Discovery of Novel Proline-Based Neuropeptide FF Receptor Antagonists

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Abstract

The neuropeptide FF (NPFF) system has been implicated in a number of physiological processes including modulating the pharmacological activity of opioid analgesics and several other classes of drugs of abuse. In this study, we report the discovery of a novel proline scaffold with antagonistic activity at the NPFF receptors through a high throuput screening campaign using a functional calcium mobilization assay. Focused structure-activity relationship studies on the initial hit **1** have resulted in several analogs with calcium mobilization potencies in the submicromolar range and modest selectivity for the NPFF1 receptor. Affinities and potencies of these compounds were confirmed in radioligand binding and functional cAMP assays. Two compounds **16** and **33** had good solubility and blood-brain barrier permeability that fall within the range of CNS permeant candidates without the liability of being a P-glycoprotein substrate. Finally, both compounds reversed fentanyl-induced hyperalgesia in rats when administered intraperitoneally. Together, these results point to the potential of these proline analogs as promising NPFF receptor antagonists.

Graphical Abstract
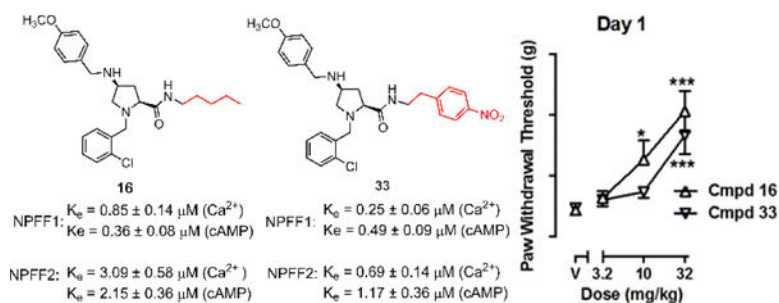
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Author Contributions

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

ASSOCIATED CONTENT

Supporting Information

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

The authors declare no competing financial interest.

Keywords

proline; neuropeptide FF; antagonist; structure-activity relationship

Introduction

Neuropeptide FF (NPFF, FLFQPQRF-amide) belongs to a family of neuropeptides called RFamide peptides, members of which all contain an Arg-Phe-NH$_2$ (RF-amide) motif at the C terminus.[1–3] NPFF is an endogenous peptide that binds to and activates two G protein-coupled receptors (GPCRs), NPFF1 and NPFF2. These receptors are members of the rhodopsin family and predominantly couple to G$\alpha_{i/o}$ proteins. Originally isolated from bovine brain,[4] NPFF and its receptors have recently been identified in the central nervous system (CNS) of various animal species. Ligand binding studies performed on rodents confirmed that both subtypes are widely expressed in the brain, whereas only NPFF2 receptors are expressed in the spine at detectable levels.[5–7] The NPFF system has been implicated in the regulation of a variety of physiological processes, such as insulin release, food intake, memory, blood pressure, electrolyte balance, and neural regeneration.[8, 9] Most interestingly, the NPFF system has also been shown to play an important role in modulating the effects of opioids and several other classes of drugs of abuse.[10–12]

It is well documented that NPFF, having no affinity for the opioid receptors, is an important modulator of opioid receptor function. Several studies have shown that the effects of NPFF on opioid modulation are dependent on the route of administration. For example, intracerebroventricular (i.c.v.) administration of NPFF in rats attenuated morphine-induced analgesia and locomotion, and precipitated opioid withdrawal syndromes in morphine-dependent rats, whereas intrathecal (i.t.) administration produced opioid-induced analgesia and also prolonged morphine-induced analgesia.[4, 13, 14] Ventricular injection of NPFF antiserum restored the analgesic response to morphine in morphine-tolerant rats but did not affect opiate-naïve rats.[15] RF9, a dipeptide NPFF½ receptor antagonist, dose-dependently blocked the long-lasting hyperalgesia produced by either acute fentanyl or chronic morphine administration and prevented the development of associated tolerance.[16, 17] This effect appears to be mainly mediated by the NPFF1 receptor, as the selective NPFF1 antagonist AC-262620 also reduced opioid tolerance.[18] Together, these results confirm the opioid-modulating properties of the NPFF system and suggest that co-administration of an NPFF antagonist with opioids may alleviate hyperalgesia and tolerance, two main side effects associated with chronic opioid use.

