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Article

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Synthesis and Pharmacological Evaluation of 1-Phenyl-3-Thiophenylurea Derivatives as Cannabinoid Type-1 Receptor Allosteric Modulators

Thuy Nguyen,[a] Thomas F. Gamage,[a] Ann M. Decker,[a] Daniel Barrus,[a] Tiffany L. Langston,[a] Jun-Xu Li,[b] Brian F. Thomas,[a] and Yanan Zhang[a,]*

[a] Research Triangle Institute, Research Triangle Park, North Carolina 27709, United States

[b] Department of Pharmacology and Toxicology, University of Buffalo, the State University of New York, Buffalo, New York 14214, United States

Abstract

We previously reported diarylurea derivatives as cannabinoid type-1 receptor (CB_1) allosteric modulators which were effective in attenuating cocaine seeking behavior. Herein, we extended the structure-activity relationships of PSNCBAM-1 (**2**) at the central phenyl ring directly connected to the urea moiety. Replacement with a thiophene ring led to **11** with improved or comparable potencies in calcium mobilization, [^{35}S]GTPγS binding, and cAMP assays, whereas substitution with non-aromatic rings led to significant attenuation of the modulatory activity. These compounds had no inverse agonism in [^{35}S]GTPγS binding, a characteristic that is often thought to contribute to adverse psychiatric effects. While **11** had good metabolic stability in rat liver microsomes, it

showed modest solubility and blood-brain barrier permeability. **11** showed an insignificant attenuation of cocaine seeking behavior in rats, most likely due to its limited CNS penetration, suggesting that pharmacokinetics and distribution play a role in translating the in vitro efficacy to in vivo behavior.

INTRODUCTION

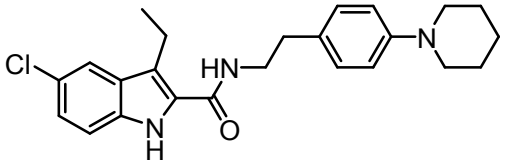
The cannabinoid type-1 and type-2 receptors (CB_1 and CB_2) belong to the class A Rhodopsin-like superfamily of G protein-coupled receptors (GPCRs), with CB_1 being one of the most abundantly expressed receptors in the brain.[1] CB_1 plays an important role in many physiological processes, such as addiction, pain, learning and memory, appetite and feeding behaviors, anxiety and depression.[2-4] CB_1 agonists such as (-)-*trans*-Δ^9-tetrahydrocannabinol (THC), the major phytocannabinoid found in marijuana, have been known to induce appetite and weight gain as well as euphoria, tolerance, dependence, and withdrawal, whereas CB_1 inhibition has been widely investigated to develop therapeutic interventions for obesity, metabolic disorders, and substance abuse.[5-11] Unfortunately, rimonabant, the first CB_1 inverse agonist/antagonist that received approval in Europe for the treatment of obesity in 2006 was subsequently withdrawn due to adverse effects including depression and suicidal ideation.

Despite this setback, the research community continues to target this important receptor system and explore different strategies to overcome the adverse psychiatric effects of CB_1 antagonism/inverse agonism while preserving the beneficial therapeutic effects. These strategies include neutral antagonists, peripherally restricted antagonists, and allosteric modulators.[12-17] Like many GPCRs, CB_1 displays a high level of constitutive activity in the absence of exogenous ligands in both neurons[18, 19] and non-neuronal cells.[20] Rimonabant's inhibition of CB_1 constitutive activity in the central nervous system (CNS) may underly its psychiatric side effects;[21-24] therefore, neutral antagonists which maintain basal CB_1 signaling may not have these side effects. In addition, peripherally restricted antagonists which do not cross the blood-brain barrier (BBB) have shown promising therapeutic efficacy in the treatment of obesity, diabetes, and nonalcoholic steatohepatitis (NASH) without the liability of the CNS side effects,[25] although these drugs are

unviable for the treatment of substance abuse for obvious reasons. Finally, CB_1 allosteric antagonists, such as Org27569 (**1**) and PSNCBAM-1 (**2**) (Fig. 1),[26-28] bind to a location that is topographically distinct from the orthosteric site and can reduce signaling in a probe dependent manner.[29]

Allosteric modulators offer several advantages compared to orthosteric ligands, including lower risk of overdosing due to the "ceiling" effect and preservation of the temporal characteristics of receptor signaling.[15] In particular with CB_1, the allosteric antagonists of the CB_1 reported so far are positive allosteric modulator (PAM)-antagonists,[29] since they increase the affinity of orthosteric agonists for the receptor but decrease their signaling capability. This unique mechanism may provide great advantages for therapeutic blockade of pathological signaling by receptors such as CB_1. Compared to competitive CB_1 antagonists/inverse agonists whose potency diminishes with increasing agonist concentration, the potency of PAM-antagonists is enhanced as agonist concentration increases due to the bidirectionality of the cooperativity vector.[29]

With our long-standing interest in developing CB_1 allosteric modulators for the treatment of drug addiction, we have conducted structure-activity relationship (SAR) studies on both **1** and **2**, two of the most characterized CB_1 allosteric antagonists (Fig. 1).[30, 31] Our initial efforts in optimization of **2** led to compound RTICBM-28 (**3**) (Fig 1), which has comparable in vitro potencies.[30] Further SAR efforts demonstrated that the pyrrolidinyl ring is not required for the CB_1 modulatory activity and the pyridinyl ring could be replaced with other substituted phenyl rings or five-membered heterocycles, leading to the discovery of RTICBM-74 (**4**) and RTICBM-229 (**5**), respectively (Fig 1).[32, 33] We found that **4** attenuated prime-induced restatement of cocaine seeking[32] and **3** reduced THC's potency in drug discrimination,[34] demonstrating the therapeutic potential of these CB_1 allosteric modulators for the treatment of the relapse of cocaine addiction and THC dependence.



Org27569 (**1**)

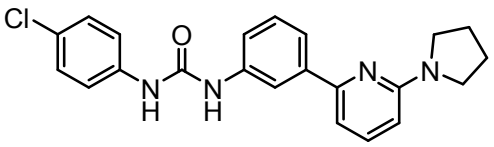
hCB_1 Ca^{2+} assay IC_{50} = 853 nM

[^{3}H]CP55,940 binding K_B = 217 nM, α = 6.9

Reduced food intake

Reduced CP55,940-induced hypothermia in rats

Attenuated cue and drug-induced reinstatement of cocaine- and methamphetamine-seeking behavior



PSNCBAM-1 (**2**)

hCB_1 Ca^{2+} assay IC_{50} = 33 nM

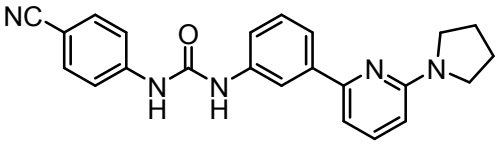
mCB_1 GTPγS assay IC_{50} = 115 nM

[^{3}H]CP55,940 binding K_B = 54 nM, α = 7.3

Rat liver microsomes $T_{1/2}$ = 13 min

Reduced food intake and body weight

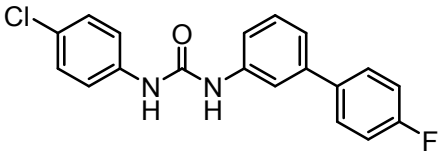
Attenuated the reinstatement of cocaine re-seeeking behavior



RTICBM-28 (**3**)

hCB_1 Ca^{2+} assay IC_{50} = 33 nM

Reduced THC's potency in drug discrimination test



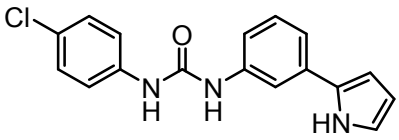
RTICBM-74 (**4**)

hCB_1 Ca^{2+} assay IC_{50} = 23 nM

mCB_1 GTPγS assay IC_{50} = 151 nM

Rat liver microsomes $T_{1/2}$ > 300 min

Attenuated the reinstatement of cocaine re-seeeking behavior



RTICBM-229 (**5**)

hCB_1 Ca^{2+} assay IC_{50} = 169 nM

mCB_1 GTPγS assay IC_{50} = 40 nM

[^{3}H]CP55,940 binding K_d = 1.4 nM

Figure 1. Structures of representative indole-based and diarylurea-based CB_1 allosteric modulators with in vitro and in vivo potency.[27, 30, 32-39]

Herein, we report our optimization effort to extend the SAR understanding of this diarylurea scaffold by structural optimization at the central phenyl ring (Fig. 2), a position that has not yet been investigated. Specifically, we replaced this phenyl ring with a variety of heterocylic rings

including pyridine, thiophene and thiazole, as well as non-aromatic rings such as cyclopropyl or piperidinyl rings. Newly synthesized compounds were evaluated in human calcium mobilization and cAMP assays as well as human and mouse [^{35}S]GTPγS binding assays for their potencies in modulating the activity of the orthosteric agonist CP55,940. The compounds were additionally assessed alone for their effects on the CB_1 basal tone in the [^{35}S]GTPγS binding assay. Select compounds were also examined in preliminary ADME assays, including metabolic stability against rat liver microsomes, blood-brain barrier permeability and aqueous solubility. Finally, the most potent compound from this series, **11**, was evaluated in the reinstatement of cocaine seeking behavior in rats.

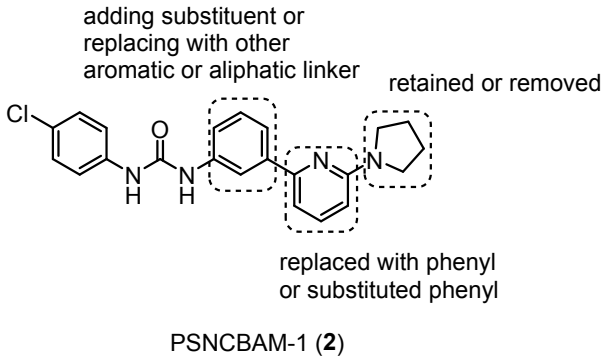


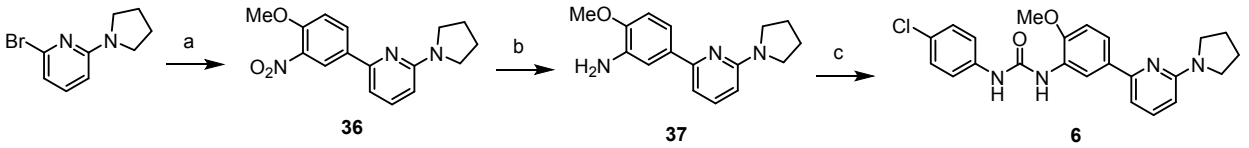
Figure 2. Strategies to investigate SAR of **2**.

RESULTS AND DISCUSSION

Chemistry

To prepare **6**, 2-bromo-6-(pyrrolidin-1-yl)pyridine[30] underwent Suzuki coupling with 4-methoxy-3-nitro-phenylboronic acid to give **36** which was subsequently reduced by Raney-nickel and hydrazine to **37**. Coupling of **37** with 4-chlorophenyl isocyanate yielded the desired product **6** (Scheme 1).

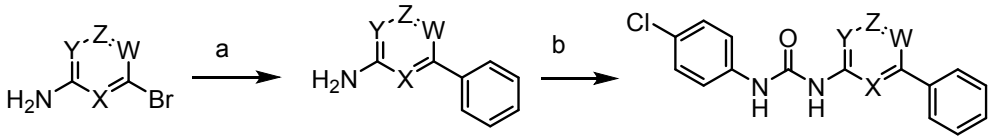
Scheme 1



Reagents and conditions: a) 4-methoxy-3-nitro-phenylboronic acid, $Pd(PPh_3)_4$, aq. $NaHCO_3$, DME, reflux, 16 h, 30%; b) $N_2H_4.H_2O$, Raney-Ni, EtOH, 60 ^oC, 1 h, 93%; c) 4-ClPhNCO, $CHCl_3$, 60 ^oC, 16 h, 64%.

To prepare the pyridinyl analogs, the corresponding bromopyridinyl amines underwent Suzuki coupling with phenylboronic acid to give intermediates **38** – **41**, which were subsequently coupled with 4-chlorophenyl isocyanate to afford compounds **7** - **10** (Scheme 2).

Scheme 2



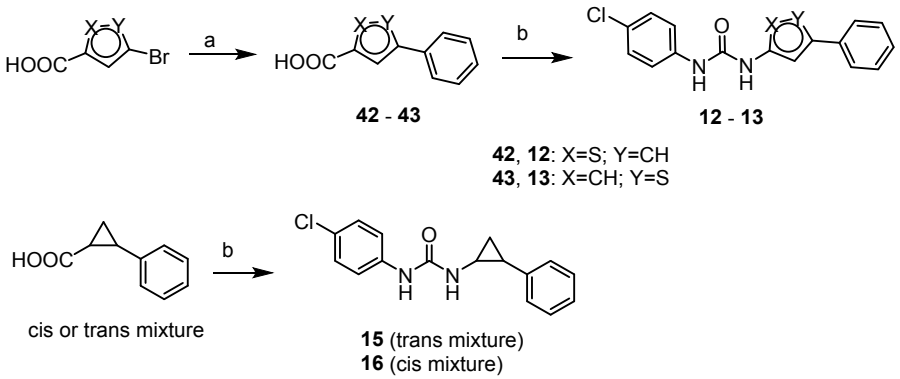
38, **7**: Y=N; X,Z,W=CH
39, **8**: X=N; Y,Z,W=CH
40, **9**: Z=N; X,Y,W=CH
41, **10**: W=N; X,Y,Z=CH

Reagents and conditions: a) phenylboronic acid, $Pd(PPh_3)_4$, aq. $NaHCO_3$, DME, reflux, 16 h, 44-89%; b) 4-ClPhNCO, $CHCl_3$, 60 ^oC, 16 h, 35-60%.

Commercially available bromothiophenecarboxylic acids underwent Suzuki coupling with phenylboronic acid to afford the intermediates **42** and **43**. The urea derivatives **12** and **13** were

obtained via a microwave-assisted coupling of these carboxylic acids via a Curtius rearrangement[40] with 4-chlorophenylamine in the presence of diphenylphosphoryl azide. Similarly, **15** and **16** were afforded from the Curtius rearrangement reaction of *cis*- or *trans*-2-phenylcyclopropane-1-carboxylic acid and 4-chlorophenylamine (Scheme 3).

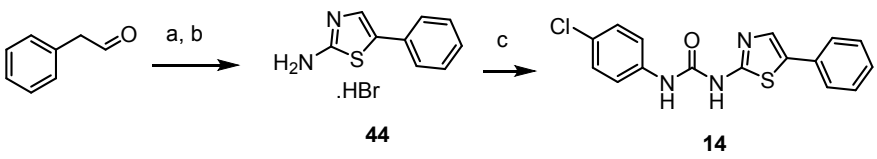
Scheme 3



Reagents and conditions: a) phenylboronic acid, $Pd(PPh_3)_4$, aq. $NaHCO_3$, DME, reflux, 16 h, 62-99%; b) 4-$ClPhNH_2$, DPPA, Et_3N, toluene, mw, 100 ^oC, 5 min, 65-83%.

As the Suzuki coupling between 2-bromo-5-nitrothiazole and phenylboronic acid failed to give the desired product **14**, a different route was sought to form the thiazole ring. Phenyl acetaldehyde was treated with bromine to give 2-bromo-2-phenylacetaldehyde, which underwent cyclization with thiourea to give the intermediate **44**.[41] Coupling of **44** with 4-chlorophenyl isocyanate yielded **14** (Scheme 4).

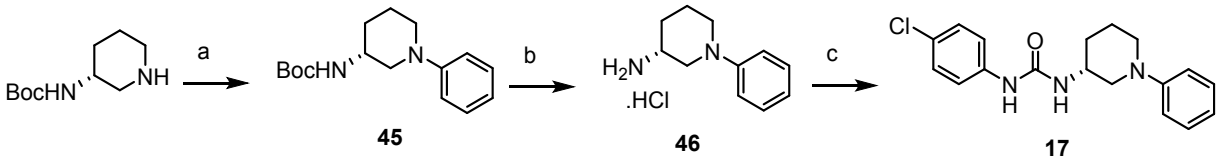
Scheme 4



Reagents and conditions: a) bromine, DCM, -10 °C, 30 min then reflux, 16 h b) thiourea, EtOH, reflux, 8 h, 2-step yield 77%; c) 4-ClPhNCO, $CHCl_3$, 60 °C, 16 h, 34%.

