Journal of Medicinal Chemistry

ACS AuthorChoice

Article

pubs.acs.org/jmc

Terms of Use

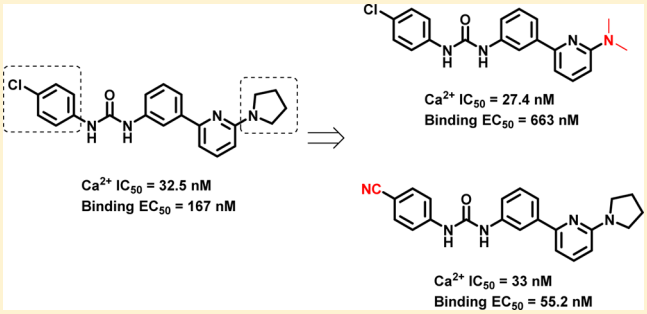
Diarylureas as Allosteric Modulators of the Cannabinoid CB1 Receptor: Structure−Activity Relationship Studies on 1-(4-Chlorophenyl)-3-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]phenyl}urea (PSNCBAM-1)

Nadezhda German, Ann M. Decker, Brian P. Gilmour, Elaine A. Gay, Jenny L. Wiley, Brian F. Thomas, and Yanan Zhang*

Research Triangle Institute, Research Triangle Park, North Carolina 27709, United States

Supporting Information

ABSTRACT: The recent discovery of allosteric modulators of the CB1 receptor including PSNCBAM-1 (**4**) has generated significant interest in CB1 receptor allosteric modulation. Here in the first SAR study on **4**, we have designed and synthesized a series of analogs focusing on modifications at two positions. Pharmacological evaluation in calcium mobilization and binding assays revealed the importance of alkyl substitution at the 2-aminopyridine moiety and electron deficient aromatic groups at the 4-chlorophenyl position for activity at the CB1 receptor, resulting in several analogs with comparable potency to **4**. These compounds increased the specific binding of [^{3}H]CP55,940, in agreement with previous reports. Importantly, **4** and two analogs dose-dependently reduced the E_{max} of the agonist curve in the CB1 calcium mobilization assays, confirming their negative allosteric modulator characteristics. Given the side effects associated with CB1 receptor orthosteric antagonists, negative allosteric modulators provide an alternative approach to modulate the pharmacologically important CB1 receptor.



INTRODUCTION

Cannabinoid CB1 and CB2 receptors are G-protein-coupled receptors (GPCRs) and are key components of the endocannabinoid system.[1−3] The CB1 receptor is one of the most highly expressed GPCRs in the central nervous system (CNS) and plays a role in retrograde neuronal signaling and attenuation of neurotransmitter release. The CB2 receptor is primarily located in immune cells, regulating cell differentiation and migration. The CB1 receptor in particular is involved in many disorders, such as obesity, mental illness, pain, multiple sclerosis, smoking, and drug addiction, and has therefore been considered a promising target for the treatment of these pathological conditions.[4−6] A plethora of selective and nonselective ligands for the CB1 receptor have been developed, some of which are used extensively as research tools.[7−10] However, use of CB1 agonists has been associated with marijuana-like psychoactivity and clinical trials have raised concerns that CB1 antagonists could promote a state of depression and anxiety.[11,12]

An alternative strategy for regulating GPCRs has recently emerged which involves the allosteric binding site, one that is topographically distinct from the orthosteric site. Allosteric modulators are ligands that bind to these sites to alter the receptor signaling properties of the orthosteric ligand, changing ligand affinity, functional efficacy, and functional potency.[13−16] Compared to traditional orthosteric drugs, allosteric modulators may offer several unique advantages. First, allosteric modulators may exhibit greater subtype selectivity because of the higher sequence divergence at extracellular allosteric binding sites,[17] in contrast to the often conserved orthosteric domains for certain GPCR subtypes. Second, allosteric modulators may have tissue selectivity, exerting effects only where endogenous ligands are present. Finally, the effect of allosteric modulators is saturable because of their dependence on endogenous ligands for signaling.[18,19]

Several allosteric ligands for the CB1 receptor, including both small molecules and peptides, have been recently reported.[20−22] Among them, two series, the Org compounds (Org27569, -27759, and -29647; **1**−**3**) and PSNCBAM-1 (**4**) (Figure 1), have been more widely studied.[23−28] Interestingly, the pharmacological profile of these allosteric modulators is complex. While these compounds all enhanced the specific binding of the CB1 agonist [^{3}H]CP55,940, they behaved as negative allosteric modulators, or allosteric antagonists, in a number of functional assays including reporter gene, GTP-γ-S, and mouse vas deferens assays.[23,24,29,30] Subsequently, using a site-directed fluorescence labeling (SDFL) approach, Fay et al.

Received: July 10, 2014
Published: August 27, 2014

   dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

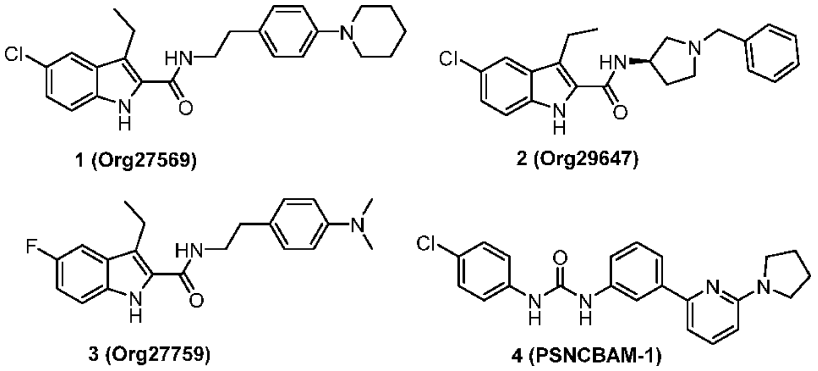


Figure 1. Structures of reported allosteric modulators of the CB1 receptor.

suggested that **1** binding to the CB1 receptor produced a unique agonist-bound conformation, one that does not lead to protein binding and/or activation, therefore behaving as a negative modulator in functional assays.[31] Compound **4** caused noncompetitive antagonism in [^{35}S]GTP-γ-S binding studies.[24,29,30] In electrophysiological studies, **4** pretreatment revealed agonist-dependent functional antagonism, abolishing CP55,940-induced reductions in miniature inhibitory postsynaptic currents (mIPSCs) frequency.[29] When tested in vivo, **4** reduced feeding and body weight in rats in an acute feeding study, confirming its allosteric antagonist character.[24] These findings indicate that the negative modulation of the CB1 receptor may provide an alternative strategy to regulate the endocannabinoid system and hence may represent a valid approach for medication development for the treatment of CB1 receptor mediated diseases.[26]

More recently, structure−activity relationship (SAR) studies have been reported by several groups on the Org compounds.[28,32−34] To the best of our knowledge, no such study has been published on **4**. Here, we report our initial SAR studies on compound **4** and the pharmacological evaluation of its analogs in calcium mobilization and radioligand binding assays. We have focused on two main structural areas: the 2-pyrrolidinyl position on the pyridine and the 4-chlorophenyl group (Figure 2).

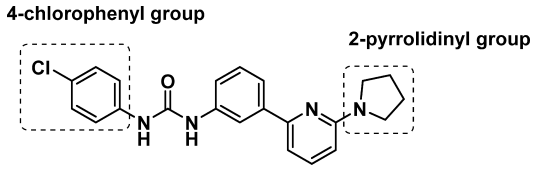


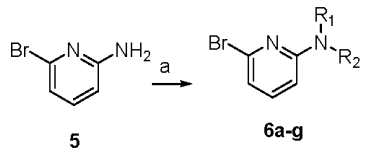
Figure 2. Proposed structural modifications on **4**.

RESULTS AND DISCUSSION

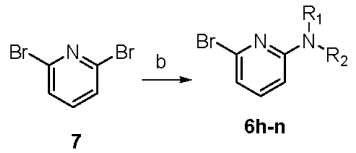
Chemistry. Compounds **4** and **10−45** were synthesized following procedures depicted in Scheme 1 and Scheme 2. 2,6-Disubstituted pyridines **6a−g** were prepared by reacting 2-amino-6-bromopyridine (**5**) with either acetic anhydride (**6a**) or the corresponding aldehydes (**6b−g**) in the presence of sodium triacetoxyborohydride using 1,2-dichloroethane as a solvent.[35,36] The synthesis of intermediates **6h−n** consisted of displacement of one of the bromides of 2,6-dibromopyridine (**7**) by refluxing in the appropriate amine.[37,38]

Suzuki coupling reactions of intermediates **6a−n** with 3-nitrophenylboronic acid under standard conditions in the

Scheme 1. Synthesis of Intermediates 6a−n[a]



6a: R1=H, R2=C(O)CH3
6b: R1=H, R2=n-Pr
6c: R1=n-Pr, R2=Pr
6d: R1=H, R2=n-Bu
6e: R1=n-Bu, R2=n-Bu
6f: R1=H, R2=Pentyl
6g:R1=H, R2=Hexyl



6h: R1, R2 =pyrrolidine
6i: R1=H, R2=Me
6j: R1=Me, R2=Me
6k: R1=H, R2=Et
6l: R1=Et; R2=Et
6m: R1,R2 = piperidine
6n: R1=H; R2=cyclopentyl

[a]Reagents and conditions: (a) acetic anhydride, DCM, rt, 16 h (for **6a**) or corresponding aldehyde, $Na(OAc)_3BH$, acetic acid, 1,2-DCE; (b) corresponding amine, reflux, 15 min to 3 h.

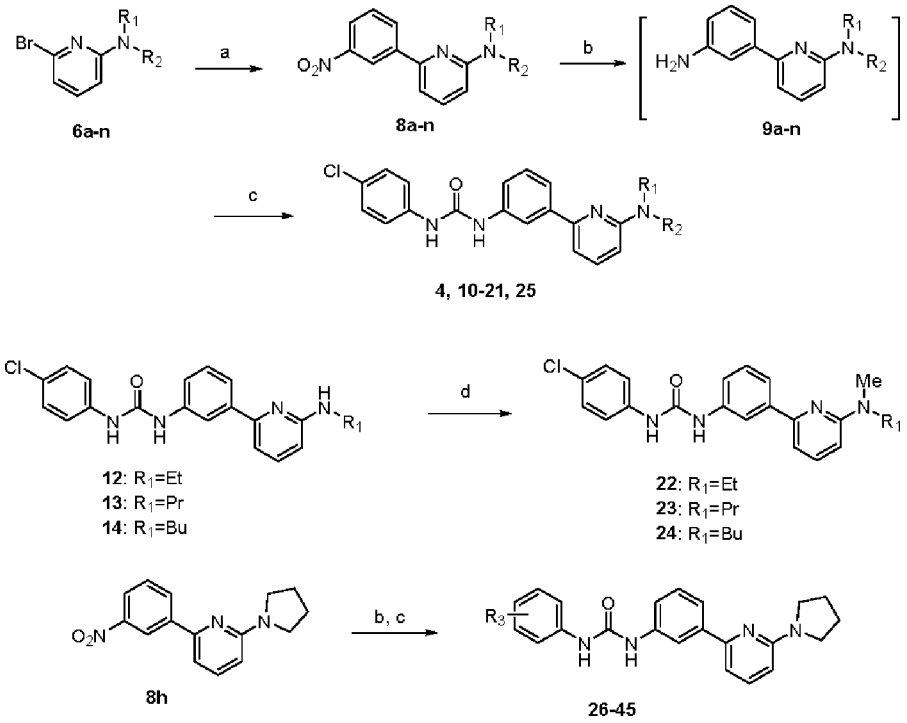
presence of $Pd(PPh_3)_4$ afforded compounds **8a−n**.[39,40] Reduction of intermediates **8a−n** with hydrazine hydrate and Raney nickel in ethanol at 50 °C provided intermediates **9a−n**, followed by final coupling of **9a−n** with the corresponding phenyl isocyanates in chloroform to give the final ureas **4**, **10−21**, and **25**.[36] Compounds **22−24** were obtained through methylation of the corresponding monoalkyl derivatives **12−14**, respectively, using reductive amination with formaldehyde. Compounds **26−45** were synthesized from intermediates **8h** using the same two-step procedure in the synthesis of **4**.