The finding that NPFF precipitates a nicotine withdrawal syndrome also suggests that NPFF participates in the processes of dependence and addiction to other drugs.[19] Chronic administration of NPFF into the lateral ventricle potentiated the behavioral sensitization to amphetamine.[20] More recently, it was demonstrated that stimulation of NPFF receptors decreased the expression of amphetamine-induced condition-placed preference, while inhibition of NPFF receptors decreased amphetamine withdrawal anxiety.[21] Moreover, it was suggested that NPFF is involved in the mechanism of expression of sensitization to cocaine hyperlocomotion, although this effect could be non-specific.[22] Consistent with these

observations, there is evidence that NPFF binding sites are abundant in the ventral tegmental area (VTA), while NPFF-like immunoreactivity was detected in the nucleus accumbens (NAc), two brain regions belonging to the mesolimbic dopamine DA projections which are known to be involved in drug addiction.[23] Together, these findings suggest that the NPFF system is a viable target for the treatment of drug addiction.

In addition to NPFF, several other neuropeptides from the RFamide family activate one or both NPFF receptors, including NPSF, NPAF and NPVF. A number of peptidomimetic NPFF ligands have also been reported, which retained the guanidine functional group (Figure 1), including the putative NPFF½ antagonist RF9.[24] In these ligands, acylation of the N-terminus of the last two amino acid residues (RF) have been reported to be critical for NPFF activities (e.g. BIBP3226 and RF9).[5, 17] Subsequent modification of RF9 led to the discovery of the potent NPFF1 selective dipeptide biphenyl-RF and peptidomimetic RF313.[25–27] These peptides or peptidomimetics were effective in preventing fentanyl-induced hyperalgesia in rats by subcutaneous or oral administration, acting as antagonists (Figure 1).[17, 25–27] However, other studies suggest that RF9 is not an NPFF receptor antagonist in some assays, as it did not reverse phosphorylation of MAPK/ERK½ and anorectic effects in fasting mice induced by [Tyr[1]]NPFF, but rather exhibited anorectic effects itself after i.c.v or subcutaneous administration.[28]

Several classes of non-peptidic NPFF ligands have recently been disclosed. Quinazolino-, quinolino-, pyrimidine-, and thiazole-guanidines were reported in several patents as NPFF ligands.[29–33] A series of N-benzylpiperidines with mixed activities as agonists/antagonists at NPFF1 and antagonists at NPFF2 were recently reported.[34] These series still contained the guanidine functionality, which is often associated with high plasma-protein binding and limited BBB penetration.[35] Despite this, some of these guanidine-containing ligands have been reported to enter the CNS, although to a relatively small extent.[32] So far, only two series of small molecule NPFF ligands, fused imidazole-pyrazines and indole derivatives (Figure 1),[24] which do not possess the guanidine functionality have been reported in patents; however, only limited biological data were presented on these compounds.[36, 37] Together, these results underscore the importance of discovering novel NPFF ligands with improved pharmacological profiles (agonist or antagonist) and properties, particularly small non-peptidic molecules that are not subject to peptidolytic degradation and thus are more favorable tools to explore the biological roles of the NPFF receptors.

In an effort to develop novel small molecule NPFF ligands, we performed a high throughput screen (HTS) of a GPCR-oriented compound library. Proline **1** emerged as a promising hit with moderate activities at the two NPFF subtypes with reasonable physiochemical properties (Figure 2). We have thus developed a synthetic route for this scaffold and prepared a focused library of proline analogs to explore the structure-activity relationships (SARs) at the carboxamide region, some of which displayed improved potency at the NPFF receptors when characterized in the functional calcium mobilization assays. Several active compounds were further evaluated for their effects in modulating cellular levels of cyclic adenosine monophosphate (cAMP) and their binding affinities to the two NPFF receptors. The drug-like properties such as solubility and blood-brain barrier permeability were then examined. Finally, the effects of these compounds in reversing fentanyl-induced

hyperalgesia were investigated. Herein, we describe the discovery and characterization of these proline-based novel NPFF antagonists.