Copper-catalyzed coupling between (*R*)-3-(Boc-amino)piperidine and phenylboronic acid yielded the intermediate **45**. Removal of the Boc protecting group gave **46**, which underwent coupling with 4-chlorophenyl isocyanate to afford **17** (Scheme 5).

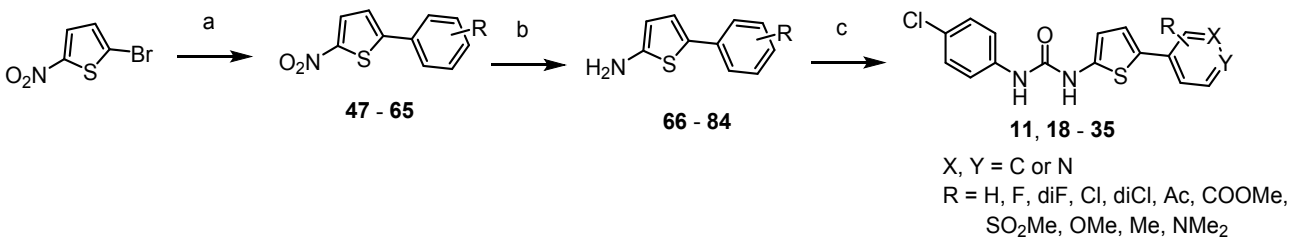
Scheme 5



Reagents and conditions: a) $PhB(OH)_2$, $Cu(OAc)_2$, Et_3N, DCM, 60 °C, 72 h, 32%; b) 4N HCl/1,4-dioxane, rt, 1 h, quant.; c) 4-ClPhNCO, $CHCl_3$, 60 °C, 16 h, 32%.

Standard procedures were followed to prepare the 5-phenyl-thiophen-2-yl analogues, starting with Suzuki coupling between 2-bromo-5-nitrothiophene with corresponding substituted phenylboronic acid to give intermediates **47** - **65**, which were reduced by Raney-Ni and hydrazine to amines **66** - **84**. Coupling of these amines with 4-chlorophenyl isocyanate afforded compounds **11** and **18** - **35** (scheme 6).

Scheme 6



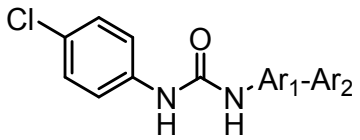
Reagents and conditions: a) corresponding phenylboronic acid, $Pd(PPh_3)_4$, aq. $NaHCO_3$, DME, reflux, 16 h, 8-85% b) $N_2H_4.H_2O$, Raney-Ni, EtOH, 60 ^oC, 1 h, 12%-quant. Yield; c) 4-ClPhNCO, $CHCl_3$, 60 ^oC, 16 h, 7-88%.

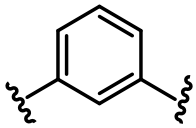
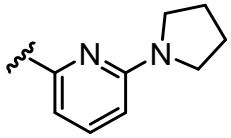
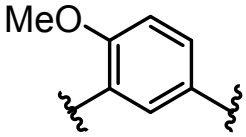
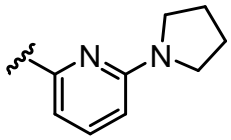
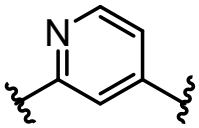
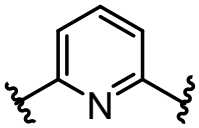
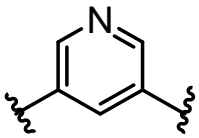
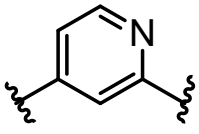
Biological Evaluations

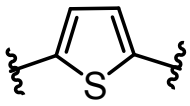
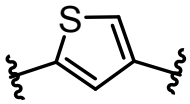
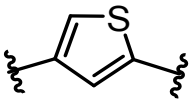
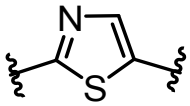
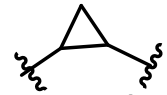
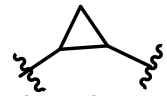
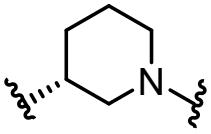
We assessed the ability of our new compounds to antagonize the CB_1-mediated responses to CP55,940 in calcium mobilization and [^{35}S]GTPγS binding assays as previously described.[30, 32, 33] The calcium mobilization assay was conducted with CHO-RD-HGA16 cells overexpressing the human CB_1 and the promiscuous $G\alpha_{16}$ protein, which enables the CB_1 to signal through mobilization of internal calcium. The [^{35}S]GTPγS binding assays were conducted in HEK293 cells overexpressing the human CB_1.[32, 33] Select compounds were also assessed in the [^{35}S]GTPγS binding assay in mouse cerebellum which has a high expression of CB_1.[33] Table 1 shows the IC_{50} values against the EC_{80} concentration of CP55,940 (100 nM) in all three assays.

SAR of the central phenyl ring of 2 (Compounds 6 – 17)

Table 1. Allosteric Modulating Activities of Compounds **2** and **6 - 17** in the CB_1 Calcium Mobilization and [^{35}S]GTPγS Binding Assays.



Compound	Ar_1	Ar_2	hCB_1 Calcium assay IC_{50} (nM)[a]	hCB_1 $[^{35}S]GTP\gamma S$ binding assay IC_{50} (nM)[b]	mCB_1 $[^{35}S]GTP\gamma S$ binding assay IC_{50} (nM)[c]
2			33 ± 8	455 (307 – 673)	288 (152 – 589)
6			1270 ± 420	>10,000	N.D.
7		Ph	156 ± 71[d]	>10,000	>10,000
8		Ph	3530 ± 240	>10,000	N.D.
9		Ph	292 ± 55	>10,000	N.D.
10		Ph	233 ± 34	2770 (1830 – 4190)	N.D.

11		Ph	6.8 ± 1	524 (283 – 969)	63 (42 – 92)
12		Ph	37 ± 2	667 (417 – 1060)	776 (546 – 1103)
13		Ph	60 ± 8	888 (621 – 1270)	N.D.
14		Ph	79 ± 10	604 (348 – 1050)	N.D.
15	 trans mixture	Ph	1220 ± 190	>10,000	>10,000
16	 cis mixture	Ph	>10,000[e]	>10,000	N.D.
17		Ph	3880 ± 140	>10,000	N.D.

[a] Against 100 nM CP55,940 in stable human CB_1-CHO-RD-HGA16 cells. Values are the mean ± SEM of at least three independent experiments in duplicate.[b] Against 100 nM CP55,940 in HEK293 cells stably expressing CB_1. Values are expressed as mean (95% confidence interval) from at least three independent experiments in duplicate. [c] Against 100 nM CP55,940 in mouse cerebellar membranes. Values are expressed as mean (95% confidence interval) from at least three independent experiments in duplicate. [d] Partial inhibition at 33%. [e] Value is the result of two independent experiments in duplicate. N.D. Not determined

First, we introduced a methoxy group at the 2-position of the central phenyl ring (**6**) to probe the electronic properties of this ring. As the diarylurea possesses a flat structure, resulting in tight packing and limited aqueous solubility, this 2-methoxy group also introduces steric hindrance, thus disrupting the planar structure and preventing the tight packing. Unfortunately, the allosteric modulating activity was significantly weakened (**6**, IC_{50} = 1270 nM) compared to **2** (IC_{50} = 33 nM).

As previously demonstrated,[32] the pyrrolidinylpyridine ring can be replaced with a phenyl ring without compromising the activity. Therefore, to simplify the synthesis effort, the SAR studies at the central phenyl ring were conducted without the pyrrolidinyl ring. The central phenyl ring was replaced with various aromatic heterocycles such as pyridine, thiophene, and thiazole. All three pyridinyl analogues (**7**, **9**, **10**) exhibited a modest drop in activity and were 4.7-fold, 8.8-fold, and 7.1-fold, respectively, less potent than **2**. The activity of the 2,6-pyridinyl analogue (**8**) was significantly diminished (IC_{50} = 3530 nM). Interestingly, the five membered ring analogues, thiophenes and thiazole, all displayed activities better than their six-membered pyridinyl counterparts. In particular, the 2,5-thiophenyl analogue (**11**, IC_{50} = 6.8 nM) had significantly improved activity compared to **2** (Fig 3). Other thiophene and thiazole (**12** - **14**) analogues had comparable potencies to **2**, with **12** being the most active of the three compounds (IC_{50} = 37 nM). When the central phenyl ring was replaced with non-aromatic rings such as cyclopropyl (**15** and **16**) or piperidinyl (**17**), the allosteric modulating activity was greatly diminished and resulted in IC_{50} values of 1220 nM (**15**), >10,000 (**16**), and 3880 nM (**17**).

The results from the [^{35}S]GTPγS binding assay against human CB_1 were generally in agreement with the calcium mobilization assays with similar potency ranking orders, albeit the potencies appeared to be weaker. In general, compounds with weak activities (**6**, **8**, **15** - **17**) in the calcium

assay were mostly inactive in the hCB_1 [^{35}S]GTPγS binding assay. The pyridinyl analogues (**7**, **9**, and **10**) with moderate activities in the calcium assay demonstrated no or weak activities in the [^{35}S]GTPγS binding assay. In contrast, the five membered ring thiophene and thiazole analogues (**11** - **14**) still maintained good activities in the [^{35}S]GTPγS binding assay comparable to that of **2**.

Evaluation in the [^{35}S]GTPγS binding assay using mouse cerebellum, which natively expresses CB_1, revealed some differences between assays and species/source of receptors. In particular, **7** had no activities in both [^{35}S]GTPγS binding assays but displayed a moderate activity in the calcium assay. **11** demonstrated better potencies in the calcium and [^{35}S]GTPγS binding assay against mouse CB_1 than **2** but appeared to be equipotent in the [^{35}S]GTPγS binding assay against human CB_1. On the other hand, **12** had comparable potencies in the calcium and [^{35}S]GTPγS binding assay against human CB_1 but weaker potency in the [^{35}S]GTPγS binding assay against mouse CB_1. Lastly, **15** had weak or no activities across three assays. These discrepancies may be attributed to the different G protein coupling ($G\alpha_{16}$ vs. $G\alpha_{i/o}$) or species (human vs. mouse) between these assays (see further discussions below).

Finally, consistent with the positive allosteric modulation, **2** increased the specific binding of [^{3}H]CP55,940, an effect that has been well-established.[27, 30, 32] Interestingly, **11** increased the % specific binding of the radioligand to a greater degree (343.1±63.94), than did **2** (190.0±29.65) following Student's t-test ($t(4) = 3.761$, $p<0.05$; Fig. 3D), suggesting greater cooperativity.

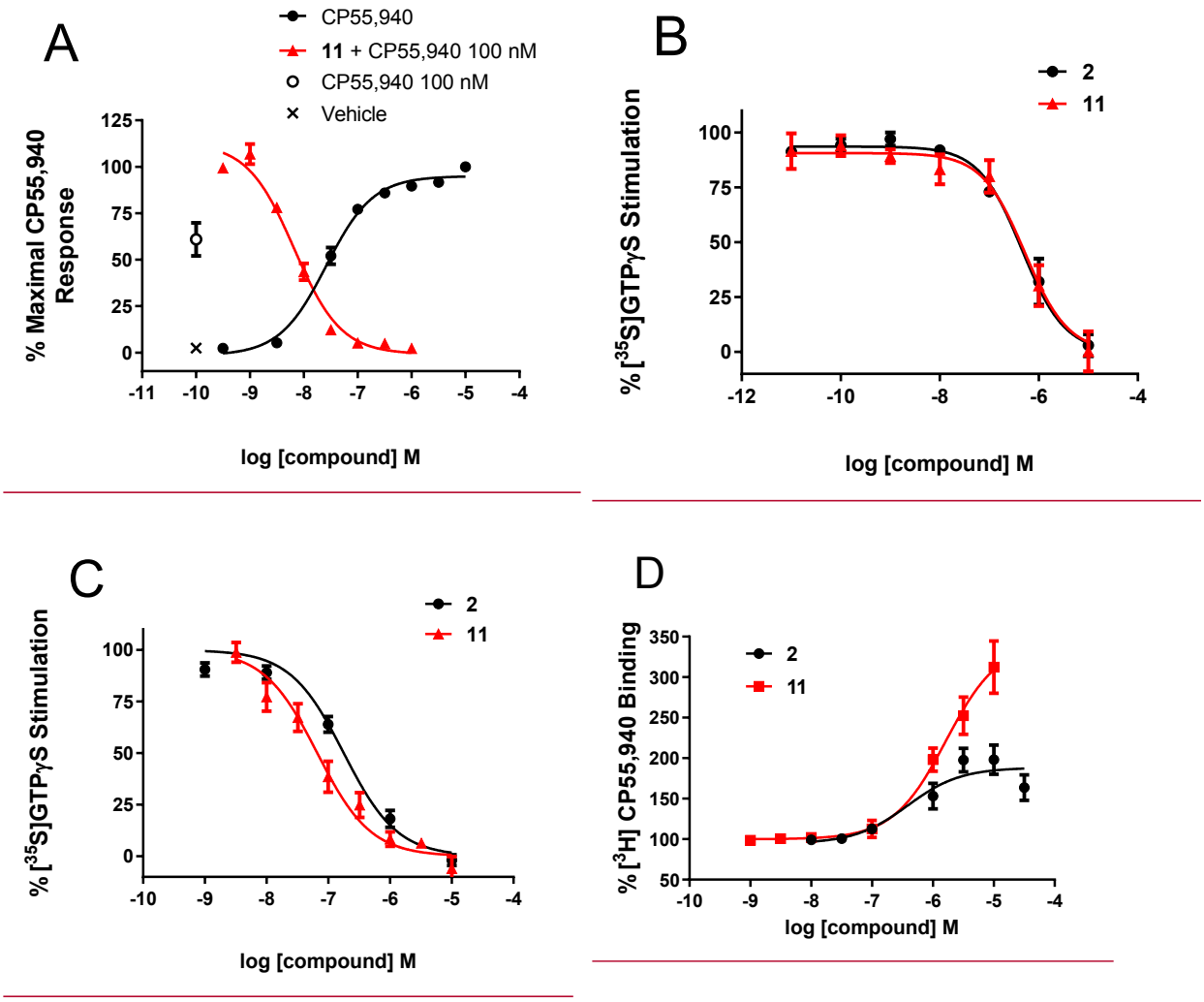
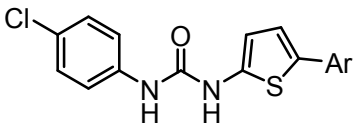
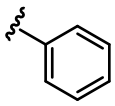
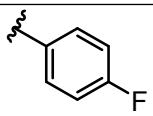
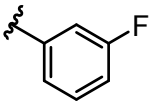
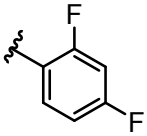
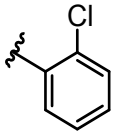


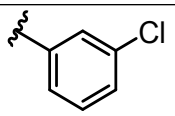
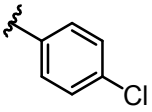
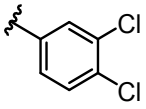
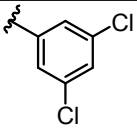
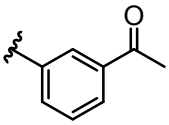
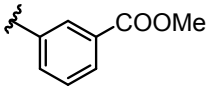
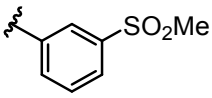
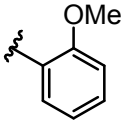
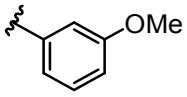
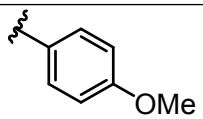
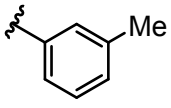
Figure 3. Activities of **2** and **11** against 100 nM CP55,940 in A) calcium mobilization assay in human CB_1-CHO-RD-HGA16 cells stably expressing the human CB_1. B) [^{35}S]GTPγS binding assay in HEK293 cells stably expressing the human CB_1. C) [^{35}S]GTPγS binding assay in cerebella of male ICR mice. D) Effects of **2** and **11** on the equilibrium binding levels of 1 nM of [^{3}H]CP55,940 to membranes of HEK293 cells stably expressing the human CB_1.