Pharmacological Evaluations. FLIPR-based CB1 and CB2 calcium mobilization assays were developed in our laboratories to characterize the target compounds. In these assays, CHO cells that have been engineered to overexpress the promiscuous G protein, $G\alpha_{16}$ (Molecular Devices), were further engineered to also stably express the CB1 and CB2 receptors, respectively. Therefore, activation of the CB1 or CB2 receptors, which are primarily coupled to $G\alpha_{i/o}$ proteins, is now coupled to the mobilization of internal calcium through the $G\alpha_{16}$ protein. These assays have shown strong correlation with other CB1 and CB2 assays such as radioligand binding assays and are routinely used in our laboratories.[41−43] These assays were also used previously by our group to characterize the CB1 receptor modulator RTI-371.[20]

In the CB1 calcium assay, **4** dose-dependently reduced the E_{max} values of CP55,940, as expected for negative allosteric modulators (Figure 3). **1** displayed a similar dose-dependent reduction in E_{max} (unpublished results). These results confirm that the CB1 calcium assay used here is a suitable system for evaluating allosteric modulators. The potencies of the

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

Scheme 2. Synthesis of Compounds 4 and 10−45[a]



[a]Reagents and conditions: (a) 3-nitrophenylboronic acid, $Pd(PPh_3)_4$, DME, $NaHCO_3$, reflux, 12 h; (b) hydrazine hydrate, Raney Ni, 50 °C, ethanol, 2 h; (c) corresponding isocyanate, chloroform, rt or 55 °C, 16 h; (d) formaldehyde (37% aq), $Na(OAc)_3BH$, acetic acid, 1,2-DCE.

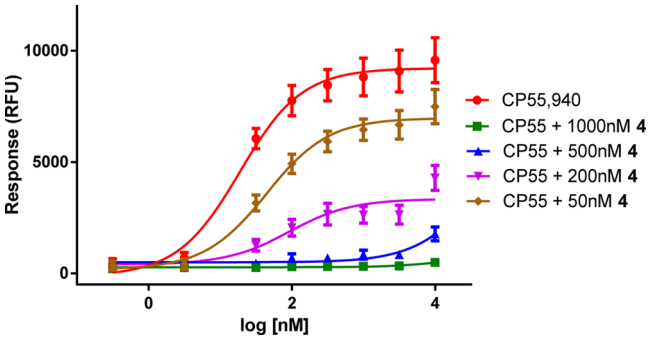


Figure 3. Allosteric modulation of **4** in CB1 Ca^{2+} assay.

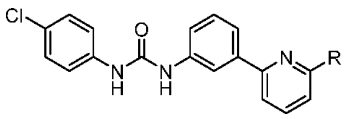
synthesized compounds at the CB1 receptor were obtained by calculating IC_{50} values for attenuation of the effects of the EC_{80} concentration of the CB1 agonist CP55,940 (Table 1). In our assays, the EC_{80} of CP55,940 averaged 100 nM. Under these conditions, **4** had IC_{50} = 32.5 nM, consistent with its potency in other assays.[24,29]

The SAR studies focused on two main structural areas of compound **4**: the 2-pyrrolidinyl position on the pyridine and the 4-chlorophenyl group. Specifically, the modifications on the pyrrolidinyl portion of the molecule were designed in order to understand the spatial requirements, as well as the necessity of the ring's presence in this region. The first analog examined was the piperidyl analog **10**. This ring expanded analog showed slightly decreased potency (~3-fold) relative to **4**. Next, a series of ring opened analogs were investigated. These efforts resulted in a series of compounds bearing various dialkyl and monoalkyl substituents on the nitrogen atom (Table 1). The diethylamino analog **12**, the ring opened analog of compound **4**, showed ~4-fold lower potency (125 nM vs 32.5 nM). Interestingly, while increasing the size of the alkyl groups to propyl and butyl resulted in further reduced potency (**13**, **14**), the smaller dimethyl analog **11** showed comparable potency to **4** (27.4 nM vs 32.5 nM). These results suggested that a ring system is not required for activity at the CB1 receptor in this series. In the *N*-monoalkyl series (**15**−**21**), potency first increases and then decreases with elongation of the alkyl substituent, except for the propyl analog **17** (IC_{50} = 311 nM). The *N*-butyl analog **18** showed the highest potency among the series (93 nM). The cyclopentyl analog (**21**) showed similar potency to that of pentyl (**19**). Comparison of the monoalkyl and dialkyl series suggests that the presence of the *N*-hydrogen is tolerated for activity. Moreover, the determining factor for CB1 activity appears to be the size of the *N*-alkyl substituent(s), as the basicity of the nitrogen remained little changed (e.g., pK_a = 3.83 (**4**) vs 3.55 (**12**) vs 3.83 (**16**), as calculated in ACD Labs). For instance, the *N*-butyl group (**18**) is similar in size to the *N*-diethyl (**12**) and they had similar potency at the CB1 receptor. To further investigate the size requirement at this position, a set of mixed alkyl analogs was examined (**22**−**24**). In these series, one of the alkyl groups is a methyl and the second substituent is an ethyl (**22**), propyl (**23**), or butyl (**24**). As expected, the smaller ethyl group showed higher potency (IC_{50} = 53.7 nM) than the propyl and butyl derivatives. Taken together, these data suggest the binding pocket prefers substitution on the nitrogen but has a limited space for size. While an NH is tolerated with another alkyl substituent of the appropriate size, the optimal substitution pattern includes a methyl group and an alkyl substituent with one to four carbons in length.

Compound **25**, the last compound of the series, was used to investigate the effect of an electron withdrawing group. Inclusion of an acetamide group (as in compound **25**) decreased CB1 antagonistic potency by roughly 8-fold when

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

Table 1. Compounds 4 and 10−25 and Inhibition of CP55,940 Activity at CB1 and CB2 Receptors



Compound	R	CB1		CB2
		IC_{50} (nM)[a]	Agonist Screen[b] (% CP55,940 E_{max})	IC_{50} (nM)[d]
4		32.5 ± 7.5	28.2 ± 4.8	> 10,000
10		95 ± 18	35 ± 18	> 10,000
11	-NMe_2	27.4 ± 5.9	c	e
12	-NEt_2	125 ± 19	c	e
13	-NPr_2	372 ± 61	29 ± 13	e
14	-NBu_2	693 ± 45	c	e
15	-NHMe	317±120	c	e
16	-NHEt	181 ± 25	c	e
17	-NHPr	311 ± 64	19.6 ± 1.7	e
18	-NHBu	93 ± 31	26.5 ± 0.9	e
19	-NHPentyl	867 ± 30	c	e
20	-NHHexyl	1360 ± 28	c	e
21		501 ± 150	35 ± 14	> 10,000
22	-NMeEt	53.7 ± 9.9	c	e
23	-NMePr	156 ± 44	c	e
24	-NMeBu	104 ± 22	22.1 ± 0.9	e
25		251 ± 37	47 ± 11	5120±1790

[a]Against EC_{80} (100 nM) of CP55,940. Values are the mean ± SEM of at least three independent experiments in duplicate. [b]Agonist screen at 10 000 nM. Values are the mean ± SEM of at least two independent experiments in duplicate. [c]Activity was less than 15% of CP55,940 E_{max} at maximum concentration tested (10 000 nM). [d]Against EC_{80} (100 nM) of CP55,940. Values are the mean ± SEM of at least two independent experiments in duplicate. [e]Less than 35% inhibition of CP55,940 EC_{80} (100 nM) at concentration of 10 000 nM.

compared to the parent compound **4**. Compound **25** also had slightly reduced potency in comparison to **22** with an ethyl group that is similar in size, suggesting that electron donating alkyl groups are preferred at this position.

All compounds were also tested for their antagonist activity at the CB2 receptor, and the percent inhibition of CP55,940 activity at this receptor is reported (Table 1). Most of the compounds showed little or no antagonism. For those compounds that exhibited over 35% inhibition, IC_{50} values against the EC_{80} concentration (100 nM) of CP55,940 at the CB2 receptor were obtained. None had an IC_{50} lower than 10 000 nM (Table 1). All compounds were also screened for agonist activity at both CB1 and CB2 receptors. At the CB1 receptor, none of the compounds showed activity greater than 35% of the CB1 agonist CP55,940 E_{max} when tested at 10 000 nM. Similarly, no compounds displayed agonist activity greater than 15% of the CP55,940 E_{max} at the maximum concentration tested (10 000 nM) at the CB2 receptor.

The next set of compounds was designed to examine the effects of substitution pattern on the 4-chlorophenyl portion of the parent compound **4** (Table 2). First, the phenyl analog (**26**), bearing no substituents, showed a 5-fold decrease in activity, suggesting that substitution at the 4-position is preferred for specific interactions with the binding pocket. To identify effects caused by electronic properties of the 4-substituent, we tested a series of compounds (**27**−**33**) bearing groups with a range of electronic characteristics. Electron withdrawing groups at the 4-position provided good potency, with the fluoro (**27**, IC_{50} = 32 nM) and the cyano analogs (**29**, IC_{50} = 33 nM) being the most active of the series, showing potency similar to **4**. Introduction of electron-donating moieties (**31**−**33**) led to reduced potency, although the reduction was only modest (5- to 6-fold). The greatest loss of potency was observed for dimethylamino analog **33** which had an IC_{50} of 1640 nM. Together, these results suggest that functional groups with electron withdrawing properties are favored at the 4-position for activity at the CB1 receptor.

We next investigated whether substitutions at other positions are tolerated. The importance of 4-phenyl substitution was immediately confirmed by moving the chloro group to position 3, where compound **40** showed a roughly 7-fold drop in activity. In addition, **41**, bearing chloro groups at the 3 and 4 positions, showed higher potency (IC_{50} = 161 nM) than **42** (IC_{50} = 716 nM) with its 3,5-dichloro substitutions. These data highlighted the importance of 4-position substitution for CB1 modulatory activity. Interestingly, none of the compounds with substitutions at the 2-position (**37**−**39**) showed activity when tested at concentrations up to 10 000 nM. While electronic properties may contribute to the loss of activity, the presence of the 2-methyl group most likely forces the structure into a nonplanar conformation which may not be favored for interaction with the binding site.

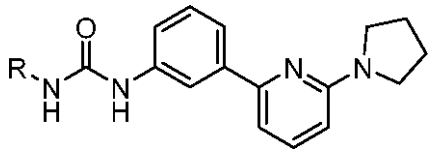
To probe the effect of substitution patterns on activity, we synthesized a series of disubstituted and trisubstituted analogs, bearing either electron withdrawing or donating functionalities. The disubstituted analogs had substitutions at the 3,4-, 3,5- and 2,6-positions with groups such as chloro, methyl, or methoxy. Among these, the 3,4-disubstitution pattern showed the highest potency (e.g., **41** vs **42**), but potencies were still lower than those of the corresponding 4-substituted analogs (**41** vs **4**). Within the same substitution pattern, electron withdrawing groups showed higher potency than the electron donating groups (**42** vs **36** and **41** vs **34**), where the dimethyl analog **36** showed ∼2-fold decreased activity compared to the dichloro analog **42**. Clearly, these data confirmed that electronic characteristics of substituents play an important role in CB1 receptor binding and activation. The trisubstituted compound **35** showed little activity.