Results and Discussion

NPFF Calcium Mobilization Assay Development.

Traditionally, NPFF activity has been examined using assays such as radioligand binding, GTP-γ-S or cAMP assays. To establish a platform allowing for low-cost HTS, we developed functional calcium mobilization assays using CHO cells simultaneously over-expressing the promiscuous $G\alpha_{16}$ protein and either human NPFF1 or NPFF2 receptors. The NPFF1 and NPFF2 stable cell lines were created by transfecting the expression plasmids into RD-HGA16 CHO cells (Molecular Devices), selecting for positive clones using antibiotic resistance, and testing for functional expression of NPFF1 and 2 following procedures previously disclosed by our group for other receptor systems.[38–40] Clones were first screened against 10 μM NPFF to identify clones that had functional NPFF receptors. Eight clones with the highest maximal NPFF response were further evaluated with NPFF concentration-response curves; the clone with the most potent and efficacious NPFF response was chosen as the working clone. Figure 3 shows the data (N=3) obtained when our stable NPFF1 and NPFF2 cell lines are treated with NPFF in 96-well format with a 1% DMSO final concentration. In the NPFF1-RD-HGA16 cells, NPFF has an EC_{50} value of 64 ± 1 nM and the signal window is 17,900 ± 1,300 relative fluorescent units (RFUs). In the NPFF2-RD-HGA16 cells, NPFF has an EC_{50} value of 49 ± 3 nM and the signal window is 9,100 ± 230 RFUs. These NPFF EC_{50} values are consistent with results from cAMP and GTP-γ-S assays.[18, 41, 42] In parental RD-HGA-16 CHO cells, there was no response to NPFF, confirming its signaling through NPFF receptors. Both NPFF functional assays tolerated DMSO up to a final concentration of 1%.

HTS of 22,000 Diverse Compound Libary.

The NPFF1 FLIPR-based calcium mobilization assay was used to screen our 22,000 ChemBridge Premium Diversity Library, which is a subset of both the ChemBridge EXPRESS-PICK and CORE library stock collections. The library is composed of highly diverse scaffolds designed to provide lead compounds for new targets as well as enable in-house scaffold hopping. The content and properties of the diversity set were critically appraised for screening against GPCRs based on a number of factors including maximum diversity, optimal ADME parameters, structural novelty (minimal overlap with scaffolds found in known GPCR ligands and drugs), and pharmacophoric compliance with characteristics prototypical of GPCR ligands.

Because the NPFF1 assay was originally developed in 96-well format, we miniaturized the assay to 384-well format and determined the cell density that provided a confluent cell monolayer and NPFF potency/efficacy consistent with the 96-well assay. We then tested the calcium mobilization assay using our automated liquid handling equipment which includes a Biotek cell dispenser, Beckman Coulter Biomek NX, and FLIPR Tetra to determine whether automation resulted in adverse effects; no changes in assay performance were observed.[43] Using the automated and miniaturized assay, we generated a Z'-factor by testing positive (10

μM final NPFF) and negative controls (1% DMSO/assay buffer) on three individual days.[44] The Z'-factor (N=3) was determined to be 0.75 with a standard deviation of 0.06; a Z'-factor greater than 0.5 is considered appropriate for HTS.

Library compounds were screened at 10 μM final concentration for both agonist and antagonist activities as part of a 3-addition protocol our laboratory developed. This protocol allows for evaluation of both modes of activity with a single assay plate thereby reducing the overall time and cost of the screen. Concentration-response curves were run with 37 cherry picked antagonists (27 with >65% inhibition and 10 with > 85% inhibition) resulting in the confirmed activities of these compounds including proline **1**.