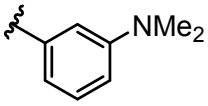
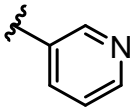
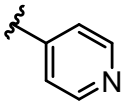
Substituted Phenylthiophenyl analogues and their in vitro pharmacological activities

Table 2. Allosteric Modulating Activities of Compounds **2**, **11**, and **18 - 35** in the hCB_1 Calcium Mobilization and hCB_1 and mCB_1 [^{35}S]GTPγS Binding, and cAMP BRET Assays.



Compound	Ar	hCB_1 Calcium assay IC_{50} (nM)[a]	hCB_1 [^{35}S]GTPγS binding assay IC_{50} (nM)[b]	mCB_1 [^{35}S]GTPγS binding assay IC_{50} (nM)[c]	hCB_1 cAMP IC_{50} (nM)[d]
2	-	33 ± 8	455 (307 – 673)	288 (152 – 589)	2620 (1690-4070)
11		6.8 ± 1	524 (283 – 969)	63 (42 – 92)	1760 (1260-2440)
18		40 ± 2	537 (319-904)	174 (78-399)	N.D.
19		39 ± 6	425 (281-644)	N.D.	N.D.
20		22 ± 4	84 (45-157)	138 (39-468)	2290 (1460 – 3590)
21		21 ± 2	272 (181-410)	363 (159-813)	1190 (675 – 2100)

22		40 ± 7	553 (326-937)	N.D.	N.D.
23		43 ± 7	774 (506-1180)	N.D.	N.D.
24		111 ± 14	1720 (941-3130)	N.D.	N.D.
25		92 ± 12	6330 (4350-9200)	3311 (1950-5630)	>10,000
26		108 ± 17	3430 (2150-5490)	N.D.	N.D.
27		345 ± 48	2350 (1600-3460)	N.D.	N.D.
28		501 ± 58	3230 (2370-4400)	N.D.	N.D.
29		58 + 5	2020 (1410-2890)	N.D.	N.D.
30		61 + 2	3280 (2390-4520)	3020 (1320-7080)	6350 (4290-9410)
31		31 ± 2	449 (278-725)	110 (48-252)	1850 (1150-2950)
32		48 ± 8	1180 (849-1650)	N.D.	N.D.

33		89 ± 14	5020 (2800-9010)	3467 (1450-8320)	>10,000
34		107 ± 6	298 (184-484)	N.D.	N.D.
35		79 ± 13	263 (133-522)	204 (67-617)	1800 (1160-2770)

[a] Against 100 nM CP55,940 in stable human CB_1-CHO-RD-HGA16 cells. Values are the mean ± SEM of at least three independent experiments in duplicate. [b] Against 100 nM CP55,940 in HEK293 cells stably expressing CB_1. Values are expressed as mean (95% confidence interval) from at least three independent experiments in duplicate. [c] Against 100 nM CP55,940 in mouse cerebellar membranes. Values are expressed as mean (95% confidence interval) from at least three independent experiments in duplicate. [d] Against 100 nM CP55,940 in HEK293 cells stably expressing the human CB_1. Replicates of at least 3 independent experiments performed in duplicate were normalized to forskolin (%) and used to calculate IC_{50} best-fit parameters following logistic non-linear regression with the top and bottom of curves globally shared. N.D. Not determined.

With the encouraging results of the thiophene **11** which exhibited better or equipotent activities in all three assays described above, we proceeded to prepare a focused series to investigate the substituent effect on the phenyl ring of the thiophene analogs and screened these compounds in all three in vitro assays. As shown in table 2, the presence of one or two fluoro substituent(s) or one chloro group (**18 - 23**) resulted in a comparable potency to **2** in the calcium assay. Particularly, **20** (IC_{50} = 84 nM) was more potent than **2** (IC_{50} = 455 nM) in the hCB_1 [^{35}S]GTPγS binding assay

with comparable potencies in the hCB_1 calcium and mCB_1 [^{35}S]GTPγS binding assays. However, inclusion of two chloro groups, as in 3,4-dichloro and 3,5-dichloro analogues (**24** and **25**), weakened the activities. Addition of electron-withdrawing groups such as acetyl (**26**), methoxycarbonyl (**27**) or methylsulfonyl (**28**) at the 3-position also dampened the activities.

Among three positional isomeric methoxy analogues, 4-methoxy analogue (**31**) was the most potent CB_1 allosteric modulator with comparable potencies to **2** in all three assays. The other two methoxy analogues (**29** and **30**) exhibited slightly lower potencies in the [^{35}S]GTPγS binding assays. The 3-methyl analogue (**32**) displayed good activity in the calcium mobilization assay (IC_{50} = 48 nM) but had a weaker potency in the hCB_1 [^{35}S]GTPγS binding assay (IC_{50} = 1180 nM). The 3-*N*,*N*-dimethylamino analogue (**33**) demonstrated diminished activities in all three assays. Although two pyridinyl analogues (**34** and **35**) had slightly weaker potencies in the calcium mobilization assay, they were equipotent to **2** in two [^{35}S]GTPγS binding assays. Overall, these results indicate that small electron-withdrawing substituents such as fluoro or chloro are tolerated on the phenyl ring. Electron-donating groups such as methoxy or methyl are preferably located at the 4-position of the phenyl ring. The inclusion of a heteroatom such as in the pyridine ring is also tolerated.

In general, the results from the two [^{35}S]GTPγS binding assays were relatively consistent with the calcium assay. Compounds with equipotent activities to **2** in the calcium assay also displayed comparable activities in the [^{35}S]GTPγS binding assay (**18**, **19**, **21 - 23**, and **31**). Compounds with weaker activities in the calcium assay also showed lower potencies in the [^{35}S]GTPγS binding assay (**24 - 29**, and **31 - 33**). Several compounds demonstrated better activities in the [^{35}S]GTPγS binding assay than in the calcium assay. For example, the two pyridinyl analogues (**34** and **35**) showed comparable potencies to **2** in the [^{35}S]GTPγS binding assay while they exhibited weaker

activities in the calcium assay. Fascinatingly, the 2,4-difluoro analogue demonstrated significantly better potencies in the [^{35}S]GTPγS binding assay compared to **2** (**20**, IC_{50} = 84 nM vs **2** IC_{50} = 455 nM).

Interestingly, the allosteric modulatory activities of some compounds appeared to be more potent at the higher CP55,940 concentration (1 μM) in the [^{35}S]GTPγS binding assays using mouse cerebellar membranes (Table 3) following two-way ANOVA [main effect of CP55,940 concentration: $F(1,58) = 18.3$, $p<0.001$; main effect of compound: $F(8,58) = 75.6$, $p<0.001$; no significant interaction, $F(8,58) = 1.15$, $p=0.345$]. A significant shift in potency was observed for **20** with a 3-fold reduction in its calculated IC_{50} value following Bonferroni post hoc analysis ($p<0.05$). **35** also exhibited a significant change in IC_{50} values, from 287 nM to 129 nM. This shift in inhibitory potencies reflects positive cooperativity characteristics of these PAM-antagonists consistent with our previous study.[34]

Table 3. CB_1 allosteric modulating activities in the [^{35}S]GTPγS binding assays using mouse cerebellum membrane at 1 μM and 100 nM CP55,940.

Compound	[CP55,940]	pIC_{50} ± S.E.M.	pIC_{50} 95% C.I.	IC_{50} (nM)	IC_{50} (nM) 95% C.I.	IC_{50} Fold Change
2	1 μM	6.91 ± 0.055	6.73 – 7.08	123	84 – 187	2.3
	100 nM	6.54 ± 0.081	6.23 – 6.82	288	152 – 589	
18	1 μM	6.84 ± 0.041	6.72 – 6.95	145	113 – 191	1.2
	100 nM	6.76 ± 0.13	6.4 – 7.11	174	78 – 399	
20	1 μM	**7.32 ± 0.1 ***	6.99 – 7.65	48	23 – 103	2.9
	100 nM	6.86 ± 0.17	6.33 – 7.41	138	39 – 468	
21	1 μM	6.69 ± 0.11	6.35 – 7.02	204	96 – 447	1.8
	100 nM	6.44 ± 0.11	6.09 – 6.8	363	159 – 813	
25	1 μM	5.62 ± 0.11	5.27 – 5.97	2399	1080 – 5380	1.4
	100 nM	5.48 ± 0.074	5.25 – 5.71	3311	1950 – 5630	

30	1 μM	5.79 ± 0.035	5.67 – 5.9	1622	1260 – 2140	1.9
	100 nM	5.52 ± 0.11	5.15 – 5.88	3020	1320 – 7080	
31	1 μM	6.92 ± 0.074	6.71 – 7.12	120	76 – 195	0.9
	100 nM	6.96 ± 0.13	6.6 – 7.32	110	48 – 252	
33	1 μM	5.59 ± 0.064	5.39 – 5.79	2570	1630 – 4080	1.3
	100 nM	5.46 ± 0.12	5.08 – 5.84	3467	1450 – 8320	
35	1 μM	6.92 ± 0.063	6.72 – 7.12	120	76 – 191	1.7
	100 nM	6.69 ± 0.15	6.21 – 7.18	204	67 – 617	

Mean IC_{50} values ± SEM of N=4-5 experiments performed in duplicate. Data were normalized (0-100%) and fit to logistic non-linear regression with top constrained to 100 and bottom constrained to 0. * $p < 0.05$ compared to respective 100 nM concentration of CP55,940 following two-way ANOVA and Bonferroni post hoc.

All compounds were screened in the calcium mobilization assay for agonist activity at the CB_1; no significant agonist effects (<30% of CP55,940 E_{max}) were observed for any of the compounds. All these compounds were also screened for agonist and antagonist activity at the CB_2 to determine receptor subtype selectivity using our previously established CB_2 calcium mobilization assay.[30, 32] None of the compounds had significant CB_2 agonist activity (<10% of CP55,940 E_{max}) or CB_2 antagonist activity (<50% inhibition of CP55,940 EC_{80} concentration at 10 μM or IC_{50} values >10 μM).

Real-time kinetic BRET CAMYEL cAMP Studies

We then assessed several representative compounds in the real-time kinetic BRET CAMYEL cAMP assay.[42] In HEK-hCB$_1$ cells, forskolin (10 μM) induced significant cAMP production that plateaued after 5 min which was inhibited by the agonist CP55,940 (10 nM), consistent with coupling to $G_{i/o}$ proteins. A one-phase exponential association model was preferred for the majority of the kinetic curves following an extra-sum-of-squares F-test, except for some of the curves for the highest concentrations of modulator for **11** (3.16 and 10; $p < 0.01$), **20** (31.6 μM; $p < 0.05$), and **31** (31.6 μM; $p < 0.01$), which could reflect inverse agonism as the accumulation of cAMP appears greater than forskolin before the curve begins to plateau. Potencies did not appear to change over the course of the assay when examined in 5 min bins (data not shown) in contrast to **1** which exhibited increases in potency over the course of the assay.[42] This can, at least in part, be explained by the fact that the allosteric modulators were pre-equilibrated in our assay whereas **1** was co-applied in the previous study; while this delay has been observed with other indole-2-carboxamides,[42] previously **2** did not exhibit a delay when it was co-applied,[43] suggesting there may be kinetic differences between these scaffolds.

Compounds **11**, **20**, **21**, **31**, and **35** were equipotent to **2** while **25**, **30** and **33** were either inactive or exerted weak activities. As seen in Fig 4, these compounds exhibited immediate dose-dependent CB$_1$ antagonism effects which reached equilibrium at around 5-6 min. In the cAMP BRET assay, compounds appeared to be less potent than in the calcium and [^{35}S]GTPγS binding assay (Table 2).

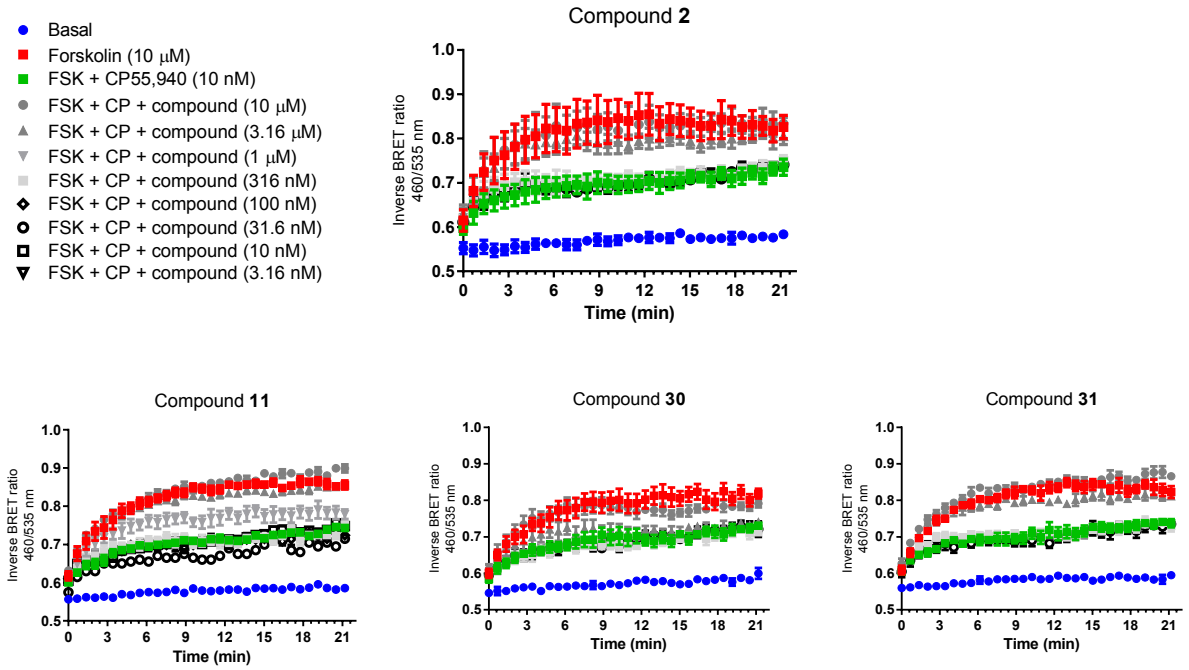


Figure 4. Temporal cAMP BRET signaling raw data of select diarylurea-based CB_1 allosteric modulators.

Potency differences between various pharmacological assays

Overall, potencies were consistent across the human [^{35}S]GTPγS, mouse [^{35}S]GTPγS, and cAMP assays as supported by significant positive correlations between IC_{50} values for human and mouse [r(10)=0.959 (0.830 – 0.990), $p < 0.0001$], human and cAMP [r(7)=0.958 (0.736 – 0.994), $p < 0.001$], and mouse and cAMP [r(7)=0.957 (0.729 – 0.994), $p < 0.0001$]. However, a significant correlation was not observed for IC_{50} values between calcium and human [^{35}S]GTPγS [r(20)=0.366 (-0.0917 – 0.696), p=0.113] or calcium and cAMP [r(7)=0.433 (-0.474 – 0.894), p=0.332]. There are several distinctions between assays that may have contributed to a lack of congruence in potency for these compounds.

While the calcium mobilization assay was performed in CHO cells with CB_1 coupling to $G\alpha_{16}$, the [^{35}S]GTPγS binding assay predominantly reflects coupling to the $G\alpha_{i/o}$ family of G proteins.[44] G-

proteins increase agonist affinity for CB_1 and agonists can exhibit biased signaling for individual G-protein subtypes.[45, 46] For example, when overexpressed, $G\alpha_{16}$ shifts the conformation of the kappa opioid receptor and increases the affinity of salvinorin A by 18-fold while not affecting the affinity of U69,593 or dynorphin A. [47] Thus, higher potencies observed in the calcium assay could reflect the contribution of this distinct G-protein subtype having a greater impact on the affinity of these PAM-antagonists.

Differences between the [^{35}S]GTPγS and cAMP assays could be due to receptor reserve. Sufficient receptor reserve exists for cAMP signaling in our HEK293 cells such that agonists of high and low efficacy in [^{35}S]GTPγS binding typically exhibit equi-efficacy in cAMP signaling, as this pathway is easily saturated due the artificially high level of CB_1. Only a fraction of receptors must be occupied to saturate the pathway and reach E_{max} in cAMP, which requires greater receptor coverage by the modulator to antagonize this response, i.e. a higher IC_{50}.