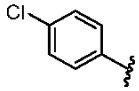
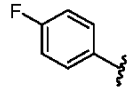
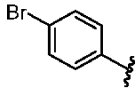
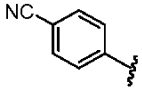
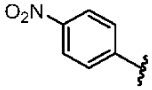
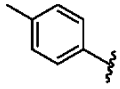
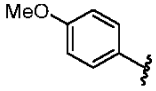
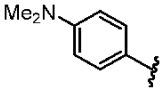
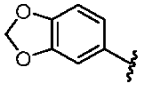
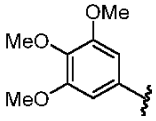
Finally, other aromatic systems were investigated. For instance, replacing the phenyl group with the electron-deficient pyridine moiety (**43**) led to a slight decline in activity, suggesting the possibility of introducing heterocyclic structures in this part of the molecule. Next, larger naphthyl groups (**44**, **45**) were used to probe possible spatial constraint of the binding pocket. The 2-naphthyl analog (**45**) showed a significant decline in activity, and the 1-naphthyl analog (**44**) had an almost total loss of activity. These results suggest that rigid bulky structures are not well tolerated at this position. Moreover, these observations are in agreement with the earlier results in the 2-substituted analogs (**37**−**39**), as the 1-naphthyl analog is most likely to adopt a more nonplanar conformation than the 2-naphthyl because of steric repulsion.

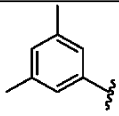
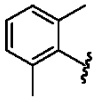
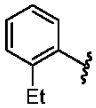
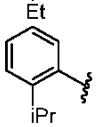
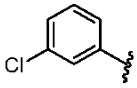
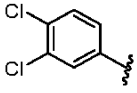
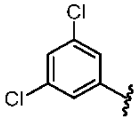
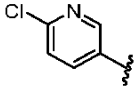
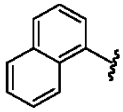
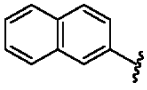
Again, all the compounds were screened at the CB2 receptor for antagonist activity, most of which showed minimal inhibition (Table 2). IC_{50} values were obtained for compounds that showed over 35% inhibition at 10 000 nM, and none showed IC_{50} values less than 10 000 nM. All compounds were also screened for agonist activity at both the CB1 and CB2 receptors. No significant agonist activity was observed for any

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

Table 2. Compounds 26−45 and Their Activity at the CB1 and CB2 Receptors



Compound	R	CB1		CB2
		IC_{50} (nM)[a]	Agonist Screen[b] (% CP55,940 E_{max})	IC_{50} (nM)[d]
4		32.5 ± 7.5	28.2 ± 4.8	e
26		166 ± 65	c	e
27		32 ± 11	23.2 ± 4.6	e
28		98 ± 30	c	e
29		33 ± 10	30.0 ± 4.8	> 10,000
30		100 ± 25	27.9 ± 5.6	e
31		203 ± 22	c	e
32		189 ± 35	c	e
33		1640 ± 610	c	e
34		312 ± 76	c	e
35		> 10,000	c	> 10,000

Compound	R	CB1		CB2
		IC_{50} (nM)[a]	Agonist Screen[b] (% CP55,940 E_{max})	IC_{50} (nM)[d]
36		1880 ± 590	c	e
37		> 10,000	c	e
38		> 10,000	c	e
39		> 10,000	c	e
40		228 ± 40	19.6 ± 9.2	e
41		161 ± 20	c	> 10,000
42		716 ± 18	22.2 ± 5.4	> 10,000
43		135 ± 46	c	e
44		> 10,000	c	e
45		888 ± 130	c	e

[a]Against EC_{80} (100 nM) of CP55,940. Values are the mean ± SEM of at least three independent experiments in duplicate. [b]Agonist screen at 10 000 nM. Values are the mean ± SEM of at least two independent experiments in duplicate. [c]Activity was less than 15% of CP55,940 E_{max} at maximum concentration tested (10 000 nM). [d]Against EC_{80} (100 nM) of CP55,940. Values are the mean ± SEM of at least two independent experiments in duplicate. [e]Less than 35% inhibition of CP55,940 EC_{80} (100 nM) at concentration of 10 000 nM.

of the compounds at either receptor (<30% and 15% of CP55,940 E_{max} at the CB1 and CB2 receptors, respectively).

Two compounds (**11** and **29**) were further evaluated for their allosteric modulation as compared to **4**. As shown in Figure 4, both compounds were able to dose-dependently lower the top of the agonist curve of CP55,940, confirming their allosteric characteristics.

A total of seven compounds that showed good potency in the calcium assay were also evaluated at the CB1 receptor in competitive radioligand binding assays against [^{3}H]CP55,940 in hCB1 cell membranes. The binding experiments on two of the compounds, **4** and **29**, are shown in Figure 5. As expected, SR141716 dose-dependently displaced the radioligand [^{3}H]-CP55,940. Similar to previously described CB1 receptor allosteric modulators, all the compounds caused significant and concentration-dependent enhancement in the binding of [^{3}H]CP55,940 (Table 3). Horswill et al. previously reported that compound **4** dose-dependently increased [^{3}H]CP55,940 binding by 58% with an EC_{50} of 14.4 nM.[24] In our hands, compound **4** had an EC_{50} of 167 nM, with an increase of E_{max} of ∼74% (E_{max} = 174%, Table 3). Compounds **11** and **27** both had similar potencies to **4** in the calcium assay. Interestingly, **11** showed a 4-fold decrease in potency, whereas **27** had almost identical EC_{50} values in the binding assay. Compounds **18** and

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

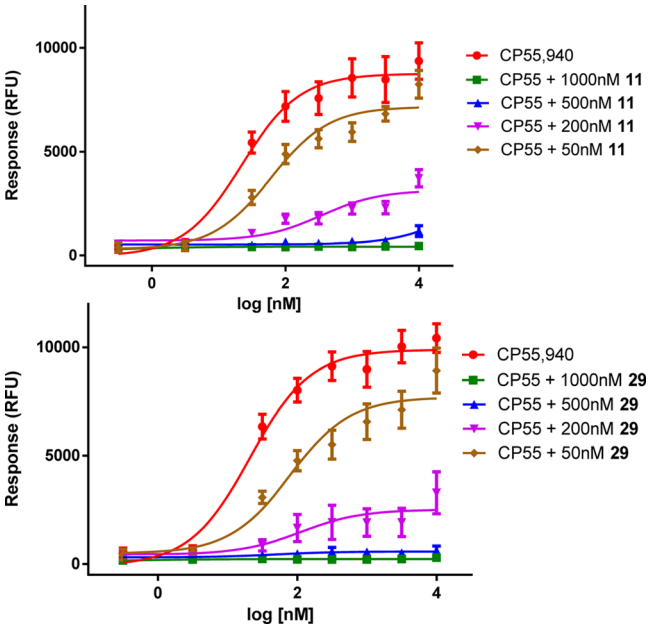


Figure 4. Allosteric modulation of compounds **11** (top) and **29** (bottom) in CB1 Ca^{2+} assay.

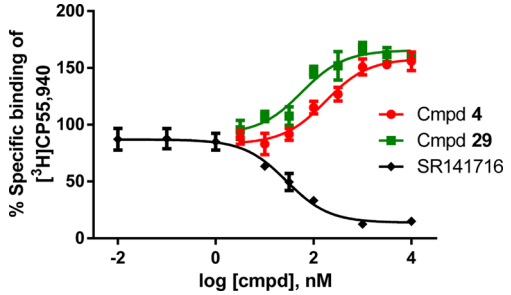


Figure 5. Competitive binding studies on compounds **4** and **29**.

Table 3. Radioligand Binding Studies at the CB1 Receptor against [^{3}H]CP55,940

compd	EC_{50} (nM)[a]	E_{max} (% specific binding)[a]
4	167 ± 37	174 ± 8
10	*b*	132 ± 6
11	663 ± 92	184 ± 3
18	1520 ± 630	161 ± 10
27	207 ± 62	172 ± 3
29	55.2 ± 2.8	172 ± 3
43	408 ± 127	196 ± 5

[a]Values are the mean ± SEM of at least three independent experiments in duplicate. [b]EC_{50} could not be obtained because of the limited E_{max} enhancement (~32%).

43 both had lower potency than compound **4** in binding enhancement, in line with the calcium results. Finally, the 4-cyano analog **29** had the highest potency among the series (EC_{50} = 55.2 nM) in increasing [^{3}H]CP55,940 binding, with a 3-fold increase in potency compared to **4**.

CONCLUSIONS

A number of recently discovered allosteric modulators of the CB1 receptor display opposing pharmacological properties, with both positive (in radiolabeled binding studies against [^{3}H]CP55,940) and negative (in several functional assays) modulation.[23,24] Results of this first SAR study of **4** revealed that a series of analogs of **4** showed a similar complex pharmacological profile, enhancing [^{3}H]CP55,940 binding while antagonizing activity of the agonist CP55,940 in the calcium assays. Our initial SAR studies showed that a cyclic system at the 2-pyrrolidylpyridine position was not required for activity. Moreover, tertiary amine substitution was more favorable than secondary and the optimal pattern for substitution was obtained when one of the alkyl groups was methyl and the other was one to four carbons in length. The most active compound of the series was the dimethyl analog **11**, which showed almost identical potency to **4** in the calcium assay and 4-fold deceased potency in the binding enhancement of [^{3}H]CP55940. At the 4-chlorophenyl position, our data suggest that the 4-position on the phenyl group tolerates structural modifications but favors electron withdrawing functionalities. Compound **29** with a 4-cyano group showed the highest potency in both CB1 calcium mobilization and radioligand binding assays.

4 has been shown to possess hypophagic activity in vivo, but its therapeutic potential has yet to be fully explored. Our study further supports the earlier finding that **4** acts as a noncompetitive, allosteric antagonist of the CB1 receptor.[29,30] Importantly, **4** has shown no adverse or toxic effects after administration in vivo,[24] as might be expected with allosteric modulators because of their dependence on endogenous ligands for signaling. Given the recent adverse effects observed with orthosteric antagonists, these allosteric antagonists represent a promising alternative to modulate CB1 function and may serve as much needed tools to identify alternative treatment strategies with reduced CNS side effects involving the CB1 receptor system.

EXPERIMENTAL SECTION

Chemistry. All solvents and chemicals were reagent grade. Unless otherwise mentioned, all reagents and solvents were purchased from commercial vendors and used as received. Flash column chromatography was carried out on a Teledyne ISCO CombiFlash Rf system using prepacked columns. Solvents used include hexane, ethyl acetate (EtOAc), dichloromethane, methanol, and chloroform/methanol/ammonium hydroxide (80:18:2) (CMA-80). Purity and characterization of compounds were established by a combination of HPLC, TLC, mass spectrometry, and NMR analyses. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-300 (300 MHz) spectrometer and were determined in chloroform-*d*, DMSO-d_6, or methanol-d_4 with tetramethylsilane (TMS) (0.00 ppm) or solvent peaks as the internal reference. Chemical shifts are reported in ppm relative to the reference signal, and coupling constant (*J*) values are reported in hertz (Hz). Thin layer chromatography (TLC) was performed on EMD precoated silica gel 60 F254 plates, and spots were visualized with UV light or iodine staining. Low resolution mass spectra were obtained using a Waters Alliance HT/Micromass ZQ system (ESI). All test compounds were greater than 95% pure as determined by HPLC on an Agilent 1100 system using an Agilent Zorbax SB-Phenyl, 2.1 mm × 150 mm, 5 μm column with gradient elution using the mobile phases (A) H_2O containing 0.1% CF_3COOH and (B) MeCN, with a flow rate of 1.0 mL/min.

1-(4-Chlorophenyl)-3-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (4). Hydrazine hydrate (3.2 mL, 66 mmol) was added to a suspension of **8h** (1 g, 4.4 mmol) in ethanol (50 mL). The mixture was stirred at 50 °C for 15 min, and a clear solution was obtained. An excess of Raney nickel (~1 g) was added portionwise. After the bubbling ceased, the mixture was cooled to room temperature and filtered. The filtrate was condensed to afford intermediate **9h** (0.6 g), which was used in the next step without further purification.