Chemistry.

Compounds **1**, **10** - **47** were synthesized following procedures depicted in Scheme 1. *trans*-4-Hydroxy-*L*-proline methyl ester (**2**) underwent reductive amination by sodium triacetoxyborohydride in 1,2-dichloroethane to give the intermediate **3** which was then converted to a tosylated derivative **4** in the presence of pyridine in dichloromethane. Substitution of the tosyl group with sodium azide provided azide **5**, which subsequently underwent Staudinger reduction with triphenylphosphine to give amine **6**. A second reductive amination with either 4-methoxybenzaldehyde or 4-(methylthio)benzaldehyde provided intermediates **7a** and **7b**, respectively. This three-step conversion from the tosylate **4** to amines **7a-b** gave higher yields (overall three-step yield 32%) than the direct displacement with 4-methoxybenzylamine or 4-(methylthio)benzylamine (<10%).[45] While the low yields of this direct conversion could be slightly improved by the employment of a large excess of the amines under elevated reaction temperatures in a polar solvent, the unreacted amines greatly complicated the purification of the product. Furthermore, the use of excess amines is not amenable to scale-up synthesis of this common intermediate. The secondary amines **7a**-**b** were then protected with a Boc group and the resulting intermediates **8a-b** were subsequently hydrolyzed under basic conditions to give acids **9a-b**. HBTU-assisted amide coupling between **9a-b** and corresponding amines followed by cleavage of the Boc protective group provided the final products (**1**, **10** - **47**). All target compounds were characterized by ^{1}H and ^{13}C NMR, HRMS, and HPLC.

Pharmacological Evaluations.

All the synthesized compounds were first characterized in NPFF1 and NPFF2 calcium mobilization assays for their ability to antagonize NPFF stimulated calcium influx. Since the NPFF receptors natively couple to $G\alpha_{i/o}$ proteins and inhibit adenylate cyclase, we chose active compounds with K_e values of 1 μM in NPFF1 or NPFF2 calcium assays, and further evaluated them in PerkinElmer's Lance cAMP assays. Figure 4 displays the concentration-response curve of a representative compound (**16**) in the NPFF1 and NPFF2 calcium mobilization assays. The concentration-response curve of compounds **16** and **33** in NPFF1 and NPFF2 cAMP assays are shown in Figure 5. In the cAMP assay, the NPFF + **16** curve had a Hill slope of 1.3 in both NPFF1 and FF2 cells while the NPFF + **33** curve had a Hill slope of 1.4 and 1 in NPFF1 and FF2 cells, respectively, thus indicating that the activity of these lead compounds is not due to the formation of aggregates.[46, 47] These results correlate with those from the calcium mobilization assays where the NPFF + **16** curve had a Hill

slope of 1.7 and 1.1 in NPFF1 and FF2 cells, respectively, and the NPFF + **33** curve had a Hill slope of 1.4 in both cell lines.

In both functional assays, the apparent dissociation constant K_e of the test compounds was calculated from compound-mediated inhibition of NPFF activity, where the EC_{50} curves of the agonist NPFF were obtained alone and together with the test compound and the right-shift of the agonist curve was measured, as previously described by our group.[48, 49] The selected compounds were also characterized in radioligand binding assays to measure affinity and confirm that the proline scaffold is a bona fide template as an NPFF ligand. All compounds were also tested for agonist activity using the calcium mobilization assay; none showed any significant agonist activity at either the NPFF1 or NPFF2 receptor (< 20% of NPFF E_{max} at 10 μM final concentration).