Other factors that may also have impacted relative potencies include differences in equilibration time and receptor:G-protein ratios. Because the calcium assay measures the response for 90 s immediately after agonist CP55,940 is added, it is conducted at hemi-equilibrium.[48] Therefore, differences in cooperativity between these allosteric modulators will impact the binding kinetics of CP55,940,[49, 50] which could result in apparent differences in relative potencies compared to assays in which equilibrium was reached or approached (e.g. cAMP is 20 min, [^{35}S]GTPγS is 60 min plus 30 min pre-equilibration with agonist and modulator). In addition, cerebellar membranes contain high levels of CB_1 with receptor/G-protein stoichiometry that is more physiologically relevant, whereas the HEK293 cells have overexpressed human CB_1 which results in a higher receptor to G-protein ratio, which means a large proportion of CB_1 will be uncoupled and exhibit low affinity for agonist.[51] Interestingly, IC_{50} values in the human CB_1 HEK293 membranes were

generally higher than in the mouse CB_1 which would be consistent with this, though differences in the human versus mouse form of the receptor cannot be discounted. While the CB_1 $G\alpha_{16}$ CHO cells have overexpressed CB_1, they also overexpress $G\alpha_{16}$ so the ratio may be favoring coupled/high affinity receptors resulting in a concomitant increase in affinity of the modulator due to cooperativity.

Intrinsic activities of CB_1 allosteric modulators

CB_1 has been demonstrated to possess constitutive activities which are critical to maintain normal physiological functions. SR141716 acts as an CB_1 inverse agonist, reducing the CB_1 signaling on its own. It has been suggested that inhibition of this basal activity results in the adverse effects of SR141716.[52] Hence, we were interested in investigating the intrinsic activities of these allosteric modulators in the absence of the CB_1 agonist CP55,940. As shown in Fig 5, SR141716 imparted significant inverse agonism with an IC_{50} of 2.8 nM. **2** reached the same level of inverse agonism produced by SR141716, but only at the highest concentration of 10 μM (IC_{50} = 1.47 μM). At 10 μM, the allosteric modulators in this new series only exhibited a modest degree of inverse agonism. Indeed, the thiophene analogue **11** produced very little inverse agonism up to 10 μM. These results imply that these CB_1 allosteric modulators may have less propensity to impart adverse effects than SR141716/rimonabant.

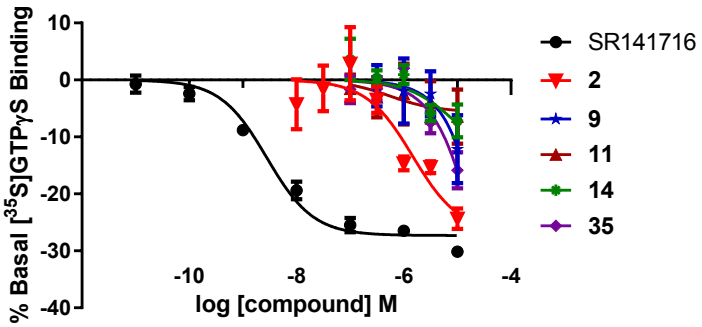


Figure 5. Intrinsic activities of the CB_1 allosteric modulators and SR141716 in the absence of the CB_1 agonist CP55,940.

ADME Studies on compounds 2 and 11

As pharmacokinetics and biodistribution plays an important role determining whether compounds reach the site of action at sufficient levels to impart in vivo activities, we examined preliminary ADME properties for the most potent compound in vitro, **11**, along with **2** (Table 4). Compound **11** ($T_{1/2}$ = 65 min) showed significantly improved metabolic stability than **2** ($T_{1/2}$ = 13 min). As expected with the diarylurea scaffold, **2** displayed poor kinetic solubilities (<1 μM) whereas **11** has slightly better solubility (1.5 μM). The slightly enhanced solubility could be attributed to the less tight packing of the five membered ring thiophene compared to the phenyl ring. Finally, we employed the bidirectional MDCK-MDR1 assay to evaluate the blood-brain barrier permeability and the probability to be a P-glycoprotein substrate. Both **2** and **11** exhibited low permeabilities in this assay, with P_{app} values of 2.6 and 1.6 x 10^{-6} cm/s (A to B), significantly lower than the commonly desired P_{app} value of 15 x 10^{-6} cm/s for CNS-penetrant compounds.[53] However, neither is a P-glycoprotein substrate (efflux ratio < 2.5).[53]

Table 4. ADME properties of **2** and **11**.

Compound	**2**	**11**
Half-life (min) [a]	13.4 ± 4.1	65.0 ± 19.1
Clearance (μL/min/mg) [a]	113.7 ± 34.4	22.2 ± 6.5
Solubility (μM) [b]	< 0.5	1.5 ± 0.1
MDCK-MDR1 P_{app} A to B (x10^{-6} cm/s)[a]	2.6 ± 0	1.6 ± 0.1
MDCK-MDR1 P_{app} B to A (x10^{-6} cm/s)[a]	2.2 ± 0.1	1.1 ± 0.4
MDCK-MDR1 efflux ratio BA/AB	0.8	0.7

[a] Values are expressed as mean ± SD from two independent experiments. [b] Values are expressed as mean ± SD from three independent experiments.

Compound 11 on reinstatement of cocaine-seeking behaviors

Blockade of the CB_1 in vivo by antagonists/inverse agonists has been demonstrated to reduce food intake and self-administration of several drugs of abuse.[54-56] We previously demonstrated that rats pretreated with **1**, **2**, and **4** were less likely to seek drugs of abuse, such as cocaine or methamphetamine, after a period of extinction.[32, 38] Herein, we examined one of the most potent compounds, **11,** in this new series in the reinstatement of cocaine seeking behavior in rats.

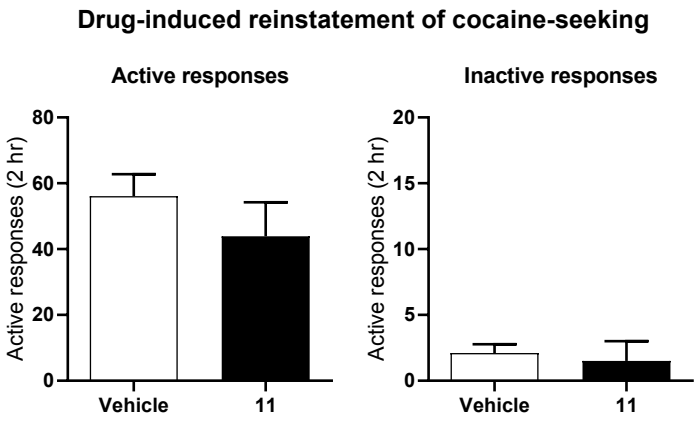


Figure 6. Behavioral effects of **11** (10 mg/kg, i.p.) in the drug-induced reinstatement of cocaine-seeking models in rats (n = 10 male Sprague-Dawley rats/group, $p > 0.05$ by Student's t-test).

As **11** had equipotent or better in vitro potencies compared to **4**, we tested the effect of the same dose of 10 mg/kg (i.p.) pretreatment on drug-induced cocaine-seeking behavior.[32] As shown in Fig. 6, this dose of **11** failed to significantly reduce drug-induced cocaine-seeking behavior ($p > 0.05$). Both active and inactive responses were not significantly altered by 10 mg/kg of **11**. This lack of efficacy is likely due to the poor brain penetration of **11**, as demonstrated in the MDCK-MDR1 studies. Future studies administering the drug directly into the brain may better assess the pharmacological effects of **11** on cocaine-seeking behavior.

Conclusions

In this study, we investigated a series of diarylurea-based CB_1 allosteric modulators centering around modifications on the central phenyl ring of **2**. SAR revealed that replacement with non-aromatic rings resulted in significant reduction of CB_1 modulating activity. Interestingly, the

presence of a five-membered ring such as thiophene led to the identification of **11** with better or comparable potencies than **2** in calcium, cAMP, and [^{35}S]GTPγS binding assays. This series of compounds do not exhibit inverse agonism in [^{35}S]GTPγS, indicating that they may not have liability of psychiatric side effects associated with the CB_1 antagonist/inverse agonist SR141716. Although **11** had good metabolic stability, its solubility and blood-brain barrier permeability need further improvement. In contrast to **4**, **11** showed little effect in attenuating cocaine seeking behavior in rats and seems to be comparable to **2** in this in vivo assay. The unsuccessful extrapolation of in vitro pharmacological potencies to in vivo efficacy in the cocaine relapse model could be due to the limited brain penetration of this compound. If follow-up pharmacokinetic studies demonstrate that **11** is indeed restricted to the periphery, it could stand as a promising candidate for peripherally CB_1-mediated conditions. More importantly, our SAR studies reveal several modifiable regions to optimize pharmacokinetic properties to achieve in vivo efficacy.

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 hexanes, ethyl acetate (EtOAc), dichloromethane, and methanol. Purity and characterization of compounds were established by a combination of high pressure liquid chromatography (HPLC), thin layer chromatography (TLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) analyses. Melting point was recorded by the Mel-Temp II instrument (laboratory Devices Inc., US). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-300 (300 MHz) spectrometer and were determined in $CDCl_3$, DMSO-d_6, or

CD_3OD 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). TLC was performed on EMD precoated silica gel 60 F254 plates, and spots were visualized with UV light or iodine staining. Nominal mass spectra were obtained using an Agilent 1260 Infinity II system (ESI). High resolution mass spectra were obtained using Agilent 1290 Infinity UHPLC-6230 TOF system (ESI). All final 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 using a 15 minute gradient elution of 5-95% solvent B at 1 mL/min followed by 10 minutes at 95% solvent B (solvent A, water with 0.1% TFA; solvent B, acetonitrile with 0.1% TFA and 5% water; absorbance monitored at 220 and 280 nm).

General procedure A. To a mixture of aryl bromide (1 eq), boronic acid (1.1 eq) in dimethoxyethane (0.1 M) was added 1M aqueous $NaHCO_3$ solution (3 eq) followed by $Pd(Ph_3)_4$ (0.075 eq). The reaction mixture was refluxed overnight under nitrogen atmosphere. The reaction mixture was diluted with ethyl acetate, washed with a saturated $NaHCO_3$ solution and brine. The combined organic layers were dried over anhydrous $MgSO_4$ and filtered. The filtrate was concentrated in vacuo and the residue was purified by column chromatography (SiO_2, ethyl acetate/hexanes) to give the desired product.

General procedure B. To a solution of nitrobenzene derivative (1 eq) in ethanol (0.1 M) was added hydrazine hydrate (15 eq). The reaction was stirred at 50 ^oC for 15 min and an excess of Raney nickel slurry in water (1.2 eq) was added slowly. After 1 h, the bubbling ceased, the mixture was cooled to room temperature and filtered through Celite. The filtrate was condensed under reduced pressured and the residue was either used for the next step without purification or purified by column chromatography (SiO_2, ethyl acetate/hexanes) to afford the desired product.

General procedure C. To a solution of aryl amine (1 eq) in anhydrous chloroform (0.04 M) was added 4-chlorophenyl isocyanate (1 eq) at room temperature. The reaction mixture was then heated at 60 ^{o}C for 16 h. The precipitated product was filtered and thoroughly washed with dichloromethane.

General procedure D. To a solution of 4-chloroaniline (1 eq) in toluene (0.2 M) in 10 ml CEM microwave vial was added acid (1.5 eq) , triethylamine (3 eq), and diphenylphosphoryl azide (1.2 eq). The reaction mixture in the capped vial was pre-stirred at room temperature for 2 min before heating at 100 ^{o}C for 5 min by microwave irradiation in the CEM Discover SP microwave reactor. Upon cooling to room temperature, the reaction mixture was diluted with ethyl acetate, acidified to pH 4-5 with 1 N HCl solution. The phases were separated, and the aqueous phase was extracted with ethyl acetate twice. The combined organic fractions were dried over anhydrous magnesium sulphate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (SiO_2, ethyl acetate:hexanes) to yield the desired product.

3-(4-Chlorophenyl)-1-{2-methoxy-5-[6-(pyrrolidin-1-yl)pyridin-2-yl]phenyl}urea (**6**) was prepared from 37 (0.02 g, 0.11 mmol) following the general procedure C as white solid (0.03 g, 64%), m.p. 216-217 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d6) δ 9.58 (s, 1H), 8.85 (d, J = 2.07 Hz, 1H), 8.38 (s, 1H), 7.75 (dd, J = 2.07, 8.48 Hz, 1H), 7.52 - 7.66 (m, 3H), 7.40 (d, J = 8.85 Hz, 2H), 7.15 (d, J = 8.67 Hz, 1H), 7.03 (d, J = 7.54 Hz, 1H), 6.42 (d, J = 8.29 Hz, 1H), 3.99 (s, 3H), 3.48 - 3.59 (m, 4H), 1.99 - 2.10 (m, 4H). 13C NMR (75 MHz, DMSO-d6) d 156.6, 154.3, 152.3, 148.3, 138.8, 137.7, 132.2, 128.6, 128.3, 125.2, 120.4, 119.5, 116.9, 110.6, 106.6, 104.4, 55.9, 46.2, 25.0. HRMS (ESI) *m/z* for $C_{23}H_{23}ClN_4O_2$ [M + H]+: calcd, 423.1582; found, 423.1589.

1-(4-Chlorophenyl)-3-(4-phenylpyridin-2-yl)urea (**7**) was prepared from **38** (0.09 g, 0.51 mmol) following the general procedure C as white solid (0.10 g, 60%), m.p. 264-265 ^{o}C. ^{1}H NMR (300

MHz, DMSO-d_6) δ 10.61 (br. s., 1H), 9.57 (s, 1H), 8.36 (d, *J* = 5.27 Hz, 1H), 7.82 (s, 1H), 7.73 (d, *J* = 6.78 Hz, 2H), 7.57 - 7.61 (m, 2H), 7.54 (d, *J* = 7.72 Hz, 2H), 7.49 - 7.52 (m, 1H), 7.37 (d, *J* = 8.48 Hz, 2H), 7.33 - 7.35 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 153.4, 152.1, 149.6, 147.6, 138.0, 137.4, 129.4, 129.2, 128.7, 126.7, 126.0, 120.3, 115.6, 109.0. HRMS (ESI) *m/z* for $C_{18}H_{14}ClN_3O$ [M + H]+: calcd, 324.0898; found, 324.0910.

1-(4-Chlorophenyl)-3-(6-phenylpyridin-2-yl)urea (**8**) was prepared from **39** (0.08 g, 0.45 mmol) following the general procedure C as white solid (0.08 g, 56%), m.p.239-240 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.73 (br. s., 1H), 9.64 (s, 1H), 8.01 (d, *J* = 7.35 Hz, 2H), 7.83 - 7.90 (m, 1H), 7.50 - 7.60 (m, 5H), 7.47 (d, *J* = 7.91 Hz, 2H), 7.39 (d, *J* = 8.67 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.0, 152.5, 152.1, 139.6, 138.3, 138.0, 129.2, 128.9, 128.8, 126.5, 126.0, 120.2, 114.3, 110.7. HRMS (ESI) *m/z* for $C_{18}H_{14}ClN_3O$ [M + H]+: calcd, 324.0898; found, 324.0901.

1-(4-Chlorophenyl)-3-(5-phenylpyridin-3-yl)urea (**9**) was prepared from **40** (0.07 g, 0.37 mmol) following the general procedure C as white solid (0.04 g, 35%), m.p. 238-240 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.05 (s, 1H), 9.00 (s, 1H), 8.59 (d, *J* = 2.07 Hz, 1H), 8.51 (d, *J* = 1.51 Hz, 1H), 8.23 (s, 1H), 7.69 (d, *J* = 7.16 Hz, 2H), 7.48 - 7.57 (m, 4H), 7.45 (d, *J* = 6.97 Hz, 1H), 7.35 (d, *J* = 8.85 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.6, 141.1, 139.1, 138.4, 137.1, 136.4, 135.5, 129.1, 128.6, 128.2, 126.8, 125.7, 123.2, 120.0. HRMS (ESI) *m/z* for $C_{18}H_{14}ClN_3O$ [M + H]+: calcd, 324.0898; found, 324.0903.

1-(4-Chlorophenyl)-3-(2-phenylpyridin-4-yl)urea (**10**) was prepared from **41** (0.05 g, 0.27 mmol) following the general procedure C as white solid (0.04 g, 45%), m.p. 242-243 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.26 (s, 1H), 9.13 (s, 1H), 8.47 (d, *J* = 5.65 Hz, 1H), 7.96 - 8.02 (m, 3H), 7.43 - 7.55 (m, 5H), 7.33 - 7.41 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 156.8, 152.1, 150.1,

147.4, 139.0, 138.1, 128.9, 128.7, 128.7, 126.4, 126.0, 120.2, 111.3, 108.6. HRMS (ESI) *m/z* for $C_{18}H_{14}ClN_3O$ [M + H]+: calcd, 324.0898; found, 324.0907.