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

4-Chlorophenyl isocyanate (19 mg, 0.12 mmol) was added to a stirred solution of **9h** (30 mg, 0.12 mmol) in anhydrous chloroform (5 mL). The reaction mixture was stirred overnight at room temperature. The formed precipitate was filtered and thoroughly washed with dichloromethane. Final product **4** was obtained as an off-white solid (20 mg, 45%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.82 (d, J = 11.87 Hz, 2H), 8.08 (s, 1H), 7.43−7.71 (m, 5H), 7.23−7.40 (m, 3H), 7.06 (d, J = 7.35 Hz, 1H), 6.42 (d, J = 8.29 Hz, 1H), 3.48 (br s, 4H), 1.97 (br s, 4H).

***N*-(6-Bromopyridin-2-yl)acetamide (6a).** Acetic anhydride was added to a solution of the commercially available 2-amino-6-bromopyridine (**5**, 0.3 g, 1.73 mmol) in DCM (7 mL), and the reaction mixture was stirred at room temperature for 16 h. Upon completion reaction mixture was diluted with DCM, washed with saturated sodium carbonate, dried over $MgSO_4$, and concentrated under reduced pressure. Final product was obtained as a white solid (0.36 g, 97%). ^{1}H NMR (300 MHz, chloroform-d) δ 8.15 (d, J = 8.10 Hz, 1H), 7.56 (t, J = 7.91 Hz, 1H), 7.21 (d, J = 7.72 Hz, 1H), 2.20 (s, 3H).

6-Bromo-*N*-propylpyridin-2-amine (6b). A solution of propanal (0.16 mL, 2.2 mmol) and 2-amino-6-bromopyridine (**5**, 0.30 g, 1.7 mmol) in 1,2-DCE (10 mL) was treated with $Na(OAc)_3BH$ (0.92 g, 4.3 mmol), and the reaction mixture was stirred at room temperature for 24 h. After completion the reaction was quenched with aqueous NaOH (1 M) and then extracted with DCM (3 × 10 mL). Combined organic layers were washed with water (2 × 15 mL), brine (15 mL), dried over $MgSO_4$, and concentrated under reduced pressure. Crude product was obtained as a clear oil (0.41 g, 92%) and used in the next step without purification. ^{1}H NMR (300 MHz, chloroform-d) δ 7.15−7.35 (m, 1H), 6.63−6.78 (m, 1H), 6.27 (d, J = 8.29 Hz, 1H), 4.71 (br s, 1H), 3.09−3.24 (m, 2H), 1.63 (sxt, J = 7.27 Hz, 2H), 0.98 (t, J = 7.44 Hz, 3H).

6-Bromo-*N*,*N*-dipropylpyridin-2-amine (6c). **6c** was prepared using the procedure for compound **6b** in 46% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.10−7.23 (m, 1H), 6.51−6.67 (m, 1H), 6.30 (d, J = 8.48 Hz, 1H), 3.31−3.41 (m, 4H), 1.51−1.66 (m, 4H), 0.88−0.96 (m, 6H).

6-Bromo-*N*-butylpyridin-2-amine (6d). **6d** was prepared using the procedure for compound **6b** in 41% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.24 (t, J = 7.91 Hz, 1H), 6.70 (d, J = 7.35 Hz, 1H), 6.27 (d, J = 8.10 Hz, 1H), 4.70 (br s, 1H), 3.20 (q, J = 6.78 Hz, 2H), 1.51−1.64 (m, 2H), 1.33−1.46 (m, 2H), 0.94 (t, J = 7.35 Hz, 3H).

6-Bromo-*N*,*N*-dibutylpyridin-2-amine (6e). **6e** was prepared using the procedure for compound **6b** in 43% yield. ^{1}H NMR (300 MHz, chloroform-d) δ ppm 7.13−7.24 (m, 1 H), 6.60 (d, J = 7.35 Hz, 1 H), 6.30 (d, J = 8.38 Hz, 1 H), 3.34−3.46 (m, 4 H), 1.47−1.63 (m, 4 H), 1.27−1.43 (m, 4 H), 0.90−1.02 (m, 6 H).

6-Bromo-*N*-pentylpyridin-2-amine (6f). **6f** was prepared using the procedure for compound **6b** in 38% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (t, J = 1.74 Hz, 1H), 8.31 (d, J = 7.82 Hz, 1H), 8.20 (dd, J = 1.32, 8.19 Hz, 1H), 7.56 (td, J = 7.89, 15.68 Hz, 2H), 7.07 (d, J = 9.70 Hz, 1H), 6.39 (d, J = 9.70 Hz, 1H), 3.35 (q, J = 6.81 Hz, 2H), 1.54−1.75 (m, 2H), 1.21−1.40 (m, 4H), 0.83−1.00 (m, 3H).

6-Bromo-*N*-hexylpyridin-2-amine (6g). **6g** was prepared using the procedure for compound **6b** in 41% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (s, 1H), 8.31 (d, J = 7.72 Hz, 1H), 8.09−8.26 (m, 1H), 7.43−7.69 (m, 2H), 7.08 (d, J = 7.44 Hz, 1H), 6.41 (d, J = 8.19 Hz, 1H), 4.65 (br s, 1H), 3.35 (q, J = 6.59 Hz, 2H), 1.56−1.75 (m, 2H), 1.26−1.49 (m, 6H), 0.91 (t, J = 6.59 Hz, 3H).

2-Bromo-6-(pyrrolidin-1-yl)pyridine (6h). A solution of 2,6-dibromopyridine (**7**, 5g, 21 mmol) in pyrrolidine (30 mL) was heated to reflux for 10 min until no starting material was detected by TLC. Pyrrolidine was removed under reduced pressure and the residue was dissolved in a 30% solution of ethyl acetate in dichloromethane. The organic portion was washed with NaOH (1 M, 20 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by chromatography on silica (0−15% EtOAc in hexane) to give the desired product as a white solid (4.0 g, 83%). ^{1}H NMR (300 MHz, chloroform-d) δ 7.19−7.25 (m, 1H), 6.64 (d, J = 7.16 Hz, 1H), 6.23 (d, J = 8.29 Hz, 1H), 3.43 (t, J = 6.59 Hz, 4H), 1.99 (dd, J = 3.39, 9.80 Hz, 4H).

6-Bromo-*N*-methylpyridin-2-amine (6i). **6i** was prepared using the procedure for compound **6h** in 63% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.23−7.31 (m, 1H), 6.73 (d, J = 7.54 Hz, 1H), 6.29 (d, J = 8.10 Hz, 1H), 4.73 (br s, 1H), 2.90 (d, J = 5.09 Hz, 3H).

6-Bromo-*N*,*N*-dimethylpyridin-2-amine (6j). **6j** was prepared using the procedure for compound **6h** in 87% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.20−7.28 (m, 1H), 6.66 (d, J = 7.54 Hz, 1H), 6.37 (d, J = 8.48 Hz, 1H), 3.06 (s, 6H).

6-Bromo-*N*-ethylpyridin-2-amine (6k). **6k** was prepared using the procedure for compound **6h** in 26% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.24 (t, J = 7.82 Hz, 1H), 6.69 (d, J = 7.44 Hz, 1H), 6.27 (d, J = 8.19 Hz, 1H), 4.74 (br s, 1H), 3.16−3.33 (m, 2H), 1.22 (t, J = 7.16 Hz, 3H).

6-Bromo-*N*,*N*-diethylpyridin-2-amine (6l). **6l** was prepared using the procedure for compound **6h** in 41% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 6.98−7.63 (m, 1H), 6.61 (d, J = 7.35 Hz, 1H), 6.33 (d, J = 8.48 Hz, 1H), 3.48 (q, J = 7.03 Hz, 4H), 1.16 (t, J = 6.97 Hz, 6H).

2-Bromo-6-(piperidin-1-yl)pyridine (6m). **6m** was prepared using the procedure for compound **6h** in 63% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.19−7.25 (m, 1H), 6.67 (d, J = 7.35 Hz, 1H), 6.50 (d, J = 8.48 Hz, 1H), 3.51 (br s, 4H), 1.64 (br s, 6H).

6-Bromo-*N*-cyclopentylpyridin-2-amine (6n). **6n** was prepared using the procedure for compound **6h** in 52% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (s, 1H), 8.30 (d, J = 7.72 Hz, 1H), 8.21 (d, J = 8.10 Hz, 1H), 7.48−7.64 (m, 2H), 7.08 (d, J = 7.54 Hz, 1H), 6.43 (d, J = 8.29 Hz, 1H), 4.71 (br s, 1H), 3.96−4.18 (m, 1H), 2.10 (dd, J = 5.65, 12.06 Hz, 2H), 1.63−1.88 (m, 4H), 1.53−1.55 (m, 2H).

***N*-[6-(3-Nitrophenyl)pyridin-2-yl]acetamide (8a).** **8a** was prepared using the procedure for compound **8h** in 80% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.88 (s, 1H), 8.19−8.32 (m, 3H), 8.05 (br s, 1H), 7.83 (t, J = 8.01 Hz, 1H), 7.60−7.68 (m, 1H), 7.55 (d, J = 7.72 Hz, 1H), 2.28 (s, 3H).

***N*-Propyl-6-(3-nitrophenyl)pyridine-2-amine (8b).** **8b** was prepared using the procedure for compound **8h** in 81% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.90 (s, 1H), 8.36 (d, J = 7.72 Hz, 1H), 8.19 (dd, J = 1.13, 8.10 Hz, 1H), 7.53 (td, J = 8.03, 19.92 Hz, 2H), 7.04 (d, J = 7.35 Hz, 1H), 6.51 (d, J = 8.67 Hz, 1H), 3.38−3.56 (m, 2H), 1.59−1.79 (m, 2H), 0.99 (t, J = 7.44 Hz, 3H).

***N*,*N*-Dipropyl-6-(3-nitrophenyl)pyridine-2-amine (8c).** **8c** was prepared using the procedure for compound **8h** in 80% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.92 (s, 1H), 8.34 (d, J = 7.72 Hz, 1H), 8.19 (dd, J = 1.13, 8.10 Hz, 1H), 7.55 (td, J = 8.03, 19.92 Hz, 2H), 7.04 (d, J = 7.35 Hz, 1H), 6.48 (d, J = 8.67 Hz, 1H), 3.43−3.55 (m, 4H), 1.61−1.78 (m, 4H), 0.99 (t, J = 7.44 Hz, 6H).

***N*-Butyl-6-(3-nitrophenyl)pyridine-2-amine (8d).** **8d** was prepared using the procedure for compound **8h** in 68% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (s, 1H), 8.36 (d, J = 7.91 Hz, 1H), 8.21 (dd, J = 1.32, 8.10 Hz, 1H), 7.58 (q, J = 7.54 Hz, 2H), 7.10 (d, J = 7.35 Hz, 1H), 6.68 (d, J = 8.48 Hz, 1H), 3.18 (t, J = 6.97 Hz, 1H), 1.53 (m, 2H), 1.24−1.41 (m, 2H), 0.81−0.95 (m, 3H).

***N*,*N*-Dibutyl-6-(3-nitrophenyl)pyridine-2-amine (8e).** **8e** was prepared using the procedure for compound **8h** in 73% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.84 (br s, 1H), 8.25 (d, J = 7.54 Hz, 1H), 8.11 (d, J = 7.25 Hz, 1H), 7.32−7.58 (m, 2H), 6.95 (d, J = 7.16 Hz, 1H), 6.39 (d, J = 8.38 Hz, 1H), 3.45 (br s, 4H), 1.57 (br s, 4H), 1.34 (d, J = 6.78 Hz, 4H), 0.92 (t, J = 7.06 Hz, 6H).

***N*-Pentyl-6-(3-nitrophenyl)pyridine-2-amine (8f).** **8f** was prepared using the procedure for compound **8h** in 79% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (t, J = 1.74 Hz, 1H), 8.31 (d, J = 7.82 Hz, 1H), 8.20 (dd, J = 1.32, 8.19 Hz, 1H), 7.56 (td, J = 7.89, 15.68 Hz, 2H), 7.07 (d, J = 9.70 Hz, 1H), 6.39 (d, J = 9.70 Hz, 1H), 3.35 (q, J = 6.81 Hz, 2H), 1.54−1.75 (m, 2H), 1.21−1.40 (m, 4H), 0.83−1.00 (m, 3H).