Analogs with a 4-methoxybenzyl group at the 4-position of the proline core, instead of the 4-(methylthio)benzyl group in the hit compound **1**, were synthesized in the SAR studies at the carboxamide region because the methoxy moiety is known to have better metabolic stability. The corresponding starting material, 4-methoxybenzaldehyde is also more readily available compared to the methylthio counterpart. As can be seen from Table 1, the NPFF1 antagonist activity of this series was sensitive to the length of the side chain R of the carboxamide functionality. Methyl and ethyl analogs (**10** and **11**) were inactive at both NPFF receptors. The antagonist activities at the NPFF1 receptor increased from *n*-propyl to *n*-pentyl (**12**, **13**, **16**), then decreased slightly with *n*-hexyl (**18**) and was completely abolished with *n*-decyl chain (**19**). Among the three butyl isomers (**13**-**15**), the NPFF1 antagonist activity slightly decreased in the order of *n*-butyl, *s*-butyl and *t*-butyl. In contrast, the NPFF2 antagonist activity increased significantly in the same order, indicating that a linear chain is preferred for NPFF1 selectivity. A similar trend was observed with *n*-pentyl and *i*-pentyl isomers (**16** and **17**). Between these two isomers, *n*-pentyl is the more potent and selective NPFF1 antagonist (**16**, NPFF1 K_e = 0.72 μM, NPFF2 K_e = 3.09 μM). Compounds **20**-**21** have aliphatic side chains with a bulkier moiety at the terminal end. The cyclohexyl analog **21** (NPFF1 K_e = 1.12 μM) is slightly more potent than the more hydrophilic morpholinyl counterpart **20** (NPFF1 K_e = 1.96 μM). The flexible N,N-diethylaminoethyl side chain (**22**) was inactive at both receptors. These results suggest that introduction of a heteroatom is not well tolerated in this region. Most of the aliphatic analogs did not show significant activities at the NPFF2 receptor except the *t*-Bu (**15**), *i*-pentyl (**17**), *n*-hexyl (**18**), and (cyclohexyl)ethyl (**21**) analogs which displayed modest antagonist activities in the micromolar range.

When the cyclohexyl (**21**) was replaced by a phenyl group (**24**), the NPFF1 activity was slightly improved while retaining selectivity over the NPFF2 receptor. The shorter benzyl group (**23**, NPFF1 K_e = 0.78 μM) has equivalent NPFF1 antagonist activity to the phenethyl analog (**24**, NPFF1 K_e = 0.85 μM). However, lengthening the side by another methylene group as in phenylpropyl (**25**) completely abolished the activity at the NPFF1 receptor. While displaying similar antagonist activity at the NPFF1 receptor, the phenethyl analog seemed to have better selectivity at the NPFF1 receptor over the NPFF2 receptor. As **24** emerged as a potent, selective NPFF1 antagonist, it is warranted to study the substituent effect on the phenyl ring of compound **24**.

Among electron-donating substituents at the para position of the phenyl ring (**26**-**31**), 4-methoxy (**26**, NPFF1 K_e = 0.67 μM) was the most potent NPFF1 antagonist. 3,4-Dimethoxy (**27**), and 4-dimethylamino (**28**) were slightly less potent. Bulky groups such as 4-acetamido (**29**), 4-(methylamino)carbonyl (**30**), and *t*-butyl (**31**) were not well tolerated, in agreement with the previous observation that there is limited space at the binding pocket. Turning to electron-withdrawing substituents, 4-nitro (**33**, NPFF1 K_e = 0.25 μM) demonstrated the best NPFF1 antagonist potency among all proline analogs. Our results indicate that strong electron-withdrawing groups are favored for good NPFF1 antagonist activity as 3,4-difluoro (**34**, NPFF1 K_e = 0.61 μM) was more potent than 4-chloro (**32**, NPFF1 K_e = 0.99 μM), 4-fluoro (**35**, NPFF1 K_e = 1.13 μM) and 4-trifluoromethyl (**36**, NPFF1 K_e = 1.67 μM). 4-Pyridinyl (**37**) which is commonly used as an isosteric replacement of 4-nitrophenyl,[50] only displayed moderate NPFF1 activity (K_e = 1.82 μM). Finally, the NPFF1 antagonist activity of the two bulky electron-withdrawing groups, acetyl (**38**) and 4-methylsulfonyl (**39**) was significantly dampened compared to the 4-nitro analog. These data imply that small, strong electron-withdrawing substituents were preferred whereas bulky groups proved to be deleterious for NPFF1 antagonist activity. Similar to the aliphatic series, these phenethyl analogs were not potent at the NPFF2 receptor except 4-methoxy (**26**), diethylamino (**28**), 4-chloro (**32**), 4-nitro (**33**), and 3,4-difluoro (**34**).