1-(4-Chlorophenyl)-3-(5-phenylthiophen-2-yl)urea (**11**) was prepared from **66** (0.05 g, 0.30 mmol) following the general procedure C as white solid (0.06 g, 57%), m.p. 232-233 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.83 (s, 1H), 8.96 (s, 1H), 7.53 (dd, *J* = 8.19, 14.22 Hz, 4H), 7.31 - 7.41 (m, 4H), 7.20 - 7.26 (m, 2H), 6.58 (d, *J* = 3.96 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 140.6, 138.3, 134.5, 132.5, 129.0, 128.6, 126.4, 125.7, 124.3, 120.8, 120.0, 110.6. HRMS (ESI) *m/z* for $C_{17}H_{13}ClN_2OS$ [M + H]+: calcd, 329.0510; found, 329.0519.

1-(4-Chlorophenyl)-3-(4-phenylthiophen-2-yl)urea (**12**) was prepared from **42** (0.03 g, 0.16 mmol) following the general procedure D as white solid (0.04 g, 65%), m.p. 240-241 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.77 (s, 1H), 9.02 (s, 1H), 7.65 (d, *J* = 7.54 Hz, 2H), 7.52 (d, *J* = 8.48 Hz, 2H), 7.31 - 7.45 (m, 4H), 7.28 (d, *J* = 7.35 Hz, 2H), 6.97 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.6, 141.5, 138.3, 137.8, 135.4, 128.7, 128.6, 126.9, 125.6, 119.9, 111.4, 108.2. HRMS (ESI) *m/z* for $C_{17}H_{13}ClN_2OS$ [M + H]+: calcd, 329.0510; found, 329.0519.

1-(4-Chlorophenyl)-3-(5-phenylthiophen-3-yl)urea (**13**) was prepared from **43** (0.03 g, 0.5 mmol) following the general procedure D as white solid (0.04 g, 65%), m.p. 220-221 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.97 (s, 1H), 8.87 (s, 1H), 7.64 (d, *J* = 1.32 Hz, 1H), 7.61 (s, 1H), 7.48 - 7.52 (m, 2H), 7.39 - 7.46 (m, 3H), 7.27 - 7.37 (m, 4H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 152.2, 141.3, 138.7, 137.7, 133.6, 129.1, 128.6, 127.7, 125.3, 125.0, 119.7, 117.6, 106.0. HRMS (ESI) *m/z* for $C_{17}H_{13}ClN_2OS$ [M + H]+: calcd, 329.0510; found, 329.0503.

1-(4-Chlorophenyl)-3-(5-phenyl-1,3-thiazol-2-yl)urea (**14**) was prepared from **44** (0.03 g, 0.2 mmol) following the general procedure C as white solid (0.02 g, 34%), m.p. 266-268 °C. ^{1}H NMR

(300 MHz, DMSO-d_6) δ 10.77 (br. s., 1H), 9.14 (s, 1H), 7.80 (s, 1H), 7.52 - 7.62 (m, 4H), 7.48 (d, *J* = 8.85 Hz, 1H), 7.33 - 7.45 (m, 5H), 7.26 - 7.32 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 138.5, 137.7, 131.6, 129.1, 128.7, 128.6, 127.3, 126.3, 125.5, 125.4, 120.2, 119.8. HRMS (ESI) *m/z* for $C_{16}H_{12}ClN_3OS$ [M + H]+: calcd, 330.0462; found, 330.0462.

***trans*-1-(4-Chlorophenyl)-3-(2-phenylcyclopropyl)urea** (**15**) was prepared from *trans*-2-phenylcyclopropane-1-carboxylic acid (0.06 g, 0.38 mmol) following the general procedure D as white solid (0.05 g, 80%), m.p. 172-173 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.53 (s, 1H), 7.44 (s, 1H), 7.41 (s, 1H), 7.23 - 7.31 (m, 4H), 7.17 (d, *J* = 7.16 Hz, 1H), 7.10 - 7.15 (m, 2H), 6.64 (d, *J* = 2.64 Hz, 1H), 2.72 (dd, *J* = 4.33, 7.16 Hz, 1H), 1.97 (ddd, *J* = 3.30, 6.36, 9.18 Hz, 1H), 1.10 - 1.21 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 155.6, 141.4, 139.3, 128.4, 128.1, 125.9, 125.5, 124.6, 119.3, 32.7, 24.5, 15.7. HRMS (ESI) *m/z* for $C_{16}H_{15}ClN_2O$ [M + H]+: calcd, 287.0946; found, 287.0955.

***cis*-1-(4-Chlorophenyl)-3-(2-phenylcyclopropyl)urea** (**16**) was prepared from cis-2-phenylcyclopropane-1-carboxylic acid (0.06 g, 0.38 mmol) following the general procedure D as white solid (0.06 g, 83%), m.p. 152-153 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26 (s, 2H), 7.17 - 7.25 (m, 3H), 7.11 - 7.16 (m, 2H), 7.00 - 7.06 (m, 2H), 6.78 (s, 1H), 4.67 (br. s., 1H), 2.84 - 2.95 (m, 1H), 2.25 - 2.37 (m, 1H), 1.38 (td, *J* = 6.50, 9.23 Hz, 1H), 1.08 (dt, *J* = 4.33, 6.40 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.1, 135.9, 134.6, 134.6, 127.8, 127.4, 127.3, 125.7, 120.3, 27.8, 21.5, 11.8. HRMS (ESI) *m/z* for $C_{16}H_{15}ClN_2O$ [M + H]+: calcd, 287.0946; found, 287.0947.

3-(4-Chlorophenyl)-1-[(3R)-1-phenylpiperidin-3-yl]urea (**17**) was prepared from **46** (0.05 g, 0.32 mmol) following the general procedure C as white solid (0.03 g, 32%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.62 (s, 1H), 7.37 - 7.44 (m, 2H), 7.23 - 7.29 (m, 2H), 7.17 - 7.22 (m, 2H), 6.95 (d, *J* = 7.91 Hz, 2H), 6.76 (t, *J* = 7.16 Hz, 1H), 6.35 (d, *J* = 7.72 Hz, 1H), 3.77 (dd, *J* = 3.67, 7.82 Hz,

1H), 3.41 - 3.49 (m, 1H), 3.25 (br. s., 1H), 2.94 - 3.06 (m, 1H), 2.84 (dd, *J* = 7.82, 11.96 Hz, 1H), 1.70 - 1.86 (m, 2H), 1.57 - 1.67 (m, 1H), 1.41 - 1.53 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.4, 151.2, 139.4, 128.9, 128.4, 124.4, 119.0, 118.7, 116.0, 54.4, 48.9, 45.1, 29.7, 22.6. HRMS (ESI) *m/z* for $C_{18}H_{20}ClN_3O$ [M + H]+: calcd, 330.1368; found, 330.1363.

1-(4-Chlorophenyl)-3-[5-(4-fluorophenyl)thiophen-2-yl]urea (**18**) was prepared from **67** (0.01 g, 0.04 mmol) following the general procedure C as white solid (0.01 g, 71%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.84 (s, 1H), 8.98 (s, 1H), 7.58 (dd, *J* = 5.46, 8.85 Hz, 2H), 7.50 (d, *J* = 8.85 Hz, 2H), 7.34 (d, *J* = 8.85 Hz, 2H), 7.15 - 7.25 (m, 3H), 6.57 (d, *J* = 3.96 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 140.6, 138.3, 131.4, 128.6, 126.3, 126.1, 125.7, 120.9, 120.0, 116.0, 115.7, 110.6. HRMS (ESI) *m/z* for $C_{17}H_{12}ClFN_2OS$ [M + H]+: calcd, 347.0416; found, 347.0417.

1-(4-Chlorophenyl)-3-[5-(3-fluorophenyl)thiophen-2-yl]urea (**19**) was prepared from **68** (0.07 g, 0.37 mmol) following the general procedure C as white solid (0.06 g, 44%), m.p. 114-116 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.91 (br. s., 1H), 8.99 (br. s., 1H), 7.51 (d, *J* = 7.91 Hz, 2H), 7.35 (d, *J* = 8.85 Hz, 6H), 6.95 - 7.12 (m, 1H), 6.49 - 6.69 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.3, 161.1, 151.4, 141.4, 138.2, 136.9, 130.9, 128.6, 125.8, 122.2, 120.3, 120.3, 120.0, 112.8, 110.7. HRMS (ESI) *m/z* for $C_{17}H_{12}ClFN_2OS$ [M + H]+: calcd, 347.0416; found, 347.0417.

1-(4-Chlorophenyl)-3-[5-(2,4-difluorophenyl)thiophen-2-yl]urea (**20**) was prepared from **69** (0.03 g, 0.14 mmol) following the general procedure C as white solid (0.04 g, 72%), m.p. 198-199 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.92 (s, 9H), 9.01 (s, 9H), 7.66 - 7.78 (m, 9H), 7.51 (d, *J* = 8.85 Hz, 18H), 7.21 - 7.41 (m, 37H), 7.07 - 7.18 (m, 9H), 6.62 (d, *J* = 3.96 Hz, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 141.9, 138.2, 128.7, 128.6, 125.8, 124.6, 123.7, 120.0, 118.8, 112.3, 112.0, 109.9, 104.6, 96.3. HRMS (ESI) *m/z* for $C_{17}H_{11}ClF_2N_2OS$ [M + H]+: calcd, 365.0321; found, 365.0319.

1-(4-Chlorophenyl)-3-[5-(2-chlorophenyl)thiophen-2-yl]urea (**21**) was prepared from **70** (0.03 g, 0.18 mmol) following the general procedure C as white solid (0.01 g, 13%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.90 (s, 1H), 8.99 (s, 1H), 7.59 (dd, *J* = 1.51, 7.72 Hz, 1H), 7.48 - 7.55 (m, 3H), 7.25 - 7.41 (m, 4H), 7.21 (d, *J* = 3.96 Hz, 1H), 6.62 (d, *J* = 3.96 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 142.3, 138.2, 132.9, 130.5, 130.5, 130.3, 128.6, 128.5, 128.2, 127.6, 125.8, 125.3, 120.0, 109.7. HRMS (ESI) *m/z* for $C_{17}H_{12}Cl_2N_2OS$ [M + H]+: calcd, 363.0120; found, 363.0122.

1-(4-Chlorophenyl)-3-[5-(3-chlorophenyl)thiophen-2-yl]urea (**22**) was prepared from **71** (0.02 g, 0.08 mmol) following the general procedure C as white solid (0.01 g, 64%), m.p. 212-213 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.94 (br. s., 1H), 9.02 (br. s., 1H), 7.61 (d, *J* = 1.70 Hz, 1H), 7.47 - 7.57 (m, 3H), 7.30 - 7.43 (m, 4H), 7.21 - 7.29 (m, 1H), 6.59 (d, *J* = 3.96 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 150.4, 140.5, 137.2, 135.6, 132.8, 129.8, 129.5, 127.6, 124.9, 124.8, 122.6, 121.8, 121.3, 119.0, 109.6. HRMS (ESI) *m/z* for $C_{17}H_{12}Cl_2N_2OS$ [M + H]+: calcd, 363.0120; found, 363.0124.

1-(4-Chlorophenyl)-3-[5-(4-chlorophenyl)thiophen-2-yl]urea (**23**) was prepared from **72** (0.02 g, 0.12 mmol) following the general procedure C as white solid (0.03 g, 60%), m.p. 237-238 ^{o}C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.88 (s, 1H), 8.98 (s, 1H), 7.54 (dd, *J* = 8.57, 18.37 Hz, 4H), 7.37 (dd, *J* = 8.57, 17.80 Hz, 4H), 7.26 (d, *J* = 3.77 Hz, 1H), 6.58 (d, *J* = 3.77 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 150.4, 140.1, 137.2, 132.4, 130.0, 129.6, 127.9, 127.6, 124.8, 120.6, 119.0, 109.6. HRMS (ESI) *m/z* for $C_{17}H_{12}Cl_2N_2OS$ [M + H]+: calcd, 363.0120; found, 363.0121.

1-(4-Chlorophenyl)-3-[5-(3,4-dichlorophenyl)thiophen-2-yl]urea (**24**) was prepared from **73** (0.01 g, 0.03 mmol) following the general procedure C as white solid (0.01 g, 67%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.97 (s, 1H), 9.02 (s, 1H), 7.82 (d, *J* = 2.07 Hz, 1H), 7.57 - 7.62 (m, 1H), 7.48 - 7.54 (m, 3H), 7.39 (d, *J* = 3.96 Hz, 1H), 7.35 (d, *J* = 8.85 Hz, 2H), 6.60 (d, *J* = 3.96 Hz, 1H). ^{13}C

NMR (75 MHz, DMSO-d_6) δ 151.4, 142.0, 138.2, 135.3, 131.7, 131.0, 129.4, 128.6, 128.2, 125.8, 125.5, 124.2, 122.9, 120.0, 110.6. HRMS (ESI) *m/z* for $C_{17}H_{11}Cl_3N_2OS$ [M + H]+: calcd, 396.9730; found, 396.9740.

1-(4-Chlorophenyl)-3-[5-(3,5-dichlorophenyl)thiophen-2-yl]urea (**25**) was prepared from **74** (0.02 g, 0.06 mmol) following the general procedure C as white solid (0.01 g, 54%), m.p. 232-233 ^oC. ^1H NMR (300 MHz, DMSO-d_6) δ 10.01 (br. s., 1H), 9.04 (br. s., 1H), 7.55 - 7.62 (m, 2H), 7.44 - 7.54 (m, 3H), 7.30 - 7.42 (m, 3H), 6.61 (s., 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 150.3, 141.4, 137.0, 137.0, 133.6, 127.7, 127.6, 124.8, 124.1, 122.6, 121.3, 119.0, 109.6. HRMS (ESI) *m/z* for $C_{17}H_{11}Cl_3N_2OS$ [M + H]+: calcd, 396.9730; found, 396.9748.

3-[5-(3-Acetylphenyl)thiophen-2-yl]-1-(4-chlorophenyl)urea (**26**) was prepared from **75** (0.06 g, 0.28 mmol) following the general procedure C as yellow solid (0.01 g, 7%). ^1H NMR (300 MHz, DMSO-d_6) δ 9.91 (br. s., 1H), 9.01 (br. s., 1H), 8.05 (s, 1H), 7.77 - 7.88 (m, 2H), 7.51 (d, J = 5.27 Hz, 3H), 7.30 - 7.42 (m, 3H), 6.56 - 6.65 (m, 1H), 2.63 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 197.8, 151.4, 141.3, 138.2, 137.5, 134.9, 131.3, 129.4, 128.7, 128.6, 126.0, 125.8, 123.4, 121.9, 120.0, 110.6, 26.8. HRMS (ESI) *m/z* for $C_{19}H_{15}ClN_2O_2S$ [M + H]+: calcd, 371.0616; found, 371.0616.

Methyl 3-(5-{[(4-chlorophenyl)carbamoyl]amino}thiophen-2-yl)benzoate (**27**) was prepared from **76** (0.04 g, 0.16 mmol) following the general procedure C as white solid (0.05 g, 85%), m.p. 228-229 ^oC. ^1H NMR (300 MHz, DMSO-d_6) δ 9.93 (s, 1H), 9.01 (s, 1H), 8.07 (s, 1H), 7.86 (d, J = 7.91 Hz, 1H), 7.78 (d, J = 7.72 Hz, 1H), 7.46 - 7.57 (m, 3H), 7.34 (d, J = 8.85 Hz, 3H), 6.60 (d, J = 3.77 Hz, 1H), 3.88 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.0, 151.4, 141.3, 138.2, 135.0, 131.0, 130.4, 129.5, 128.7, 128.6, 126.7, 125.8, 124.4, 121.8, 120.0, 110.6, 52.2. HRMS (ESI) *m/z* for $C_{19}H_{15}ClN_2O_3S$ [M + H]+: calcd, 387.0565; found, 387.0581.