***N*-Hexyl-6-(3-nitrophenyl)pyridine-2-amine (8g).** **8g** was prepared using the procedure for compound **8h** in 82% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (s, 1H), 8.31 (d, J = 7.72 Hz, 1H), 8.09−8.26 (m, 1H), 7.43−7.69 (m, 2H), 7.08 (d, J = 7.44 Hz,

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

1H), 6.41 (d, J = 8.19 Hz, 1H), 4.65 (br s, 1H), 3.35 (q, J = 6.59 Hz, 2H), 1.56−1.75 (m, 2H), 1.26−1.49 (m, 6H), 0.91 (t, J = 6.59 Hz, 3H).

2-(3-Nitrophenyl)-6-(pyrrolidine-1-yl)pyridine (8h). Nitrogen was bubbled through a mixture of 3-nitrophenylboronic acid (0.81 g, 4.84 mmol), **6h** (1.00 g, 4.4 mmol), and $NaHCO_3$ (1.10 g, 13.20 mmol) in DME (60 mL) and water (25 mL) for 15 min. $Pd(Ph_3)_4$ (0.38 g, 0.33 mmol) was added, and reaction mixture was refluxed overnight under nitrogen atmosphere. Reaction solvent was removed under reduced pressure and the resulting residue was purified by chromatography on silica (0−10% EtOAc in hexane) to give the desired product (1.0 g, 84%). ^{1}H NMR (300 MHz, chloroform-d) δ 7.27−7.46 (m, 3H), 7.06−7.20 (m, 1H), 6.88 (d, J = 7.54 Hz, 1H), 6.61 (d, J = 7.72 Hz, 1H), 6.21 (d, J = 8.29 Hz, 1H), 3.48 (br s, 4H), 1.79−2.04 (m, 4H).

***N*-Methyl-6-(3-nitrophenyl)pyridine-2-amine (8i).** **8i** was prepared using the procedure for compound **8h** in 79% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (s, 1H), 8.32 (d, J = 7.91 Hz, 1H), 8.21 (dd, J = 0.94, 7.16 Hz, 1H), 7.57 (q, J = 7.79 Hz, 2H), 7.10 (d, J = 7.54 Hz, 1H), 6.44 (d, J = 8.29 Hz, 1H), 4.69 (br s, 1H), 3.01 (d, J = 4.90 Hz, 3H).

***N*,*N*-Dimethyl-6-(3-nitrophenyl)pyridine-2-amine (8j).** **8j** was prepared using the procedure for compound **8h** in 85% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.90 (s, 1H), 8.38 (d, J = 7.72 Hz, 1H), 8.21 (d, J = 8.29 Hz, 1H), 7.47−7.70 (m, 2H), 7.09 (d, J = 7.35 Hz, 1H), 6.56 (d, J = 8.48 Hz, 1H), 3.19 (s, 6H).

***N*-Ethyl-6-(3-nitrophenyl)pyridine-2-amine (8k).** **8k** was prepared in using the procedure for compound **8h** in 81% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (t, J = 1.84 Hz, 1H), 8.31 (d, J = 7.82 Hz, 1H), 8.20 (dd, J = 1.32, 8.19 Hz, 1H), 7.56 (td, J = 7.94, 13.99 Hz, 2H), 7.09 (d, J = 7.44 Hz, 1H), 6.41 (d, J = 8.29 Hz, 1H), 4.61 (br s, 1H), 3.34−3.46 (m, 2H), 1.31 (t, J = 7.16 Hz, 3H).

***N*,*N*-Diethyl-6-(3-nitrophenyl)pyridine-2-amine (8l).** **8l** was prepared using the procedure for compound **8h** in 73% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.88 (s, 1H), 8.38 (s, 1H), 8.20 (d, J = 7.91 Hz, 1H), 7.47−7.63 (m, 2H), 7.04 (d, J = 7.35 Hz, 1H), 6.50 (d, J = 8.48 Hz, 1H), 3.61 (q, J = 6.97 Hz, 4H), 1.25 (t, J = 7.06 Hz, 6H).

2-(3-Nitrophenyl)-6-(piperidin-1-yl)pyridine (8m). **8m** was prepared using the procedure for compound **8h** in 67% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.86 (s, 1H), 8.36 (d, J = 7.91 Hz, 1H), 8.21 (dd, J = 1.32, 8.10 Hz, 1H), 7.58 (q, J = 7.54 Hz, 3H), 7.10 (d, J = 7.35 Hz, 1H), 6.68 (d, J = 8.48 Hz, 1H), 3.65 (br s, 4H), 1.69 (s, 6H).

***N*-Cyclopentyl-6-(3-nitrophenyl)pyridine-2-amine (8n).** **8n** was prepared using the procedure for compound **8h** in 65% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 8.85 (s, 1H), 8.30 (d, J = 7.72 Hz, 1H), 8.21 (d, J = 8.10 Hz, 1H), 7.48−7.64 (m, 2H), 7.08 (d, J = 7.54 Hz, 1H), 6.43 (d, J = 8.29 Hz, 1H), 4.71 (br s, 1H), 3.96−4.18 (m, 1H), 2.10 (dd, J = 5.65, 12.06 Hz, 2H), 1.63−1.88 (m, 4H), 1.53−1.55 (m, 2H).

3-(4-Chlorophenyl)-1-{3-[6-(piperidin-1-yl)pyridin-2-yl]-phenyl}urea (10). 10 was prepared from **9m** using the procedure for compound **4** in 42% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.03 (d, J = 9.80 Hz, 2H), 8.03 (s, 1H), 7.44−7.68 (m, 4H), 7.23−7.40 (m, 2H), 7.09 (d, J = 7.54 Hz, 1H), 6.78 (d, J = 8.48 Hz, 1H), 3.61 (m, 4H), 1.61 (m, 6H).

3-(4-Chlorophenyl)-1-{3-[6-(dimethylamino)pyridin-2-yl]-phenyl}urea (11). 11 was prepared from **9j** using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.11−9.52 (m, 2H), 8.11 (s, 1H), 7.42−7.69 (m, 5H), 7.28−7.40 (m, 3H), 7.08 (d, J = 7.35 Hz, 1H), 6.62 (d, J = 8.48 Hz, 1H), 3.11 (s, 6H).

3-(4-Chlorophenyl)-1-{3-[6-(diethylamino)pyridin-2-yl]-phenyl}urea (12). 12 was prepared from **9l** using the procedure for compound **4** in 46% yield. ^{1}H NMR (300 MHz, methanol-d_4) δ 8.06 (t, J = 1.79 Hz, 1H), 7.69 (d, J = 7.72 Hz, 1H), 7.41−7.55 (m, 4H), 7.23−7.38 (m, 3H), 7.02 (d, J = 7.35 Hz, 1H), 6.52 (d, J = 8.48 Hz, 1H), 3.63 (q, J = 7.16 Hz, 4H), 1.23 (t, J = 6.97 Hz, 6H).

3-(4-Chlorophenyl)-1-{3-[6-(dipropylamino)pyridin-2-yl]-phenyl}urea (13). 13 was prepared from **9c** using the procedure for compound **4** in 43% yield. ^{1}H NMR (300 MHz, methanol-d_4) δ 8.11 (s, 1H), 7.68 (d, J = 7.72 Hz, 1H), 7.37−7.53 (m, 4H), 7.17−7.36 (m, 4H), 7.00 (d, J = 7.35 Hz, 1H), 6.47 (d, J = 8.48 Hz, 1H), 3.43−3.58 (m, 4H), 1.58−1.78 (m, 4H), 0.97 (t, J = 7.35 Hz, 6H).

3-(4-Chlorophenyl)-1-{3-[6-(dibutylamino)pyridin-2-yl]-phenyl}urea (14). 14 was prepared from **9e** using the procedure for compound **4** in 54% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.87 (s, 1H), 8.77 (s, 1H), 8.18 (s, 1H), 7.62 (d, J = 7.54 Hz, 1H), 7.51 (d, J = 8.90 Hz, 3H), 7.28−7.43 (m, 4H), 7.01 (d, J = 7.39 Hz, 1H), 6.53 (d, J = 8.52 Hz, 1H), 3.51 (t, J = 7.32 Hz, 4H), 1.47−1.66 (m, 4H), 1.21−1.43 (m, 4H), 0.93 (t, J = 7.32 Hz, 6H).

3-(4-Chlorophenyl)-1-{3-[6-(methylamino)pyridin-2-yl]-phenyl}urea (15). 15 was prepared from **9i** using the procedure for compound **4** in 53% in yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.83 (s, 1H), 8.80 (s, 1H), 7.61 (d, J = 7.72 Hz, 1H), 7.47−7.55 (m, 4H), 7.30−7.38 (m, 3H), 6.99 (d, J = 7.35 Hz, 1H), 6.42 (d, J = 8.19 Hz, 1H), 2.87 (d, J = 4.71 Hz, 3H).

3-(4-Chlorophenyl)-1-{3-[6-(ethylamino)pyridin-2-yl]-phenyl}urea (16). 16 was prepared from **9k** using the procedure for compound **4** in 47% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.10 (s, 1H), 7.76 (s, 3H), 7.63−7.70 (m, 3H), 7.59 (br s, 3H), 7.36 (d, J = 15.82 Hz, 1H), 7.08 (s, 1H), 6.42 (d, J = 8.29 Hz, 1H), 3.41−3.57 (m, 2H), 1.87−2.10 (m, 3H).

3-(4-Chlorophenyl)-1-{3-[6-(propylamino)pyridin-2-yl]-phenyl}urea (17). 17 was prepared from **9b** using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.81 (d, J = 16.77 Hz, 2H), 8.05 (s, 1H), 7.60 (d, J = 7.72 Hz, 1H), 7.39−7.55 (m, 4H), 7.27−7.38 (m, 3H), 6.96 (d, J = 7.35 Hz, 1H), 6.56 (t, J = 5.46 Hz, 1H), 6.43 (d, J = 8.29 Hz, 1H), 1.50−1.69 (m, 2H), 0.96 (t, J = 7.35 Hz, 3H).

3-(4-Chlorophenyl)-1-{3-[6-(butylamino)pyridin-2-yl]-phenyl}urea (18). 18 was prepared from **9d** using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, chloroform-d) δ 7.40 (t, J = 7.82 Hz, 4H), 7.05−7.20 (m, 2H), 6.91 (d, J = 7.35 Hz, 1H), 6.62 (dd, J = 1.51, 6.97 Hz, 1H), 6.24 (d, J = 8.29 Hz, 1H), 3.13−3.30 (m, 2H), 1.46−1.66 (m, 2H), 1.27−1.45 (m, 2H), 0.89 (t, J = 7.35 Hz, 3H).

3-(4-Chlorophenyl)-1-{3-[6-(pentylamino)pyridin-2-yl]-phenyl}urea (19). 19 was prepared from **9f** using the procedure for compound **4** in 57% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.84 (s, 1H), 8.78 (s, 1H), 8.07 (s, 1H), 7.60 (d, J = 7.72 Hz, 1H), 7.38−7.54 (m, 5H), 7.27−7.37 (m, 3H), 6.96 (d, J = 7.35 Hz, 1H), 6.42 (d, J = 8.29 Hz, 1H), 1.51−1.64 (m, 2H), 1.18−1.44 (m, 6H), 0.86 (t, J = 6.50 Hz, 3H).

3-(4-Chlorophenyl)-1-{3-[6-(hexylamino)pyridin-2-yl]-phenyl}urea (20). 20 was prepared from **9g** using the procedure for compound **4** in 40% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.84 (s, 1H), 8.78 (s, 1H), 7.60 (d, J = 7.72 Hz, 1H), 7.40 (s, 4H), 7.28−7.37 (m, 3H), 6.96 (d, J = 7.35 Hz, 1H), 6.53 (t, J = 5.32 Hz, 1H), 6.42 (d, J = 8.29 Hz, 1H), 1.48−1.66 (m, 2H), 1.17−1.45 (m, 8H), 0.86 (t, J = 6.45 Hz, 3H).