Next, the effect of disubstituted amides at this region was also investigated. Diethylamino and dipropylamino analogs (**40**, NPFF1 K_e = 2.90 μM and **41**, NPFF1 K_e = 0.88 μM) were more potent at the NPFF1 receptor compared to their monosubstituted amide counterparts (**11**, NPFF1 K_e >10 μM and **12**, NPFF1 K_e = 2.60 μM). Compound **42** (NPFF1 K_e = 1.33 μM) with a rigid spacer between the phenyl ring and the amide was less active at the NPFF1 receptor compared to **24** (NPFF1 K_e = 0.85 μM) with a flexible ethylene linker.

Since the initial hit **1** has a 4-methylthio at the 4-benzyl group on the proline scaffold, after exploring various substituents at the carboxamide region with a 4-(4-methoxybenzyl) substitution of the proline scaffold, we selected several of the more potent 4-methoxybenzyl analogs and examined the effects of the 4-methylthio substitution (Table 2). The pentyl analog at the carboxamide of the (methylthio)benzyl series was a slightly more potent NPFF1 antagonist than its methoxy counterpart in the calcium mobilization assay (**43** K_e = 0.36 μM vs. **16** K_e = 0.72 μM). Similarly, phenethyl (**44**) and 3,4-difluorophenethyl (**47**) analogs demonstrated slightly better NPFF1 activities in the thioether series than their methoxy counterparts (**44** K_e = 0.53 μM vs. **24** K_e = 0.85 μM; **47** K_e = 0.47 μM vs. **34** K_e = 0.61 μM). On the other hand, the 4-methoxy (**45**) and 4-nitro (**46**) analogs were similar in potency as their methoxy equivalents (**45** K_e = 0.87 μM vs. **26** K_e = 0.67 μM, **46** K_e = 0.37 μM vs. **33** K_e = 0.25 μM). At the NPFF2 receptor, this series appeared to be more active and thus, less selective for the NPFF1 receptor compared the 4-(4-methoxybenzyl)amino analogs except for **45** and **46**.

Throughout the course of these studies, most of the compounds we tested displayed competitive antagonism in the curve-shift assays; however, several compounds (**23**, **32**, **33**, **41**, **42**, **45**) showed evidence of insurmountable antagonism by shifting the curve to the right and also depressing the maximal NPFF signal. While allosteric modulators commonly

produce such a response, this type of antagonism can also be observed with competitive orthosteric antagonists with slow dissociation rates. Our group has previously reported that by performing the curve-shift assays with longer antagonist-receptor incubation periods, the system reaches equilibrium, and hence the compounds produce a typical competitive antagonist profile.[51] Indeed, when we applied the longer incubations to the NPFF assays, the compounds displayed the typical competitive antagonist activity profile.

Several compounds were further characterized in radioligand binding and cAMP assays (Table 3). In general, the data obtained from all three assays are in good concordance with each other. Compounds that were potent antagonists in the calcium mobilization assays also showed good antagonist potency in the secondary cAMP assay that measures native G-protein coupling (Fig. 5). The binding assays confirm that all compounds bind to the NPFF receptors and that potent compounds (**33** and **34**) in the NPFF1 functional assays have potent binding affinities as well.

Collectively, these results highlight the importance of the substituent size, a preference for lipophilicity, and some flexibility at the NPFF binding pocket around the carboxamide region. Several ligands with moderate activity against NPFF1 and no/weak NPFF2 activity were identified. The most potent NPFF1 compounds of the series were the pentyl (**16**) and 4-nitro (**33**) analog based on their potency and affinity in all three assays. These compounds were then further evaluated for the preliminary ADME properties and in vivo activities in a hyperalgesia model.