1-(4-Chlorophenyl)-3-[5-(3-methanesulfonylphenyl)thiophen-2-yl]urea (**28**) was prepared from **77** (0.04 g, 0.15 mmol) following the general procedure C as white solid (0.05 g, 75%), m.p. 175-176 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.98 (br. s., 1H), 9.03 (br. s., 1H), 8.03 (s, 1H), 7.90 (s, 1H), 7.70 - 7.79 (m, 1H), 7.59 - 7.68 (m, 1H), 7.48 - 7.57 (m, 2H), 7.40 - 7.46 (m, 1H), 7.28 - 7.39 (m, 2H), 6.63 (s, 1H), 3.29 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 142.0, 141.7, 138.2, 135.7, 130.2, 130.1, 128.8, 128.6, 125.9, 124.2, 122.8, 122.0, 120.0, 110.6, 43.4. HRMS (ESI) *m/z* for $C_{18}H_{15}ClN_2O_3S_2$ [M + H]+: calcd, 407.0285; found, 407.0293.

1-(4-Chlorophenyl)-3-[5-(2-methoxyphenyl)thiophen-2-yl]urea (**29**) was prepared from **78** (0.02 g, 0.15 mmol) following the general procedure C as white solid (0.04 g, 78%), m.p. 177-178 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.72 (br. s., 1H), 8.93 (br. s., 1H), 7.58 - 7.68 (m, 1H), 7.46 - 7.57 (m, 2H), 7.26 - 7.39 (m, 3H), 7.15 - 7.24 (m, 1H), 7.04 - 7.13 (m, 1H), 6.92 - 7.02 (m, 1H), 6.53 - 6.63 (m, 1H), 3.89 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 154.7, 151.4, 141.4, 138.4, 128.6, 128.5, 127.3, 126.8, 125.6, 123.1, 122.7, 120.9, 119.9, 112.1, 109.7, 55.6. HRMS (ESI) *m/z* for $C_{18}H_{15}ClN_2O_2S$ [M + H]+: calcd, 359.0616; found, 359.0625.

1-(4-Chlorophenyl)-3-[5-(3-methoxyphenyl)thiophen-2-yl]urea (**30**) was prepared from **79** (0.04 g, 0.28 mmol) following the general procedure C as white solid (0.06 g, 55%), m.p. 141-142 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.84 (s, 1H), 8.98 (s, 1H), 7.52 (d, *J* = 8.67 Hz, 2H), 7.35 (d, *J* = 8.85 Hz, 2H), 7.21 - 7.28 (m, 2H), 7.06 - 7.17 (m, 2H), 6.75 - 6.85 (m, 1H), 6.58 (d, *J* = 3.77 Hz, 1H), 3.80 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 159.7, 151.4, 140.7, 138.3, 135.8, 132.4, 130.0, 128.6, 125.8, 121.2, 120.0, 116.8, 112.1, 110.5, 109.7, 55.0. HRMS (ESI) *m/z* for $C_{18}H_{15}ClN_2O_2S$ [M + H]+: calcd, 359.0616; found, 359.0617.

1-(4-Chlorophenyl)-3-[5-(4-methoxyphenyl)thiophen-2-yl]urea (**31**) was prepared from **80** (0.02 g, 0.10 mmol) following the general procedure C as white solid (0.03 g, 86%), m.p. 228-229

°C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.73 (br. s., 1H), 8.93 (br. s., 1H), 7.49 (t, *J* = 8.95 Hz, 4H), 7.34 (d, *J* = 8.48 Hz, 2H), 7.07 (d, *J* = 3.01 Hz, 1H), 6.94 (d, *J* = 8.29 Hz, 2H), 6.54 (d, *J* = 3.01 Hz, 1H), 3.76 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 158.6, 151.9, 140.0, 138.8, 133.3, 129.1, 127.7, 126.2, 126.1, 120.4, 119.9, 114.9, 111.1, 55.6. HRMS (ESI) *m/z* for $C_{18}H_{15}ClN_2O_2S$ [M + H]+: calcd, 359.0616; found, 359.0598.

1-(4-Chlorophenyl)-3-[5-(3-methylphenyl)thiophen-2-yl]urea (**32**) was prepared from **81** (0.02 g, 0.12 mmol) following the general procedure C as white solid (0.03 g, 62%), m.p. 186-187 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.81 (br. s., 1H), 8.96 (br. s., 1H), 7.46 - 7.57 (m, 2H), 7.30 - 7.43 (m, 4H), 7.17 - 7.29 (m, 2H), 6.98 - 7.07 (m, 1H), 6.51 - 6.63 (m, 1H), 2.33 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 140.4, 138.3, 138.1, 134.4, 132.7, 128.8, 128.6, 127.1, 125.7, 124.9, 121.5, 120.7, 119.9, 110.6, 21.0. HRMS (ESI) *m/z* for $C_{18}H_{15}ClN_2OS$ [M + H]+: calcd, 343.0666; found, 343.0680.

1-(4-Chlorophenyl)-3-{5-[3-(dimethylamino)phenyl]thiophen-2-yl}urea (**33**) was prepared from **82** (0.06 g, 0.26 mmol) following the general procedure C as white solid (0.06 g, 58%), m.p. 130-131 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.79 (br. s., 1H), 8.97 (br. s., 1H), 7.47 - 7.58 (m, 2H), 7.35 (d, *J* = 6.59 Hz, 2H), 7.10 - 7.22 (m, 2H), 6.79 - 6.92 (m, 2H), 6.51 - 6.65 (m, 2H), 2.93 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 150.7, 140.1, 138.3, 135.0, 133.7, 129.4, 128.6, 125.7, 120.4, 119.9, 112.8, 110.9, 110.4, 108.1. HRMS (ESI) *m/z* for $C_{19}H_{18}ClN_3OS$ [M + H]+: calcd, 372.0932; found, 372.0939.

1-(4-Chlorophenyl)-3-[5-(pyridin-3-yl)thiophen-2-yl]urea (**34**) was prepared from **83** (0.08 g, 0.05 mmol) following the general procedure C as white solid (0.02 g, 88%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.94 (s, 1H), 9.01 (s, 1H), 8.81 (d, *J* = 2.07 Hz, 1H), 8.40 (dd, *J* = 1.32, 4.71 Hz, 1H), 7.93 (td, *J* = 1.81, 8.05 Hz, 1H), 7.51 (d, *J* = 9.04 Hz, 2H), 7.33 - 7.41 (m, 4H), 6.62 (d, *J* = 3.96

Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 151.4, 147.2, 145.2, 141.7, 138.2, 131.3, 130.5, 128.6, 125.8, 123.9, 122.4, 120.0, 119.8, 110.6. HRMS (ESI) *m/z* for $C_{16}H_{12}ClN_3OS$ [M + H]+: calcd, 330.0462; found, 330.0474.

1-(4-Chlorophenyl)-3-[5-(pyridin-4-yl)thiophen-2-yl]urea (**35**) was prepared from **84** (0.004 g, 0.03 mmol) following the general procedure C as yellow solid (0.007 g, 75%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.07 (br. s., 1H), 9.05 (br. s., 1H), 8.42 - 8.53 (m, 2H), 7.43 - 7.61 (m, 5H), 7.36 (d, *J* = 7.91 Hz, 2H), 6.62 - 6.68 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 150.0, 143.2, 141.4, 138.1, 128.9, 128.6, 125.9, 124.3, 120.1, 119.8, 118.3, 110.7. HRMS (ESI) *m/z* for $C_{16}H_{12}ClN_3OS$ [M + H]+: calcd, 330.0462; found, 330.0472.

2-(4-Methoxy-3-nitrophenyl)-6-(pyrrolidin-1-yl)pyridine (**36**) was prepared from 2-bromo-6-(pyrrolidin-1-yl)pyridine (0.30 g, 1.32 mmol)[30] and 4-methoxy-3-nitrophenylboronic acid (0.29 g, 1.45 mmol) following the general procedure A as yellow solid (0.12 g, 30%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.23 (dd, *J* = 1.22, 8.76 Hz, 1H), 7.46 - 7.56 (m, 1H), 7.13 (d, *J* = 8.85 Hz, 1H), 6.91 - 7.00 (m, 2H), 6.34 (d, *J* = 8.48 Hz, 1H), 3.54 (t, *J* = 6.50 Hz, 4H), 1.99 - 2.07 (m, 4H). MS (ESI) *m/z* $[M+H]^+$ calcd: 300.1; found: 300.4.

2-Methoxy-5-[6-(pyrrolidin-1-yl)pyridin-2-yl]aniline (**37**) was prepared from **36** (0.12 g, 0.4 mmol) following the general procedure B as white solid (0.10 g, 93%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (d, *J* = 2.07 Hz, 1H), 7.44 - 7.48 (m, 1H), 7.39 - 7.43 (m, 1H), 6.92 (d, *J* = 7.54 Hz, 1H), 6.83 (d, *J* = 8.48 Hz, 1H), 6.25 (d, *J* = 8.29 Hz, 1H), 3.89 (s, 3H), 3.84 (br. s., 2H), 3.54 (t, *J* = 6.59 Hz, 4H), 2.00 (t, *J* = 6.59 Hz, 4H). MS (ESI) *m/z* $[M+H]^+$ calcd: 270.1; found: 270.3.

4-Phenylpyridin-2-amine (**38**) was prepared from phenylboronic acid (0.10 g, 0.58 mmol) and 4-bromopyridin-2-amine (0.08 g, 0.64 mmol) following the general procedure A as white solid (0.09

g, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (d, J = 5.27 Hz, 1H), 7.62 - 7.73 (m, 2H), 7.51 - 7.61 (m, 3H), 7.38 - 7.49 (m, 5H), 6.88 (d, J = 5.27 Hz, 1H), 6.70 (s, 1H), 4.57 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 171.1; found: 171.1.

6-Phenylpyridin-2-amine (**39**) was prepared from 6-bromopyridin-2-amine (0.10 g, 0.58 mmol) and phenyl boronic acid (0.08 g, 0.64 mmol) following the general procedure A as yellow liquid (0.10 g, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (dd, J = 1.22, 8.19 Hz, 2H), 7.32 - 7.52 (m, 4H), 7.07 (d, J = 7.35 Hz, 1H), 6.43 (d, J = 8.10 Hz, 1H), 4.55 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 171.1; found: 171.2.

5-Phenylpyridin-3-amine (**40**) was prepared from 5-bromopyridin-3-amine (0.10 g, 0.58 mmol) and phenyl boronic acid (0.08 g, 0.64 mmol) following the general procedure A as white solid (0.10 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.24 (d, J = 1.70 Hz, 1H), 8.06 (d, J = 2.45 Hz, 1H), 7.62 - 7.71 (m, 3H), 7.42 - 7.47 (m, 3H), 7.15 (dd, J = 1.88, 2.64 Hz, 1H), 3.89 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 171.1; found: 171.0.

2-Phenylpyridin-4-amine (**41**) was prepared from 2-bromopyridin-4-amine (0.10 g, 0.58 mmol) and phenyl boronic acid (0.08 g, 0.64 mmol) following the general procedure A as yellow liquid (0.04 g, 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (d, J = 5.46 Hz, 1H), 7.91 (s, 2H), 7.32 - 7.49 (m, 3H), 6.93 (s, 1H), 6.47 (dd, J = 2.17, 5.56 Hz, 1H), 4.25 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 171.1; found: 171.2.

4-Phenylthiophene-2-carboxylic acid (**42**) was prepared from 4-bromo-thiophene-2-carboxylic acid (0.21 g, 1.0 mmol) and phenylboronic acid (0.13 g, 1.1 mmol) following the general procedure A as white solid (0.13 g, 62%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.28 (br. s., 1H), 8.11 - 8.32 (m, 1H), 7.71 - 7.96 (m, 1H), 7.62 (d, J = 19.40 Hz, 2H), 7.31 - 7.54 (m, 2H), 7.30 - 7.53 (m, 2H), 7.09

- 7.29 (m, 1H). MS (ESI) *m/z* $[M-H]^-$ calcd: 203.1; found: 203.3. MS (ESI) *m/z* $[M-H]^-$ calcd: 203.1; found: 203.3.

5-Phenylthiophene-3-carboxylic acid (**43**) was prepared from 5-bromothiophene-3-carboxylic acid (0.10 g, 0.5 mmol) and phenylboronic acid (0.07 g, 0.55 mmol) following the general procedure A as white solid (0.10 g, 99%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.16 (s, 1H), 7.75 (s, 1H), 7.63 (d, *J* = 7.16 Hz, 2H), 7.38 - 7.46 (m, 2H), 7.30 - 7.37 (m, 1H). MS (ESI) *m/z* $[M-H]^-$ calcd: 203.1; found: 203.3.

5-Phenyl-1,3-thiazol-2-amine hydrobromide (**44**). To a solution of phenylacetaldehyde (0.49 ml, 4.16 mmol) in dichloromethane (1.5 ml) was added dropwise 15 ml solution of bromine (0.21 ml) at -10 ^{o}C. The reaction mixture was warmed to room temperature and then refluxed for 16 h. After cooling to room temperature, the reaction mixture was quenched with a saturated solution of sodium bicarbonate and extracted with dichloromethane (3x). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and concentrated to afford crude 2-bromo-2-phenylacetaldehyde which was added to a suspension of thiourea (0.38 g, 5 mmol) in ethanol (10 ml). The reaction mixture was refluxed for 8 h. After cooling to room temperature, solvent was evaporated in vacuo and the residue was purified by column chromatography (silica gel, MeOH/dichloromethane) to provide the product as white solid (0.56 g, 77%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.75 (br. s., 2H), 7.36 - 7.46 (m, 5H), 7.21 (s, 1H). MS (ESI) *m/z* $[M+H]^+$ calcd: 177.1; found: 177.4.

***tert*-Butyl N-[(*3R*)-1-phenylpiperidin-3-yl]carbamate** (**45**) To a solution of (*R*)-3-(Boc-amino)piperidine (0.16 g, 1 mmol) in dichloromethane (4 ml) in a sealed tube was added triethylamine (0.28 ml, 2 mmol), copper acetate (0.20 g, 1.1 mmol), and phenylboronic acid (0.27 g, 2.2 mmol). The reaction was purged with nitrogen, sealed, and heated at 60 ^{o}C for 3 days. After

cooling to room temperature, the reaction mixture was filtered through Celite, washed with 10%v/v MeOH/DCM. The filtrate was concentrated in vacuo, and the residue was purified by column chromatography (silica gel, MeOH/DCM) to yield the product as colorless liquid (0.09 g, 32%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.27 - 7.29 (m, 1H), 7.22 - 7.25 (m, 1H), 6.94 (d, J = 7.72 Hz, 2H), 6.82 - 6.89 (m, 1H), 4.93 (br. s., 1H), 3.87 (br. s., 1H), 3.32 (d, J = 11.11 Hz, 1H), 2.94 - 3.21 (m, 3H), 1.54 - 1.88 (m, 4H), 1.46 (s, 9H).

(*3R*)-1-Phenylpiperidin-3-amine hydrochloride (**46**) A solution of 4 N HCl in 1,4-dioxane was added to **45** (0.09 g, 0.32 mmol) and stirred at room temperature for 1 h. Then the reaction mixture was concentrated under reduced pressure to yield the desired product as white solid (0.07 g, quant.). MS (ESI) *m/z* $[M+H]^+$ calcd: 176.1; found: 176.3.

2-Nitro-5-phenylthiophene (**47**) was prepared from 2-bromo-5-nitrothiophene (0.20 g, 0.96 mmol) phenyl boronic acid (0.13 g, 1.06 mmol) following the general procedure A as yellow liquid (0.06 g, 28%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.91 (d, J = 4.10 Hz, 1H), 7.61-7.64 (m, 2H), 7.42-7.48 (m, 3H), 7.24 (d, J = 5.20 Hz, 1H) ppm. MS (ESI) *m/z* $[M-H]^-$ calcd: 203.1; found: 203.3.

2-(4-Fluorophenyl)-5-nitrothiophene (**48**) was prepared from 2-bromo-5-nitrothiophene (0.20 g, 0.96 mmol) and 4-fluorophenylboronic acid (0.15 g, 1.06 mmol) following the general procedure A as white solid (0.03 g, 12%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, J = 4.33 Hz, 1H), 7.62 (dd, J = 5.18, 8.76 Hz, 2H), 7.11 - 7.21 (m, 3H).