3-(4-Chlorophenyl)-1-{3-[6-(cyclopentylamino)pyridin-2-yl]-phenyl}urea (21). 21 was prepared from **9n** using the procedure for compound **4** in 56% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.74 (s, 1H), 8.46 (s, 1H), 7.83 (s, 1H), 7.68 (t, J = 8.10 Hz, 1H), 7.61 (br s, 1H), 7.43 (d, J = 7.91 Hz, 1H), 7.04−7.35 (m, 6H), 6.86 (d, J = 6.78 Hz, 1H), 6.59 (d, J = 9.04 Hz, 1H), 3.85−3.98 (m, 1H), 1.97−2.17 (m, 2H), 1.78−1.93 (m, 2H), 1.60−1.77 (m, 4H).

3-(4-Chlorophenyl)-1-(3-{6-[ethyl(methyl)amino]pyridin-2-yl}phenyl)urea (22). A solution of 37% aqueous formaldehyde solution (0.01 mL, 0.11 mmol) and **12** (0.03 g, 0.082 mmol) in 1,2-DCE (3 mL) was treated with $Na(OAc)_3BH$ (0.063 g, 0.3 mmol), and the reaction mixture was stirred at room temperature for 24 h. After completion the reaction was quenched with aqueous NaOH (1 M) and then extracted with DCM (3 × 20 mL). Combined organic layers were washed with water (2 × 15 mL), brine (15 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The resulting slurry was purified on silica to give **22** (14 mg, 43%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.86 (s, 1H), 8.81 (s, 1H), 8.04−8.13 (m, 1H), 7.44−7.68 (m, 5H), 7.28−7.39 (m, 3H), 7.05 (d, J = 7.25 Hz, 1H), 6.59 (d, J

dx.doi.org/10.1021/jm501042u | J. Med. Chem. 2014, 57, 7758−7769

= 8.48 Hz, 1H), 3.65 (q, *J* = 7.19 Hz, 2H), 3.00−3.08 (m, 3H), 1.12 (t, *J* = 7.02 Hz, 3H).

3-(4-Chlorophenyl)-1-(3-{6-[propyl(methyl)amino]pyridin-2-yl}phenyl)urea (23). **23** was prepared from **13** using the procedure for compound **22** in 41% yield. ^{1}H NMR (300 MHz, chloroform-*d*) δ 7.91 (s, 1H), 7.69 (d, *J* = 6.88 Hz, 1H), 7.37−7.49 (m, 1H), 7.27 (d, *J* = 6.03 Hz, 4H), 7.04−7.15 (m, 5H), 6.88 (d, *J* = 7.35 Hz, 1H), 6.42 (d, *J* = 8.48 Hz, 1H), 3.49 (t, *J* = 7.30 Hz, 2H), 3.08 (s, 3H), 1.54−1.70 (m, 2H), 0.89 (t, *J* = 7.35 Hz, 3H).

3-(4-Chlorophenyl)-1-(3-{6-[butyl(methyl)amino]pyridin-2-yl}phenyl)urea (24). **24** was prepared from **14** using the procedure for compound **22** in 61% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.25 (s, 1H), 7.95 (t, *J* = 8.01 Hz, 1H), 7.60 (d, *J* = 7.72 Hz, 1H), 7.42−7.54 (m, 4H), 7.29 (dd, *J* = 5.56, 8.57 Hz, 5H), 4.01 (t, *J* = 7.25 Hz, 2H), 3.26 (br s, 3H), 1.64 (t, *J* = 6.97 Hz, 2H), 1.27−1.45 (m, 2H), 0.92 (t, *J* = 7.25 Hz, 3H).

***N*-[6-(3-{[(4-Chlorophenyl)carbamoyl]amino}phenyl)pyridin-2-yl]acetamide (25).** **25** was prepared from **9a** using the procedure for compound **4** in 47% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.47 (s, 1H), 8.90 (d, *J* = 15.64 Hz, 2H), 8.15 (s, 1H), 8.03 (d, *J* = 8.10 Hz, 1H), 7.85 (t, *J* = 7.91 Hz, 1H), 7.65 (d, *J* = 7.54 Hz, 1H), 7.29−7.58 (m, 6H), 2.14 (s, 3H).

3-Phenyl-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]phenyl}urea (26). **26** was prepared using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.68 (s, 1H), 8.07 (br s, 1H), 7.51−7.69 (m, 3H), 7.46 (d, *J* = 7.91 Hz, 2H), 7.21−7.39 (m, 3H), 7.06 (d, *J* = 7.35 Hz, 1H), 6.97 (t, *J* = 7.11 Hz, 1H), 6.42 (d, *J* = 8.29 Hz, 1H), 3.48 (br s, 4H), 1.97 (br s, 4H).

3-(4-Fluorophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (27). **27** was prepared using the procedure for compound **4** in 61% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.81 (d, *J* = 9.14 Hz, 2H), 7.96−8.14 (m, 1H), 7.41−7.73 (m, 5H), 7.25−7.41 (m, 1H), 7.13 (s, 3H), 6.30−6.52 (m, 1H), 3.48 (br s, 4H), 1.83−2.10 (m, 4H).

3-(4-Bromophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (28). **28** was prepared using the procedure for compound **4** in 53% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.82 (d, *J* = 12.06 Hz, 2H), 8.07 (s, 1H), 7.51−7.70 (m, 3H), 7.45 (s, 4H), 7.28−7.39 (m, 1H), 7.06 (d, *J* = 7.35 Hz, 1H), 6.42 (d, *J* = 8.29 Hz, 1H), 3.48 (br s, 4H), 1.98 (br s, 4H).

3-(4-Cyanophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (29). **29** was prepared using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.31 (br s, 1H), 9.04 (br s, 1H), 8.10 (s, 1H), 7.68−7.81 (m, 2H), 7.62−7.69 (m, 3H), 7.57 (t, *J* = 7.72 Hz, 2H), 7.31−7.42 (m, 1H), 7.06 (d, *J* = 7.35 Hz, 1H), 6.42 (d, *J* = 8.29 Hz, 1H), 3.40−3.55 (m, 4H), 1.97 (t, *J* = 6.31 Hz, 4H).

3-(4-Nitrophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (30). **30** was prepared using the procedure for compound **4** in 45% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.55 (br s, 1H), 9.10 (br s, 1H), 8.22 (s, 2H), 8.12 (s, 1H), 7.71 (d, *J* = 9.42 Hz, 3H), 7.49−7.62 (m, 2H), 7.30−7.43 (m, 1H), 7.07 (d, *J* = 7.35 Hz, 1H), 6.42 (d, *J* = 8.48 Hz, 1H), 3.48 (br s, 4H), 1.98 (br s, 4H).

3-(4-Methylphenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (31). **31** was prepared using the procedure for compound **4** in 63% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.78 (s, 1H), 8.64 (s, 1H), 8.06 (s, 1H), 7.46−7.74 (m, 3H), 7.20−7.41 (m, 3H), 6.90−7.15 (m, 3H), 6.42 (d, *J* = 8.29 Hz, 1H), 3.48 (br s, 4H), 2.25 (s, 3H), 1.98 (br s, 4H).

3-(4-Methoxyphenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (32). **32** was prepared using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.79 (s, 1H), 8.60 (s, 1H), 8.16 (s, 1H), 7.57−7.79 (m, 3H), 7.34−7.53 (m, 3H), 7.16 (d, *J* = 7.35 Hz, 1H), 6.98 (d, *J* = 8.85 Hz, 2H), 6.52 (d, *J* = 8.29 Hz, 1H), 3.83 (s, 3H), 3.58 (br s, 4H), 2.08 (br s, 4H).

3-[4-(Dimethylamino)phenyl]-1-{3-[6-(pyrrolidin-1-yl)-pyridin-2-yl]phenyl}urea (33). **33** was prepared using the procedure for compound **4** in 56% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.60 (s, 1H), 8.30 (s, 1H), 8.05 (s, 1H), 7.45−7.70 (m, 3H), 7.17−7.38 (m, 3H), 7.05 (d, *J* = 7.35 Hz, 1H), 6.71 (d, *J* = 8.67 Hz, 2H), 6.41 (d, *J* = 8.29 Hz, 1H), 3.48 (br s, 4H), 2.84 (s, 6H), 1.98 (br s, 4H).

3-(2*H*-1,3-Benzodioxol-5-yl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]phenyl}urea (34). **34** was prepared using the procedure for compound **4** in 52% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.64 (s, 1H), 8.06 (s, 1H), 7.48−7.67 (m, 3H), 7.26−7.40 (m, 1H), 7.22 (s, 1H), 7.05 (d, *J* = 7.35 Hz, 1H), 6.69−6.89 (m, 2H), 6.41 (d, *J* = 8.29 Hz, 1H), 5.97 (s, 2H), 3.48 (br s, 4H), 1.98 (br s, 4H).

1-{3-[6-(Pyrrolidin-1-yl)pyridin-2-yl]phenyl}-3-(3,4,5-trimethoxyphenyl)urea (35). **35** was prepared using the procedure for compound **4** in 46% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.45−8.78 (m, 2H), 7.90−8.14 (m, 1H), 7.44−7.70 (m, 3H), 7.25−7.40 (m, 1H), 6.91−7.17 (m, 1H), 6.81 (s, 2H), 6.31−6.54 (m, 1H), 3.76 (s, 6H), 3.62 (s, 3H), 3.42−3.53 (m, 4H), 1.91−2.03 (m, 4H).

3-(3,5-Dimethylphenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (36). **36** was prepared using the procedure for compound **4** in 50% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.80 (s, 1H), 8.60 (s, 1H), 8.06 (s, 1H), 7.46−7.73 (m, 3H), 7.22−7.42 (m, 1H), 6.97−7.16 (m, 3H), 6.62 (s, 1H), 6.42 (d, *J* = 8.48 Hz, 1H), 3.48 (br s, 4H), 2.24 (s, 6H), 1.98 (br s, 4H).

3-(2,6-Dimethylphenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (37). **37** was prepared using the procedure for compound **4** in 60% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.95 (br s, 1H), 8.10 (s, 1H), 7.86 (s, 1H), 7.56 (q, *J* = 7.03 Hz, 2H), 7.22−7.37 (m, 1H), 6.91−7.14 (m, 3H), 6.40 (d, *J* = 8.29 Hz, 1H), 3.47 (br s, 4H), 2.23 (s, 6H), 1.97 (br s, 4H).

3-(2-Ethylphenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (38). **38** was prepared using the procedure for compound **4** in 41% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.10 (s, 1H), 8.07 (s, 1H), 7.93 (s, 1H), 7.77 (d, *J* = 7.82 Hz, 1H), 7.49−7.66 (m, 3H), 7.30−7.39 (m, 1H), 7.11−7.24 (m, 2H), 6.92−7.08 (m, 2H), 6.42 (d, *J* = 8.29 Hz, 1H), 3.43−3.61 (m, 4H),2.62 (q, *J* = 7.47 Hz, 2H), 1.96 (t, *J* = 6.31 Hz, 4H), 1.18 (t, *J* = 7.49 Hz, 3H).

3-[2-(Propan-2-yl)phenyl]-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]phenyl}urea (39). **39** was prepared using the procedure for compound **4** in 49% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.04−8.15 (m, 1H), 7.91−8.00 (m, 1H), 7.50−7.70 (m, 4H), 7.24−7.40 (m, 2H), 7.01−7.20 (m, 3H), 6.33−6.48 (m, 1H), 3.43−3.61 (m, 4H), 3.09−3.21 (m, 1H), 1.88−2.06 (m, 4H), 1.12−1.28 (m, 6H).

