Progress in Molecular Biology and Translational Science.* Vol 115: Academic Press; 2013:61-121.

21. German N, Decker AM, Gilmour BP, Gay EA, Wiley JL, Thomas BF, et al. Diarylureas as allosteric modulators of the cannabinoid CB1 receptor: structure–activity relationship studies on 1-(4-Chlorophenyl)-3-{3-[6-(pyrrolidin-1-yl) pyridin-2-yl] phenyl} urea (PSNCBAM-1). *J Med Chem.* 2014;57(18):7758-7769.

22. Khurana L, Fu B-Q, Duddupudi AL, Liao Y-H, Immadi SS, Kendall, DA et al. Pyrimidinyl biphenylureas: identification of new lead compounds as allosteric modulators of the cannabinoid receptor CB1. *J Med Chem.* 2017;60(3):1089-1104.

23. Bertini S, Chicca A, Gado F, Arena C, Nieri D, Digiacomo M, et al. Novel analogs of PSNCBAM-1 as allosteric modulators of cannabinoid CB1 receptor. *Bioorg Med Chem.* 2017; 25(24):6427-6434.

24. Ghose AK, Herbertz T, Hudkins RL, Dorsey BD, Mallamo JP. Knowledge-Based, Central Nervous System (CNS) Lead Selection and Lead Optimization for CNS Drug Discovery. *ACS Chem Neurosci.* 2012;3(1):50-68.

25. Lipinski CA. Lead-and drug-like compounds: the rule-of-five revolution. *Drug Discov Today Technol.* 2004;1(4):337-341.

26. Chen C, Okayama H. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol.* 1987;7(8):2745-2752.

27. Qiao C-J, Ali HI, Ahn KH, Kolluru S, Kendall DA, Lu D. Synthesis and biological evaluation of indole-2-carboxamides bearing photoactivatable functionalities as novel allosteric modulators for the cannabinoid CB1 receptor. *Eur J Med Chem.* 2016;121:517-529.

28. Jagla CAD, Scott CE, Tang Y, et al. Pyrimidinyl Biphenylureas Act as Allosteric Modulators to Activate Cannabinoid Receptor 1 and Initiate β-Arrestin–Dependent Responses. *Mol Pharmacol.* 2019;95(1):1-10.

Figure Legends

Figure 1

Structures of PSNCBAM-1 and the analogs tested for allosteric modulator binding.

Figure 2

The influence of allosteric modulators on orthosteric agonist [^{3}H]CP55940 specific binding to CB_1. Shown are binding assays with [^{3}H]CP55940 as a tracer with varying concentrations of (A) PSNCBAM-1, (B) LDK1324 (6b), (C) LDK1320 (12b), (D) LDK1317 (12a), (E) LDK1323 (8a), and (F) LDK1321 (6a). Results determined from at least three experiments performed in duplicate, and data are presented as the mean ± S.E. (error bars).

Figure 3

The influence of allosteric modulators on orthosteric inverse agonist [^{3}H]SR141716A specific binding to CB_1. Shown are equilibrium binding assays with [^{3}H]SR141716A as a tracer with varying concentrations of (A) LDK1321 (6a), (B) LDK1323 (8a), and (C) LDK1324 (6b). Results are determined from at least three experiments performed in duplicate, and data are presented as the mean ± S.E. (error bars).

Table 1. Binding parameters of PSNCBAM-1 analogs.

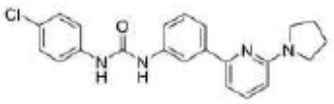
Compound code	K_B (nM)[a]	α[b]
PSNCBAM-1	55 (26-120)	3.6 (2.6-6.1)
6a, LDK1321	85 (41-180)	5.9 (2.8-12)
6b, LDK1324	300 (150-630)	5.6 (3.4-9.4)
8a, LDK1323	200 (19-1,900)	2.4 (1.4-4.2)
8b, LDK1325	NB[c]	NB[c]
10a, LDK1319	NB[c]	NB[c]
10b, LDK1318	NB[c]	NB[c]
12a, LDK1317	110 (24-490)	2.3 (1.6-3.3)
12b, LDK1320	830 (240-3,000)	5.4 (2.7-11)

[a] K_B: equilibrium dissociation.
[b] α: cooperativity factor for the allosteric modulator tested.
The allosteric parameters, K_B and α, were determined using [^{3}H]CP55,940 as the orthosteric ligand. K_B and α values are given with 95% confidence intervals in parentheses.
[c] NB: no detectable binding of the orthosteric agonist [^{3}H]CP55,940 in the presence of the test compound up to 10 μM.

Table 2. Calculated physicochemical properties and solubility of the synthesized analogs of PSNCBAM-1.[a]

Compound Code	Rings	Lipinski Rule of Five satisfaction	Log D (pH = 7.4)	Log D (pH = 1.7)	Log P (pH = 7.4)	Intrinsic Solubility[b] (pH = 7.4)
PSNCBAM-1	4	No	5.64	3.69	5.64	0.000203 mg/mL
6a, LDK1321	3	Yes	3.99	3.97	3.99	0.000207 mg/mL
6b, LDK1324	3	Yes	4.61	4.59	4.61	0.000219 mg/mL
8a, LDK1323	3	Yes	2.55	1.14	2.55	0.00381 mg/mL
8b, LDK1325	3	Yes	3.15	1.21	3.15	0.00402 mg/mL
10a, LDK1319	3	Yes	0.94	0.94	0.94	0.00758 mg/mL
10b, LDK1318	3	Yes	0.94	0.94	0.94	0.00758 mg/mL
12a, LDK1317	2	Yes	0.08	0.08	0.08	0.0493 mg/mL
12b, LDK1320	2	Yes	0.95	0.99	0.95	0.0873 mg/mL

[a]The parameters were obtained from computational prediction using the ChemAxon program.
[b]The intrinsic solubility is the equilibrium solubility of the compound at the pH where it is fully unionized.



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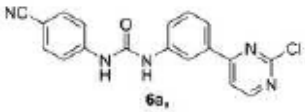

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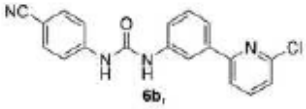
LDK1321



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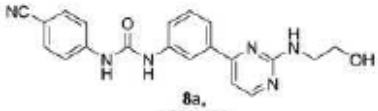

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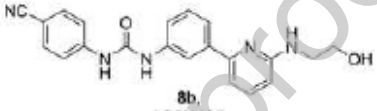

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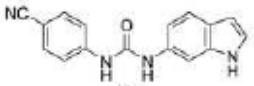

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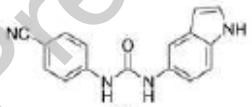

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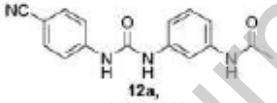

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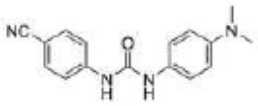

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