2-(3-Fluorophenyl)-5-nitrothiophene (**49**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-fluorophenylboronic acid (0.15 g, 1.1 mmol) following the general procedure A as yellow solid (0.10 g, 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (d, J = 4.33 Hz, 1H), 7.39 - 7.49 (m, 2H), 7.29 - 7.36 (m, 1H), 7.25 (d, J = 4.33 Hz, 1H), 7.09 - 7.18 (m, 1H).

2-(2,4-Difluorophenyl)-5-nitrothiophene (**50**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 2,4-fluorophenylboronic acid (0.20 g, 1.0 mmol) following the general procedure A as yellow solid (0.07 g, 29%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (d, *J* = 0.75 Hz, 1H), 7.64 (dt, *J* = 6.03, 8.85 Hz, 1H), 7.34 (d, *J* = 4.33 Hz, 1H), 6.93 - 7.05 (m, 2H).

2-(2-Chlorophenyl)-5-nitrothiophene (**51**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 2-chlorophenylboronic acid (0.21 g, 1.0 mmol) following the general procedure A as yellow solid (0.08 g, 33%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (d, *J* = 4.33 Hz, 1H), 7.50 - 7.57 (m, 2H), 7.34 - 7.40 (m, 2H), 7.29 (d, *J* = 4.33 Hz, 1H).

2-(3-Chlorophenyl)-5-nitrothiophene (**52**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-chlorophenylboronic acid (0.17 g, 1.0 mmol) following the general procedure A as yellow solid (0.09 g, 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (d, *J* = 4.33 Hz, 1H), 7.59 - 7.63 (m, 1H), 7.48 - 7.53 (m, 1H), 7.38 - 7.44 (m, 2H), 7.23 - 7.27 (m, 1H).

2-(4-Chlorophenyl)-5-nitrothiophene (**53**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 4-chlorophenylboronic acid (0.17 g, 1.0 mmol) following the general procedure A as yellow solid (0.09 g, 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (d, *J* = 4.33 Hz, 1H), 7.54 - 7.59 (m, 2H), 7.41 - 7.46 (m, 2H), 7.22 (d, *J* = 4.33 Hz, 1H).

2-(3,4-Dichlorophenyl)-5-nitrothiophene (**54**) was prepared from 2-bromo-5-nitrothiophene (0.20 g, 1 mmol) and 2,4-dichlorophenylboronic acid (0.20 g, 1.1 mmol) following the general procedure A as yellow solid (0.02 g, 8%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 (d, *J* = 4.33 Hz, 1H), 7.72 (d, *J* = 2.07 Hz, 1H), 7.55 (s, 1H), 7.43 - 7.47 (m, 1H), 7.24 (d, *J* = 4.33 Hz, 1H).

2-(3,5-Dichlorophenyl)-5-nitrothiophene (**55**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3,5-dichlorophenylboronic acid (0.21 g, 1.1 mmol) following the general

procedure A as yellow solid (0.05 g, 16%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 - 7.94 (m, 1H), 7.50 (br. s., 2H), 7.40 - 7.45 (m, 1H), 7.24 - 7.29 (m, 1H).

1-[3-(5-Nitrothiophen-2-yl)phenyl]ethan-1-one (**56**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-(methoxycarbonyl)phenylboronic acid (0.20 g, 1.1 mmol) following the general procedure A as yellow solid (0.09 g, 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (s, 1H), 8.00 (d, *J* = 6.59 Hz, 1H), 7.91 - 7.96 (m, 1H), 7.77 - 7.86 (m, 1H), 7.53 - 7.63 (m, 1H), 7.23 - 7.37 (m, 2H), 2.67 (s, 3H).

Methyl 3-(5-nitrothiophen-2-yl)benzoate (**57**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-acetoxyphenylboronic acid (0.18 g, 1.1 mmol) following the general procedure A as red solid (0.09 g, 33%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (s, 1H), 8.10 (d, *J* = 7.91 Hz, 1H), 7.91 - 7.96 (m, 1H), 7.81 (d, *J* = 7.91 Hz, 1H), 7.51 - 7.59 (m, 1H), 7.33 (d, *J* = 4.33 Hz, 1H), 3.97 (s, 3H).

2-(3-Methanesulfonylphenyl)-5-nitrothiophene (**58**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-(methylsulfonyl)phenylboronic acid (0.22 g, 1.1 mmol) following the general procedure A as red solid (0.08 g, 28%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (t, *J* = 1.60 Hz, 1H), 8.01 (d, *J* = 7.91 Hz, 1H), 7.95 (d, *J* = 4.33 Hz, 1H), 7.90 (d, *J* = 7.91 Hz, 1H), 7.66 - 7.73 (m, 1H), 7.37 (d, *J* = 4.33 Hz, 1H), 3.12 (s, 3H).

2-(2-Methoxyphenyl)-5-nitrothiophene (**59**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 2-methoxyphenylboronic acid (0.17 g, 1.1 mmol) following the general procedure A as yellow solid (0.20 g, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, *J* = 4.52 Hz, 1H), 7.73 (dd, *J* = 1.22, 7.82 Hz, 1H), 7.38 - 7.44 (m, 2H), 7.01 - 7.11 (m, 2H), 4.01 (s, 3H).

2-(3-Methoxyphenyl)-5-nitrothiophene (**60**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-methoxyphenylboronic acid (0.17 g, 1.1 mmol) following the general procedure A as yellow solid (0.15 g, 66%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, *J* = 4.33 Hz, 1H), 7.31 - 7.35 (m, 1H), 7.18 - 7.25 (m, 2H), 7.13 (t, *J* = 1.98 Hz, 1H), 6.98 (dd, *J* = 1.98, 8.19 Hz, 1H), 3.87 (s, 3H).

2-(4-Methoxyphenyl)-5-nitrothiophene (**61**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 4-methoxyphenylboronic acid (0.17 g, 1.1 mmol) following the general procedure A as yellow solid (0.15 g, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 - 7.91 (m, 1H), 7.57 (d, *J* = 8.10 Hz, 2H), 7.10 - 7.17 (m, 1H), 6.97 (d, *J* = 6.41 Hz, 2H), 3.86 (s, 3H).

2-(3-Methylphenyl)-5-nitrothiophene (**62**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-methylphenylboronic acid (0.17 g, 1.1 mmol) following the general procedure A as yellow solid (0.08 g, 35%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, *J* = 4.33 Hz, 1H), 7.41 - 7.46 (m, 2H), 7.34 (t, *J* = 7.82 Hz, 1H), 7.21 - 7.27 (m, 2H), 2.42 (s, 3H).

***N*,*N*-Dimethyl-3-(5-nitrothiophen-2-yl)aniline** (**63**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-(*N*,*N*-dimethylamino)phenylboronic acid (0.18 g, 1.1 mmol) following the general procedure A as orange solid (0.06 g, 26%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.87 (d, *J* = 4.33 Hz, 1H), 7.25 - 7.32 (m, 1H), 7.21 (d, *J* = 4.33 Hz, 1H), 6.95 (d, *J* = 7.72 Hz, 1H), 6.86 (s, 1H), 6.78 (dd, *J* = 1.88, 8.29 Hz, 1H), 3.01 (s, 6H).

3-(5-Nitrothiophen-2-yl)pyridine (**64**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 3-pyridylboronic acid (0.14 g, 1.1 mmol) following the general procedure A as orange solid (0.07 g, 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.92 (d, *J* = 1.88 Hz, 1H), 8.68 (d, *J* = 3.96 Hz,

1H), 7.89 - 7.97 (m, 2H), 7.42 (dd, *J* = 4.90, 7.91 Hz, 1H), 7.32 (d, *J* = 4.14 Hz, 1H). MS (ESI) *m/z* $[M+H]^+$ calcd: 207.1; found: 207.1.

4-(5-Nitrothiophen-2-yl)pyridine (**65**) was prepared from 2-bromo-5-nitrothiophene (0.21 g, 1 mmol) and 4-pyridylboronic acid (0.14 g, 1.1 mmol) following the general procedure A as yellow solid (0.07 g, 17%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.69 - 8.76 (m, 2H), 7.95 (d, *J* = 4.14 Hz, 1H), 7.48 - 7.54 (m, 2H), 7.43 (d, *J* = 4.33 Hz, 1H). MS (ESI) *m/z* $[M+H]^+$ calcd: 207.1; found: 207.2.

5-Phenylthiophen-2-amine (**66**) was prepared from **47** (0.06 g, 0.3 mmol) following the general procedure B as white solid (0.05 g, 96%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46 (s, 2H), 7.31 (d, *J* = 15.26 Hz, 2H), 7.18 (d, *J* = 7.54 Hz, 1H), 6.93 (s, 1H), 6.15 (d, *J* = 3.77 Hz, 1H), 3.82 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 176.1; found: 176.1.

5-(4-Fluorophenyl)thiophen-2-amine (**67**) was prepared from **48** (0.03 g, 0.12 mmol) following the general procedure B as white solid (0.01 g, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 - 7.46 (m, 2H), 6.95 - 7.07 (m, 2H), 6.83 (d, *J* = 2.45 Hz, 1H), 6.11 - 6.19 (m, 1H), 3.82 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 194.1; found: 194.2.

5-(3-Fluorophenyl)thiophen-2-amine (**68**) was prepared from **49** (0.10 g, 0.45 mmol) following the general procedure B as white solid (0.07 g, 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (d, *J* = 9.42 Hz, 2H), 7.14 (d, *J* = 9.80 Hz, 1H), 6.91 - 7.00 (m, 1H), 6.80 - 6.90 (m, 1H), 6.10 - 6.20 (m, 1H), 3.88 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 194.1; found: 194.3.

5-(2,4-Difluorophenyl)thiophen-2-amine (**69**) was prepared from **50** (0.07 g, 0.68 mmol) following the general procedure B as white solid (0.03 g, 49%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 (dt, *J* = 6.41, 8.57 Hz, 15H), 7.01 (dd, *J* = 1.13, 3.77 Hz, 14H), 6.79 - 6.90 (m, 29H), 6.17 (d, *J* = 3.77 Hz, 14H), 3.88 (br. s., 28H). MS (ESI) *m/z* $[M+H]^+$ calcd: 212.1; found: 212.1.

5-(2-Chlorophenyl)thiophen-2-amine (**70**) was prepared from **51** (0.08 g, 0.33 mmol) following the general procedure B as white solid (0.04 g, 56%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45 (dd, *J* = 1.51, 7.72 Hz, 1H), 7.41 (dd, *J* = 1.32, 7.72 Hz, 1H), 7.19 - 7.24 (m, 1H), 7.16 (dd, *J* = 1.51, 7.54 Hz, 1H), 7.03 (s, 1H), 6.19 (d, *J* = 3.77 Hz, 1H), 3.87 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 210.1; found: 210.1.

5-(3-Chlorophenyl)thiophen-2-amine (**71**) was prepared from **52** (0.09 g, 0.38 mmol) following the general procedure B as white solid (0.04 g, 20%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (t, *J* = 1.70 Hz, 1H), 7.28 - 7.34 (m, 1H), 7.19 - 7.24 (m, 1H), 7.09 - 7.16 (m, 1H), 6.94 (d, *J* = 3.77 Hz, 1H), 6.15 (d, *J* = 3.77 Hz, 1H), 3.89 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 210.0; found: 210.1.

5-(4-Chlorophenyl)thiophen-2-amine (**72**) was prepared from **53** (0.10 g, 0.40 mmol) following the general procedure B as white solid (0.03 g, 29%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33 - 7.40 (m, 2H), 7.23 - 7.30 (m, 2H), 6.90 (d, *J* = 3.77 Hz, 1H), 6.15 (d, *J* = 3.58 Hz, 1H), 3.86 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 210.0; found: 210.2.

5-(2,4-Dichlorophenyl)thiophen-2-amine (**73**) was prepared from **54** (0.02 g, 0.07 mmol) following the general procedure B as white solid (0.01 g, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50 (d, *J* = 2.07 Hz, 1H), 7.33 - 7.39 (m, 1H), 7.23 (d, *J* = 2.26 Hz, 1H), 6.92 (d, *J* = 3.77 Hz, 1H), 6.15 (d, *J* = 3.77 Hz, 1H), 3.91 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 244.0; found: 244.0.

5-(3,5-Dichlorophenyl)thiophen-2-amine (**74**) was prepared from **55** (0.05 g, 0.16 mmol) following the general procedure B as light yellow solid (0.01 g, 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.29 (d, *J* = 1.70 Hz, 2H), 7.13 (s, 1H), 6.95 (d, *J* = 3.77 Hz, 1H), 6.14 (d, *J* = 3.77 Hz, 1H), 3.95 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 244.0; found: 244.1.

1-[3-(5-Aminothiophen-2-yl)phenyl]ethan-1-one (**75**) was prepared from **56** (0.10 g, 0.40 mmol) following the general procedure B as yellow solid (0.06 g, 68%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 - 8.05 (m, 1H), 7.74 (d, *J* = 6.40 Hz, 1H), 7.63 (d, *J* = 5.84 Hz, 1H), 7.41 (d, *J* = 7.16 Hz, 1H), 6.96 - 7.05 (m, 1H), 6.13 - 6.24 (m, 1H), 3.91 (br. s., 2H), 2.62 (s, 3H). MS (ESI) *m/z* $[M+H]^+$ calcd: 218.1; found: 218.2.

Methyl 3-(5-aminothiophen-2-yl)benzoate (**76**) was prepared from **57** (0.09 g, 0.33 mmol) following the general procedure B as yellow solid (0.04 g, 49%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 - 8.16 (m, 1H), 7.83 (d, *J* = 6.22 Hz, 1H), 7.57 - 7.67 (m, 1H), 7.32 - 7.44 (m, 1H), 6.98 - 7.05 (m, 1H), 6.13 - 6.21 (m, 1H), 3.93 (s., 3H), 3.82 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 234.1; found: 234.3.

5-(3-Methanesulfonylphenyl)thiophen-2-amine (**77**) was prepared from **58** (0.08 g, 0.28 mmol) following the general procedure B as yellow solid (0.04 g, 54%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.98 (t, *J* = 1.79 Hz, 1H), 7.64 - 7.73 (m, 2H), 7.45 - 7.54 (m, 1H), 7.05 (d, *J* = 3.77 Hz, 1H), 6.17 (d, *J* = 3.77 Hz, 1H), 3.99 (br. s., 2H), 3.07 (s, 3H). MS (ESI) *m/z* $[M+H]^+$ calcd: 254.1; found: 254.3.

5-(2-Methoxyphenyl)thiophen-2-amine (**78**) was prepared from **59** (0.20 g, 0.85 mmol) following the general procedure B as white solid (0.04 g, 18%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51 (dd, *J* = 1.51, 7.72 Hz, 1H), 7.09 - 7.21 (m, 2H), 6.89 - 6.99 (m, 2H), 6.17 (d, *J* = 3.77 Hz, 1H), 3.90 (s, 3H), 3.80 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 206.1; found: 206.2.

5-(3-Methoxyphenyl)thiophen-2-amine (**79**) was prepared from **60** (0.15 g, 0.65 mmol) following the general procedure B as yellow solid (0.06 g, 43%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21 (d, *J* = 7.91 Hz, 1H), 7.05 (d, *J* = 7.72 Hz, 1H), 6.99 (t, *J* = 1.98 Hz, 1H), 6.92 (d, *J* = 3.77

Hz, 1H), 6.71 - 6.76 (m, 1H), 6.15 (d, *J* = 3.77 Hz, 1H), 3.80 (s, 3H). MS (ESI) *m/z* $[M+H]^+$ calcd: 206.1; found: 206.2.

5-(4-Methoxyphenyl)thiophen-2-amine (**80**) was prepared from **61** (0.19 g, 0.80 mmol) following the general procedure B as white solid (0.02 g, 12%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (d, *J* = 8.67 Hz, 2H), 6.87 (d, *J* = 8.67 Hz, 2H), 6.79 (d, *J* = 3.77 Hz, 1H), 6.15 (d, *J* = 3.58 Hz, 1H), 3.81 (s, 3H). MS (ESI) *m/z* $[M+H]^+$ calcd: 206.1; found: 206.2.

5-(3-Methylphenyl)thiophen-2-amine (**81**) was prepared from **62** (0.08 g, 0.35 mmol) following the general procedure B as orange liquid (0.02 g, 35%). ^{1}H NMR (300 MHz, $CHCl_3$) δ 7.24 - 7.29 (m, *J* = 5.70 Hz, 2H), 7.21 (d, *J* = 7.35 Hz, 1H), 7.00 (d, *J* = 7.35 Hz, 1H), 6.91 (d, *J* = 3.58 Hz, 1H), 6.15 (d, *J* = 3.77 Hz, 1H), 3.80 (br. s., 2H), 2.35 (s, 3H). MS (ESI) *m/z* $[M+H]^+$ calcd: 190.1; found: 190.3.