3-(3-Chlorophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (40). **40** was prepared using the procedure for compound **4** in 64% yield. 1H NMR (300 MHz, DMSO-d_6) δ 9.05 (s, 1H), 8.98 (s, 1H), 8.18 (s, 1H), 7.83 (s, 1H), 7.75 (d, *J* = 7.72 Hz, 1H), 7.62−7.71 (m, 2H), 7.34−7.51 (m, 3H), 7.07−7.22 (m, 2H), 6.53 (d, *J* = 8.29 Hz, 1H), 3.58 (br s, 4H), 2.08 (br s, 4H).

3-(3,4-Dichlorophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (41). **41** was prepared using the procedure for compound **4** in 43% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.02 (s, 1H), 8.90 (s, 1H), 8.08 (s, 1H), 7.90 (d, *J* = 2.26 Hz, 1H), 7.66 (d, *J* = 7.72 Hz, 1H), 7.45−7.59 (m, 3H), 7.27−7.42 (m, 2H), 7.06 (d, *J* = 7.35 Hz, 1H), 6.42 (d, *J* = 8.29 Hz, 1H), 3.48 (br s, 4H), 1.97 (br s, 4H).

3-(3,5-Dichlorophenyl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (42). **42** was prepared using the procedure for compound **4** in 52% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.08 (s, 1H), 8.97 (s, 1H), 8.08 (s, 1H), 7.66 (d, *J* = 7.72 Hz, 1H), 7.48−7.61 (m, 3H), 7.26−7.41 (m, 1H), 7.06 (d, *J* = 7.35 Hz, 1H), 6.42 (d, *J* = 8.48 Hz, 1H), 3.47 (br s, 4H), 1.97 (br s, 4H).

3-(6-Chloropyridin-3-yl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]phenyl}urea (43). **43** was prepared using the procedure for compound **4** in 62% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.99 (d, *J* = 19.03 Hz, 2H), 8.48 (d, *J* = 2.45 Hz, 1H), 8.09 (s, 1H), 8.01 (dd, *J* = 2.64, 8.67 Hz, 1H), 7.66 (d, *J* = 7.72 Hz, 1H), 7.50−7.61 (m, 2H), 7.44 (d, *J* = 8.67 Hz, 1H), 7.31−7.40 (m, 1H), 7.06 (d, *J* = 7.54 Hz, 1H), 6.42 (d, *J* = 8.48 Hz, 1H), 3.48 (br s, 4H), 1.98 (br s, 4H).

3-(Naphthalen-1-yl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (44). **44** was prepared using the procedure for compound **4** in 36% yield. ^{1}H NMR (300 MHz, DMF) δ 9.22 (s, 1H), 8.84 (s, 1H), 8.09−8.23 (m, 2H), 8.04 (d, *J* = 7.44 Hz, 1H), 7.94 (d, *J* = 7.54 Hz, 1H), 7.44−7.71 (m, 5H), 7.29−7.42 (m, 1H), 7.08 (d,

dx.doi.org/10.1021/jm501042u | *J. Med. Chem.* 2014, 57, 7758−7769

J = 7.35 Hz, 1H), 6.42 (d, J = 8.29 Hz, 1H), 3.43−3.56 (m, 4H), 1.83−2.07 (m, 4H).

3-(Naphthalen-2-yl)-1-{3-[6-(pyrrolidin-1-yl)pyridin-2-yl]-phenyl}urea (45). **45** was prepared using the procedure for compound **4** in 34% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.90 (s, 1H), 8.12 (d, J = 4.05 Hz, 2H), 7.75−7.92 (m, 4H), 7.42−7.71 (m, 5H), 7.37 (t, J = 7.86 Hz, 2H), 7.07 (d, J = 7.44 Hz, 1H), 6.43 (d, J = 8.29 Hz, 1H), 3.43−3.61 (m, 4H), 1.98 (t, J = 6.40 Hz, 4H).

CB1 Calcium Mobilization Assay. RD-HGA16 cells (Molecular Devices) stably expressing the human CB1 receptor were used. The day before the assay, cells were plated into 96-well black-walled assay plates at 25 000 cells/well in Ham's F12 supplemented with 10% fetal bovine serum, 100 units of penicillin, 100 units of streptomycin, and 100 μg/mL Normocin. The cells were incubated overnight at 37 °C, 5% CO_2. Prior to the assay, Calcium 5 dye (Molecular Devices) was reconstituted according to the manufacturer's instructions. The reconstituted dye was diluted 1:40 in prewarmed (37 °C) assay buffer (1× HBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4 at 37 °C). Growth medium was removed, and the cells were gently washed with 100 μL of prewarmed (37 °C) assay buffer. The cells were incubated for 45 min at 37 °C, 5% CO_2 in 200 μL of the diluted Calcium 5 dye. For antagonist (IC_{50}) assays, the EC_{80} concentration of CP55,940 was prepared at 10× the desired final concentration in 0.25% BSA/0.5% DMSO/0.5% EtOH/assay buffer, aliquoted into 96-well polypropylene plates, and warmed to 37 °C. Serial dilutions of the test compounds were prepared at 10× the desired final concentration in 2.25% BSA/4.5% DMSO/4.5% EtOH/assay buffer. After the dye-loading incubation period, the cells were pretreated with 25 μL of the test compound serial dilutions and incubated for 15 min at 37 °C. After the pretreatment incubation period, the plate was read with a FLIPR Tetra (Molecular Devices). Calcium-mediated changes in fluorescence were monitored every 1 s over a 90 s time period, with the Tetra adding 25 μL of the CP55,940 EC_{80} concentration at the 10 s time point (excitation at 470−495 nm, detection at 515−575 nm). Maximum kinetic reduction (ScreenWorks, Molecular Devices) relative fluorescence units (RFU) were plotted against log compound concentration. Data were fit to a three-parameter logistic curve to generate IC_{50} values (GraphPad Prism 6.0, GraphPad Software, Inc., San Diego, CA). For the modulation experiments, the above procedure was followed except that cells were pretreated with a single concentration of test compound (prepared at 10× the desired concentration in 2.25% BSA/4.5% DMSO/4.5% EtOH/assay buffer) and the Tetra added serial dilutions of CP55,940 (prepared at 10× the desired concentration in 0.25% BSA/0.5% DMSO/0.5% EtOH/assay buffer). For agonist screens, the above procedure was followed except that cells were pretreated with 2.25% BSA/4.5% DMSO/4.5% EtOH/assay buffer and the Tetra added single concentration dilutions of the test compounds prepared at 10× the desired final concentration in 0.25% BSA/0.5% DMSO/0.5% EtOH/assay buffer. Test compound RFUs were compared to the CP55,940 E_{max} RFUs to generate % E_{max} values.

CB2 Calcium Mobilization Assay. CHO-RD-HGA16 (Molecular Devices) cells stably expressing the human CB2 receptor were used. The day before the assay, cells were plated into 96-well black-walled assay plates at 30 000 cells/well in Ham's F12 supplemented with 10% fetal bovine serum, 100 units of penicillin and streptomycin, and 100 μg/mL Normocin. The cells were incubated overnight at 37 °C, 5% CO_2. Prior to the assay, Calcium 5 dye (Molecular Devices) was reconstituted according to the manufacturer's instructions. The reconstituted dye was diluted 1:40 in prewarmed (37 °C) assay buffer (1× HBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4 at 37 °C). Growth medium was removed, and the cells were gently washed with 100 μL of prewarmed (37 °C) assay buffer. The cells were incubated for 45 min at 37 °C, 5% CO_2 in 200 μL of the diluted Calcium 5 dye. For antagonist screens, the EC_{80} concentration of CP55,940 was prepared at 10× the desired final concentration in 0.25% BSA/0.5% DMSO/0.5% EtOH/assay buffer, aliquoted into 96-well polypropylene plates, and warmed to 37 °C. Single concentration dilutions of the test compounds were prepared at 10× the desired final concentration in 2.25% BSA/4.5% DMSO/4.5% EtOH/assay buffer. After the dye-loading incubation period, the cells were pretreated with 25 μL of the test compound dilutions and incubated for 15 min at 37 °C. After the pretreatment incubation period, the plate was read with a FlexStation II (Molecular Devices). Calcium-mediated changes in fluorescence were monitored every 1.52 s over a 60 s time period, with the FlexStation II adding 25 μL of the CP55,940 EC_{80} concentration at the 19 s time point (excitation at 485 nm, detection at 525 nm). Peak kinetic reduction (SoftMax, Molecular Devices) relative fluorescence units (RFUs) were generated. Test compound RFUs were compared to the CP55,940 EC_{80} RFUs to generate % inhibition values. For agonist screens, the above procedure was followed except that cells were pretreated with 2.25% BSA/4.5% DMSO/4.5% EtOH/assay buffer and the FlexStation II added single concentration dilutions of the test compounds prepared at 10× the desired final concentration in 0.25% BSA/0.5% DMSO/0.5% EtOH/assay buffer. Test compound RFUs were compared to the CP55,940 E_{max} RFUs to generate % E_{max} values. For IC_{50} assays, the above procedure was followed except that cells were pretreated with serial dilutions of the test compounds (prepared at 10× the desired final concentration in 2.25% BSA/4.5% DMSO/4.5% EtOH/assay buffer) and the FlexStation II added the EC_{80} concentration of CP55,940 (prepared at 10× the desired concentration in 0.25% BSA/0.5% DMSO/0.5% EtOH/assay buffer). Peak kinetic reduction (SoftMax, Molecular Devices) relative fluorescence units were plotted against log compound concentration (nM). Data were fit to a three-parameter logistic curve to generate IC_{50} values (GraphPad Prism 6.0, GraphPad Software, Inc., San Diego, CA).

[^{3}H]CP55,940 Competitive Binding Assay. Binding assays were preformed to determine the effect of test compounds on the binding of [^{3}H]CP55,940 to the human CB1 receptor. Assays were conducted with 0.62 nM [^{3}H]CP55,944, varying concentrations of unlabeled test compounds, and membranes from HEK293 cells expressing human CB1 receptors (PerkinElmer) in a final volume of 500 mL of assay buffer (50 mM TRIZMA HCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% BSA, 1% DMSO, pH 7.4). Specific binding was defined as the difference between [^{3}H]CP55,940 binding in the absence and presence of 10 μM nonradiolabeled CP55,940. Eight concentrations of each test compound were run in duplicate. Binding was initiated by the addition of CB1 membranes (8 mg protein). Assay tubes were incubated at 30 °C in a shaking water bath for 1 h. The binding assay was terminated by vacuum filtration onto a 96-well Unifilter GF/B glass-fiber filter plate using a cell harvester (Brandel), followed by four washes with ice-cold wash buffer (50 mM TRIZMA HCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, pH 7.4). The filter plate was presoaked in 0.1% PEI for half an hour and rinsed with cold wash buffer just before filtration of the assay tubes. The filter plate was allowed to dry, and 35 mL of MicroScint 20 (PerkinElmer) was added to each well. Radioactivity was measured using a Top Count NXT (Packard). The exact concentration of [^{3}H]CP55,940 used in each assay was determined (0.644−0.804 nM) using a TriCarb 2200CA liquid scintillation analyzer. Binding data were expressed as a percentage of specific [^{3}H]CP55,940 binding.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC analysis results of target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yzhang@rti.org. Phone: 919-541-1235. Fax: 919-541-6499.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Tiffany Langston and Allyson Smith for technical assistance and David Perrey for the critical reading of the manuscript. This work was supported by National Institute on Drug Abuse, National Institutes of Health, U.S. (Grant DA032837 to Y.Z. and Grant DA003672 to J.L.W.).