5-[3-(Dimethylamino)phenyl]thiophen-2-amine (**82**) was prepared from **63** (0.06 g, 0.26 mmol) following the general procedure B as orange liquid (0.06 g, quant.). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.18 (t, *J* = 8.01 Hz, 1H), 6.90 (d, *J* = 3.77 Hz, 1H), 6.84 (d, *J* = 7.72 Hz, 1H), 6.80 (t, *J* = 1.98 Hz, 1H), 6.59 (dd, *J* = 2.17, 8.38 Hz, 1H), 6.13 (d, *J* = 3.77 Hz, 1H), 3.77 (br. s., 2H), 2.95 (s, 6H). MS (ESI) *m/z* $[M+H]^+$ calcd: 219.1; found: 219.3.

5-(Pyridin-3-yl)thiophen-2-amine (**83**) was prepared from **64** (0.07 g, 0.34 mmol) following the general procedure B as white liquid (0.06 g, 14%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.73 (d, *J* = 2.07 Hz, 1H), 8.40 (dd, *J* = 1.32, 4.71 Hz, 1H), 7.70 (td, *J* = 1.88, 8.10 Hz, 1H), 7.20 - 7.25 (m, 1H), 6.99 (d, *J* = 3.77 Hz, 1H), 6.19 (d, *J* = 3.77 Hz, 1H), 3.94 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 177.1; found: 177.4.

5-(Pyridin-4-yl)thiophen-2-amine (**84**) was prepared from **65** (0.04 g, 0.17 mmol) following the general procedure B as white liquid (0.005 g, 16%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 - 8.50 (m, 2H), 7.27 - 7.31 (m, 2H), 7.17 (d, *J* = 3.77 Hz, 1H), 6.18 (d, *J* = 3.77 Hz, 1H), 4.06 (br. s., 2H). MS (ESI) *m/z* $[M+H]^+$ calcd: 177.1; found: 177.3.

Calcium Mobilization Assay. CHO-RD-HGA16 cells (Molecular Devices, CA) stably expressing the human CB_1 receptor were plated into 96-well black-walled assay plates at 25,000 cells/well in 100 μL of Ham's F12 (supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin, and 100 μg/mL Normocin) and incubated overnight at 37 °C, 5% CO_2. Calcium 5 dye (Molecular Devices, CA) was reconstituted according to the manufacturer's instructions. The reconstituted dye was diluted 1:40 in prewarmed (37 °C) assay buffer (1x 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 solution. For antagonist assays to determine IC_{50} values, the EC_{80} concentration of CP55,940 was prepared at 10x 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 10x 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, CA). 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 10s time point (excitation/emission: 485/525 nm). Relative fluorescence units (RFU) were plotted against the log of compound concentrations. 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 10x 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 the CB_2 agonist and antagonist assays, the same procedures were followed except that stable human CB_2-CHO-RD-HGA16 cells were used. IC_{50} values were determined from at least three independent experiments in duplicate.

[^{35}S]GTPγS Binding Assay. Source of CB_1 came from cerebella of male ICR mice (6-8 weeks old; Enviga International, Indianapolis, IN) or HEK293 cells stably expressing the human CB_1 (see following section for cell culture methods). Mouse cerebellar membranes were preincubated in assay buffer (50 mM Tris, 3 mM $MgCl_2$, 0.2 mM EGTA, pH 7.4) for 10 min with 3 units/ml adenosine deaminase (Sigma Aldrich, St. Louis, MO) to remove endogenous adenosine. Membranes (10 μg protein) from both sources were pre-equilibrated in assay buffer with 0.5% bovine serum album (BSA), 10 or 100 nM CP55,940, allosteric modulator, and 30 μM GDP in a total volume of 490 μL for 30 min. 0.1 nM [^{35}S]GTPγS (Perkin Elmer Life Sciences, Boston, MA) was added to bring the final volume to 500 μL and start the reaction which lasted for 60 min at 30°C. Non-specific binding was determined by including 30 μM unlabeled GTPγS. For inverse agonism experiments, compounds were incubated for 60 min in the absence of CP55,940. Reactions were terminated by vacuum filtration through GF/C filterplates (Perkin Elmer) and cold rinse buffer (50 mM Tris, 0.1% BSA, pH 7.4). Plates were dried, 35 μL of Microscint-20 (Perkin Elmer) was added to each well, sealed with TopSeal-A, and counted on a Packard TopCount NXT. IC_{50} values were determined from at least three independent experiments in duplicate.

cAMP Assay. Forskolin (FSK)-stimulated cyclic adenosine monophosphate (cAMP) production was measured in real-time using HEK293 cells stably expressing the human CB_1 and a transiently transfected bioluminescence resonance energy transfer (BRET) biosensor containing a cAMP binding domain (Epac1) flanked by yellow fluorescent protein (YFP) and Renilla Luciferase (RLuc) assay (CAMYEL).[57] Cells were grown were grown on 100 mm culture dishes to 80-90% confluence in Dulbecco's Modified Eagle's Media/F12 (10-092-CV; Corning Cellgro, Manassas, VA) with 10% fetal bovine serum (FBS-BBT; Rocky Mountain Biological Laboratory, CO, USA), and 50 unit/ml penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA) under 5% CO_2 atmosphere at 37°C A plate reader measures both wavelengths and their ratio, 460/535, is calculated to quantify cAMP levels where increases in the ratio indicate increases in cAMP. Human Embryonic Kidney 293 (HEK293) cells stably transfected with the human cannabinoid type-1 (CB_1) were maintained at 37°C at 5% CO_2 and seeded in 100 mM dishes for transfection. The next day, cells were given fresh growth media and transfected with 5 µg of pcDNA3L-His-CAMYEL using linear polyethyleneimine (25 kDa, Polysciences, Warrington, PA) in 1:6 DNA:PEI (ATCC, Manassas, VA, USA) ratio. The next day, cells were lifted using 1 mM EDTA in PBS and centrifuged at 200 x *g* for 5 min. The supernatant was removed, and cells were resuspended in growth media and plated on poly-D-lysine (Sigma Aldrich, St. Louis, MO) coated white 96 well plates (Perkin Elmer, Waltham, MA) at 60,000 cells per well, filling 2 columns of 8 wells each per plate, i.e. 8 samples in duplicate per plate. The following day, the media was removed, cells were rinsed with PBS and buffers/reagents/drugs added as following: At 0 min, 175 µL of stimulation buffer (5 mg/ml bovine serum albumin in HBSS including Ca^{2+} and Mg^{2+}); at 10 min, 25 µL of allosteric modulators added; at 15 min: 25 µL coelenterazine added (5 µM final); at 25 min, 25 µL of forskolin (10 µM final) with or without CP55,940 (100 nM final)

added. Immediately following addition of forskolin and the probe agonist CP55,940, luminescence was measured at 460 nm and 535 nm simultaneously for 1 s per well for ~22 min at 37°C using a Clariostar plate reader (BMG Labtech, Ortenberg, Germany). IC_{50} values were determined from at least three independent experiments in duplicate.

[^{3}H]CP55,940 equilibrium binding assay. Experiments were performed similarly to [^{35}S]GTPγS binding with the following specifications. Membranes from HEK293 cells stably expressing the human CB_1 were incubated in assay buffer for 90 min at 30°C with 1 nM [^{3}H]CP55,940 and multiple concentrations of **2** or **11**. Non-specific binding was determined by adding 1 μM unlabeled CP55,940. Data are from at least three independent experiments performed in duplicate.

Reinstatement of extinguished cocaine-seeking behavior. Adult (12 weeks old at the start of the experiment) male Sprague-Dawley rats (Envigo, Chicago, IL) weighing 280-300 g were used in the study. Animals were housed individually on a 12/12 hr light/dark cycle (behavioral experiments were conducted during the light period) with free access to water and food except during experimental sessions. Animals were maintained and experiments conducted in accordance with the Institutional Animal Care and Use Committee, University at Buffalo, and with the 2011 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington DC).

Drug self-administration, extinction and reinstatement: The reinstatement procedure was described in detail elsewhere.[38, 58] Briefly, rats were surgically implanted with a chronic indwelling jugular catheter. After one-week recovery, rats were trained to press the active lever (left lever) for infusion of cocaine (0.75 mg/kg/inf) under a fixed ratio [FR] schedule (starting FR =1, which was increased to FR 5 within 5 training sessions) schedule during daily 2-hr sessions for 14 days.

Reinforcer deliveries were accompanied by the presentation of a stimulus light over the active lever followed by a 30-s time-out period during which lever presses had no programmed consequence. Following acquisition of cocaine self-administration, extinction of drug-seeking behavior took place during 2-hr daily sessions in which lever pressing produced no consequence. All other conditions remained unchanged. After 7 days of extinction, all rats reached the extinction criteria (total responses less than 20% of the training sessions).

Drug-induced reinstatement test was conducted on the day following the last extinction session. Rats were pretreated with vehicle (10 rats) or compound **11** (10 mg/kg, 10 rats) 10 min prior to a priming injection of cocaine (10 mg/kg, i.p.) administered immediately before the start of the reinstatement session. Data are expressed as mean ± S.E.M.

Data Analysis

IC_{50} values for calcium mobilization, cAMP and [^{35}S]GTPγS were calculated by non-linear regression fitting a three-parameter logistic curve using Prism 6 (Graphpad Software, San Diego, CA). For [^{35}S]GTPγS experiments, data were normalized to maximal CP55,940 stimulation in the absence of test compound (i.e., vehicle = 100%) or normalized to basal (i.e., vehicle = 0%) stimulation for experiments assessing inverse agonism. For cAMP data, the ratio of 460/535 was calculated for each time point and plotted across time and area under the curve analysis was conducted for each replicate and averaged by condition/day where each day served as an independent experiment. The cAMP data were calculated as %FSK using the formula [(sample – basal) / (forskolin – basal) x 100] with curve-fit top and bottom shared. Data are plotted as the mean ± SEM of at least N=3 independent experiments. Time course data for cAMP were calculated as the 460/535 BRET ratio and an extra-sum-of-squares F-test was used to determine if a one- or two-phase association model fit the data better. Differences between inhibitory potencies against

100 nM and 1 μM of CP55,940 in [^{35}S]GTPγS were analyzed by two-way ANOVA with Bonferroni post hoc test using Prism 6. Radioligand binding data were normalized to the specific binding of [^{3}H]CP55,940 in the absence of modulator as percent and fit 3 parameter logistic non-linear regression. The effects of compounds **11** on reinstatement were analyzed by Student's t-test. $P < 0.05$ is considered statistically significant.

Metabolic stability assessment was performed by Paraza Pharma Inc. (Montreal, Canada). Compounds were incubated with rat liver microsomes at 37 °C for a total of 45 minutes. The reaction was performed at pH 7.4 in 100 mM potassium phosphate buffer containing 0.5 mg/mL of rat liver microsomal protein. Phase I metabolism was assessed by adding NADPH to a final concentration of 1 mM and collecting samples at time points 0, 5, 15, 30 and 45 minutes. All collected samples were quenched 1:1 with ice-cold stop solution (1 μM labetalol and 1 μM glyburide in acetonitrile), and centrifuged to remove precipitated protein. Resulting supernatants were further diluted 1:4 with acetonitrile:water (1:1). Samples were analyzed by LC/MS/MS and calculations for half-life, and *in-vitro* clearance were accomplished using Microsoft Excel (2007). Half-life and clearance were determined from two independent experiments in duplicate.

Kinetic solubility assessment was performed by Paraza Pharma Inc. (Montreal, Canada). A 10 μL of test compound stock solution (20 mM DMSO) was combined with 490 μL of phosphate buffer solution to reach a targeted concentration of 400 μM. The solution was agitated on a VX-2500 multi-tube vortexer (VWR) for 2 hours at room temperature. Following agitation, the sample was filtrated on a glass-fiber filter (1 μm) and the eluate was diluted 400-fold with a mixture of acetonitrile: water (1:1). On each experimental occasion, nicardipine and imipramine were assessed as reference compounds for low and high solubility, respectively. All samples were assessed in triplicate and analyzed by LC-MS/MS using electrospray ionization against standards

prepared in the same matrix. Solubility was determined from three independent experiments in duplicate.

Bidirectional MDCK-MDR1 permeability assay was performed by Paraza Pharma Inc. (Montreal, Canada). MDCK-mdr1 cells at passage 5 were seeded onto permeable polycarbonate supports in 12-well Costar Transwell plates and allowed to grow and differentiate for 3 days. On day 3, culture medium (DMEM supplemented with 10% FBS) was removed from both sides of the transwell inserts and cells were rinsed with warm HBSS. After the rinse step, the chambers were filled with warm transport buffer (HBSS containing 10 mM HEPES, 0.25% BSA, pH 7.4) and the plates were incubated at 37 ^{o}C for 30 min prior to TEER (Trans Epithelial Electric Resistance) measurements.

The buffer in the donor chamber (apical side for A-to-B assay, basolateral side for B-to-A assay) was removed and replaced with the working solution (10 μM test article in transport buffer). The plates were then placed at 37 ^{o}C under light agitation. At designated time points (30, 60 and 90 min), an aliquot of transport buffer from the receiver chamber was removed and replenished with fresh transport buffer. Samples were quenched with ice-cold ACN containing internal standard and then centrifuged to pellet protein. Resulting supernatants are further diluted with 50/50 ACN/H_2O (H_2O only for Atenolol) and submitted for LC-MS/MS analysis. Reported apparent permeability (Papp) values were calculated from single determination. Atenolol and propranolol were tested as low and moderate permeability references. Bidirectional transport of digoxin was assessed to demonstrate Pgp activity/expression.

The apparent permeability (Papp, measured in cm/s) of a compound is determined according to the following formula from two indendepent experiments in duplicate.

$$\text{Papp} = \frac{(\text{dQ})/(\text{dt})}{\text{A} * \text{Ci} * 60}$$

dQ/dt is the net rate of appearance in the receiver compartment

A is the area of the Transwell measured in cm^2 (1.12 cm^2)

Ci is the initial concentration of compound added to the donor chamber

60 is the conversion factor for minute to second

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

HPLC analysis results of target compounds.

AUTHOR INFORMATION

Corresponding Author

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

ORCID
Thuy Nguyen: 0000-0002-3961-0901
Yanan Zhang: 0000-0001-7153-4358

Author Contributions

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ADME: Absorption-Distribution-Metabolism-Elimination; 2-AG: 2-arachidonoylglycerol; BBB: blood-brain barrier; CB_1: cannabinoid type-1 receptor; CB_2: cannabinoid type-2 receptor; CNS: central nervous system; FLIPR: fluorometric imaging plate reader; GPCR: G-protein-coupled receptor; HPLC: high performance liquid chromatography; IC50: half-maximum inhibitory concentration; MS: mass spectrometry; NAM: negative allosteric modulator; NMR: nuclear magnetic resonance; NASH: non-alcoholic steatohepatitis; PAM: positive allosteric modulator; SAR: structure−activity relationship; THC (-)-: trans-Δ^9-tetrahydrocannabinol; TLC: thin-layer chromatography.

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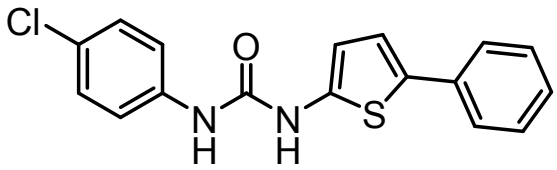

11

hCB_1 Calcium assay: IC_{50} = 6.8 nM

hCB_1 [^{35}S]GTPγS binding: IC_{50} = 524 nM

mCB_1 [^{35}S]GTPγS binding: IC_{50} = 63 nM

hCB_1 cAMP assay: IC_{50} = 1760 nM

No/little inverse agonism

$T_{1/2}$ (RLM) = 65 ± 19 min

CL = 22.2 ± 6.5 µL/min/mg

Aqueous solubility at pH 7.4 = 1.5 µM

MDCK-MDR1 P_{app} A to B ($x10^{-6}$ cm/s) = 1.6

MDCK-MDR1 P_{app} B to A ($x10^{-6}$ cm/s) = 1.1

MDCK-MDR1 efflux ratio BA/AB = 0.7