■ ABBREVIATIONS USED

CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; GPCR, G-protein-coupled receptor; SAR, structure−activity relationship; IC_{50}, half-maximum inhibitory concentration; DCM, dichloromethane; 1,2-DCE, 1,2-dichloroethane; DME, 1,2-dimethoxyethane; TLC, thin-layer chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography; FLIPR, fluorometric imaging plate reader

■ REFERENCES

(1) Pertwee, R. G. The therapeutic potential of drugs that target cannabinoid receptors or modulate the tissue levels or actions of endocannabinoids. *AAPS J.* **2005**, *7*, E625−E654.
(2) Howlett, A. C.; Breivogel, C. S.; Childers, S. R.; Deadwyler, S. A.; Hampson, R. E.; Porrino, L. J. Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* **2004**, *47* (Suppl. 1), 345−358.
(3) Pertwee, R. G. Cannabinoid pharmacology: the first 66 years. *Br. J. Pharmacol.* **2006**, *147* (Suppl. 1), S163−S171.
(4) Jagerovic, N.; Hernandez-Folgado, L.; Alkorta, I.; Goya, P.; Martin, M. I.; Dannert, M. T.; Alsasua, A.; Frigola, J.; Cuberes, M. R.; Dordal, A.; Holenz, J. Structural−activity relationship study on C-4 carbon atom of the CB1 antagonist SR141716: synthesis and pharmacological evaluation of 1,2,4-triazole-3-carboxamides. *Eur. J. Med. Chem.* **2006**, *41*, 114−120.
(5) Mackie, K. Cannabinoid receptors as therapeutic targets. *Annu. Rev. Pharmacol. Toxicol.* **2006**, *46*, 101−122.
(6) Pacher, P.; Batkai, S.; Kunos, G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol. Rev.* **2006**, *58*, 389−462.
(7) Di Marzo, V. Targeting the endocannabinoid system: to enhance or reduce? *Nat. Rev. Drug Discovery* **2008**, *7*, 438−455.
(8) Makriyannis, A. 2012 Division of Medicinal Chemistry Award Address. Trekking the cannabinoid road: a personal perspective. *J. Med. Chem.* **2014**, *57*, 3891−3911.
(9) Muccioli, G. G.; Lambert, D. M. Current knowledge on the antagonists and inverse agonists of cannabinoid receptors. *Curr. Med. Chem.* **2005**, *12*, 1361−1394.
(10) Jagerovic, N.; Fernandez-Fernandez, C.; Goya, P. CB1 cannabinoid antagonists: structure−activity relationships and potential therapeutic applications. *Curr. Top. Med. Chem.* **2008**, *8*, 205−230.
(11) Moreira, F. A.; Grieb, M.; Lutz, B. Central side-effects of therapies based on CB1 cannabinoid receptor agonists and antagonists: focus on anxiety and depression. *Best Pract. Res., Clin. Endocrinol. Metab.* **2009**, *23*, 133−144.
(12) Pertwee, R. G. Targeting the endocannabinoid system with cannabinoid receptor agonists: pharmacological strategies and therapeutic possibilities. *Philos. Trans. R. Soc. London, Ser. B* **2012**, *367*, 3353−3363.
(13) Melancon, B. J.; Hopkins, C. R.; Wood, M. R.; Emmitte, K. A.; Niswender, C. M.; Christopoulos, A.; Conn, P. J.; Lindsley, C. W. Allosteric modulation of seven transmembrane spanning receptors: theory, practice, and opportunities for central nervous system drug discovery. *J. Med. Chem.* **2012**, *55*, 1445−1464.
(14) Kenakin, T.; Miller, L. J. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. *Pharmacol. Rev.* **2010**, *62*, 265−304.
(15) Christopoulos, A.; Kenakin, T. G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* **2002**, *54*, 323−374.
(16) Conn, P. J.; Christopoulos, A.; Lindsley, C. W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discovery* **2009**, *8*, 41−54.
(17) Shore, D. M.; Baillie, G. L.; Hurst, D. H.; Navas, F., III; Seltzman, H. H.; Marcu, J. P.; Abood, M. E.; Ross, R. A.; Reggio, P. H. Allosteric modulation of a cannabinoid G protein-coupled receptor: binding site elucidation and relationship to G protein signaling. *J. Biol. Chem.* **2014**, *289*, 5828−5845.
(18) Christopoulos, A. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 198−210.
(19) Bridges, T. M.; Lindsley, C. W. G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS Chem. Biol.* **2008**, *3*, 530−541.
(20) Navarro, H. A.; Howard, J. L.; Pollard, G. T.; Carroll, F. I. Positive allosteric modulation of the human cannabinoid (CB) receptor by RTI-371, a selective inhibitor of the dopamine transporter. *Br. J. Pharmacol.* **2009**, *156*, 1178−1184.
(21) Pamplona, F. A.; Ferreira, J.; Menezes de Lima, O., Jr.; Duarte, F. S.; Bento, A. F.; Forner, S.; Villarinho, J. G.; Bellocchio, L.; Wotjak, C. T.; Lerner, R.; Monory, K.; Lutz, B.; Canetti, C.; Matias, I.; Calixto, J. B.; Marsicano, G.; Guimaraes, M. Z.; Takahashi, R. N. Anti-inflammatory lipoxin A4 is an endogenous allosteric enhancer of CB1 cannabinoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 21134−21139.
(22) Bauer, M.; Chicca, A.; Tamborrini, M.; Eisen, D.; Lerner, R.; Lutz, B.; Poetz, O.; Pluschke, G.; Gertsch, J. Identification and quantification of a new family of peptide endocannabinoids (Pepcans) showing negative allosteric modulation at CB1 receptors. *J. Biol. Chem.* **2012**, *287*, 36944−36967.
(23) Price, M. R.; Baillie, G. L.; Thomas, A.; Stevenson, L. A.; Easson, M.; Goodwin, R.; McLean, A.; McIntosh, L.; Goodwin, G.; Walker, G.; Westwood, P.; Marrs, J.; Thomson, F.; Cowley, P.; Christopoulos, A.; Pertwee, R. G.; Ross, R. A. Allosteric modulation of the cannabinoid CB1 receptor. *Mol. Pharmacol.* **2005**, *68*, 1484−1495.
(24) Horswill, J. G.; Bali, U.; Shaaban, S.; Keily, J. F.; Jeevaratnam, P.; Babbs, A. J.; Reynet, C.; Wong Kai In, P. PSNCBAM-1, a novel allosteric antagonist at cannabinoid CB1 receptors with hypophagic effects in rats. *Br. J. Pharmacol.* **2007**, *152*, 805−814.
(25) Ross, R. A. Allosterism and cannabinoid CB(1) receptors: the shape of things to come. *Trends Pharmacol. Sci.* **2007**, *28*, 567−572.
(26) Ross, R. A. Tuning the endocannabinoid system: allosteric modulators of the CB1 receptor. *Br. J. Pharmacol.* **2007**, *152*, 565−566.
(27) Ahn, K. H.; Mahmoud, M. M.; Kendall, D. A. Allosteric modulator ORG27569 induces CB1 cannabinoid receptor high affinity agonist binding state, receptor internalization, and Gi protein-independent ERK1/2 kinase activation. *J. Biol. Chem.* **2012**, *287*, 12070−12082.
(28) Ahn, K. H.; Mahmoud, M. M.; Samala, S.; Lu, D.; Kendall, D. A. Profiling two indole-2-carboxamides for allosteric modulation of the CB1 receptor. *J. Neurochem.* **2013**, *124*, 584−589.
(29) Wang, X.; Horswill, J. G.; Whalley, B. J.; Stephens, G. J. Effects of the allosteric antagonist 1-(4-chlorophenyl)-3-[3-(6-pyrrolidin-1-ylpyridin-2-yl)phenyl]urea (PSNCBAM-1) on CB1 receptor modulation in the cerebellum. *Mol. Pharmacol.* **2011**, *79*, 758−767.
(30) Baillie, G. L.; Horswill, J. G.; Anavi-Goffer, S.; Reggio, P. H.; Bolognini, D.; Abood, M. E.; McAllister, S.; Strange, P. G.; Stephens, G. J.; Pertwee, R. G.; Ross, R. A. CB(1) receptor allosteric modulators display both agonist and signaling pathway specificity. *Mol. Pharmacol.* **2013**, *83*, 322−338.
(31) Fay, J. F.; Farrens, D. L. A key agonist-induced conformational change in the cannabinoid receptor CB1 is blocked by the allosteric ligand Org 27569. *J. Biol. Chem.* **2012**, *287*, 33873−33882.
(32) Piscitelli, F.; Ligresti, A.; La Regina, G.; Coluccia, A.; Morera, L.; Allara, M.; Novellino, E.; Di Marzo, V.; Silvestri, R. Indole-2-

carboxamides as allosteric modulators of the cannabinoid CB(1) receptor. *J. Med. Chem.* **2012**, *55*, 5627−5631.
(33) Mahmoud, M. M.; Ali, H. I.; Ahn, K. H.; Damaraju, A.; Samala, S.; Pulipati, V. K.; Kolluru, S.; Kendall, D. A.; Lu, D. Structure−activity relationship study of indole-2-carboxamides identifies a potent allosteric modulator for the cannabinoid receptor 1 (CB1). *J. Med. Chem.* **2013**, *56*, 7965−7975.
(34) Khurana, L.; Ali, H. I.; Olszewska, T.; Ahn, K. H.; Damaraju, A.; Kendall, D. A.; Lu, D. Optimization of chemical functionalities of indole-2-carboxamides to improve allosteric parameters for the cannabinoid receptor 1 (CB1). *J. Med. Chem.* **2014**, *57*, 3040−3052.
(35) Stuart, D. R.; Bertrand-Laperle, M.; Burgess, K. M.; Fagnou, K. Indole synthesis via rhodium catalyzed oxidative coupling of acetanilides and internal alkynes. *J. Am. Chem. Soc.* **2008**, *130*, 16474−16475.
(36) Perrey, D. A.; German, N. A.; Gilmour, B. P.; Li, J. X.; Harris, D. L.; Thomas, B. F.; Zhang, Y. Substituted tetrahydroisoquinolines as selective antagonists for the orexin 1 receptor. *J. Med. Chem.* **2013**, *56*, 6901−6916.
(37) Sammakia, T.; Hurley, T. B. Studies on the mechanism of action of 2-formyl-4-pyrrolidinopyridine: isolation and characterization of a reactive intermediate. *J. Org. Chem.* **1999**, *64*, 4652−4664.
(38) Tsukamoto, I.; Koshio, H.; Kuramochi, T.; Saitoh, C.; Yanai-Inamura, H.; Kitada-Nozawa, C.; Yamamoto, E.; Yatsu, T.; Shimada, Y.; Sakamoto, S.; Tsukamoto, S. Synthesis and structure−activity relationships of amide derivatives of (4,4-difluoro-1,2,3,4-tetrahydro-5*H*-1-benzazepin-5-ylidene)acetic acid as selective arginine vasopressin V2 receptor agonists. *Bioorg. Med. Chem.* **2009**, *17*, 3130−3141.
(39) Handy, S. T.; Zhang, Y.; Bregman, H. A modular synthesis of the lamellarins: total synthesis of lamellarin G trimethyl ether. *J. Org. Chem.* **2004**, *69*, 2362−2366.
(40) Handy, S. T.; Zhang, Y. N. Approaches to the synthesis of the lamellarins and related natural products. A review. *Org. Prep. Proced. Int.* **2005**, *37*, 411−445.
(41) Zhang, Y.; Gilliam, A.; Maitra, R.; Damaj, M. I.; Tajuba, J. M.; Seltzman, H. H.; Thomas, B. F. Synthesis and biological evaluation of bivalent ligands for the cannabinoid 1 receptor. *J. Med. Chem.* **2010**, *53*, 7048−7060.
(42) Fulp, A.; Bortoff, K.; Zhang, Y.; Seltzman, H.; Mathews, J.; Snyder, R.; Fennell, T.; Maitra, R. Diphenyl purine derivatives as peripherally selective cannabinoid receptor 1 antagonists. *J. Med. Chem.* **2012**, *55*, 10022−10032.
(43) Fulp, A.; Bortoff, K.; Seltzman, H.; Zhang, Y.; Mathews, J.; Snyder, R.; Fennell, T.; Maitra, R. Design and synthesis of cannabinoid receptor 1 antagonists for peripheral selectivity. *J. Med. Chem.* **2012**, *55*, 2820−2834.