Physicochemical Properties and Preliminary ADME Studies.

The proline scaffold has favorable physicochemical properties with a balance between lipophilic and hydrophilic groups (Table 4, calculated in ACD Labs), which would normally lead to good solubility and blood-brain barrier (BBB) permeability required for successful CNS drugs.[52, 53] Therefore, we tested two potent NPFF1 antagonists, **16** and **33**, as free base forms in the kinetic solubility and bidirectional MDCK-MDR1 permeability assays. Both assays were performed by Paraza Pharma Inc (Montreal, Canada) according to their standard protocols.

As seen from table 4, **16** was soluble in aqueous solutions, displaying a kinetic solubility of 146.8 ± 6.9 μM (Mean ± %CV) which falls in the range of compounds with good solubility. [54] **33** has a lower solubility of 45.9 ± 7.7 μM, which is expected for a bigger molecule with higher molecular weight. Moreover, these compounds contain multiple protonable nitrogen atoms which can be converted to salt forms to enhance solubility and bioavailability.

One of the major challenges for CNS drugs is their ability to cross the blood-brain barrier (BBB) and reach the CNS. For the majority of drugs, the BBB permeability is affected by two factors: the ability to permeate through the BBB passively and the avoidance of being effluxed out by the transport proteins such as the P-glycoprotein. **16** and **33** were evaluated in the bidirectional transport assay using MDCK-MDR1 cells which are stably transfected with human MDR1 cDNA and express a higher level of the P-glycoprotein (Pgp) compared to the wild type. Table 4 showed that **16** traversed the cell barrier from the apical (A) to basolateral (B) at a rate of 7.6×10^{-6} cm/s, and the reverse direction B to A at a rate of $6.7 \times$

10^{-6} cm/s, demonstrating a moderate BBB permeability (within the range of 3–6×10^{-6} cm/s).[55] **33** could also penetrate the BBB albeit at lower permeability compared to **16**. Both compounds were not Pgp substrates as indicated by the efflux ratio ($P_{B \to A}/P_{A \to B}$).[54] Together, the data demonstrated the encouraging potential of these compounds as CNS positive ligands.

Anti-hyperalgesia effects of 16 and 33 in rats

The NPFF1/2 antagonist RF9 has been shown to block the long-lasting hyperalgesia induced by heroin and fetanyl.[16, 17] Several other NPFF antagonists have also been demonstrated to show the same effects.[26] We therefore tested **16** and **33** in a fentanyl-induced hyperalgesia model in rats.

Prior to fentanyl treatment, rats displayed a mean paw withdrawal threshold (PWT) of 24.2 ± 1.8 g, which decreased to 5.7 ± 0.8 g on day 1 after fentanyl administration. One-way repeated measures ANOVA, with time entered as the repeated measure factor, revealed a significant main effect of fentanyl treatment on paw withdrawal threshold ($F_{(6, 35)} = 23.42$, $p < 0.0001$). Bonferroni's *post hoc* tests revealed significant differences on days 1–3 as compared to day 0.

Both **16** and **33** dose-dependently increased PWT over a dose range of 3.2–32 mg/kg when tested on day 1 (Fig. 6). Treatment with **16** produced a significant main effect as determined by one-way repeated measures ANOVA, with treatment entered as the within subject factor: $F_{(3, 20)} = 15.10$, $p < 0.0001$. Additionally, Bonferroni's *post hoc* tests revealed significant differences at 10 and 32 mg/kg of **16** as compared to vehicle. Similarly, treatment with **33** produced a significant main effect ($F_{(3, 20)} = 12.45$, $p < 0.001$) and Bonferroni's *post hoc* tests revealed significant differences at 32 mg/kg of **33** as compared to vehicle.

Conclusions

The NPFF system has been implicated in a number of important physiological functions, particularly the modulation of opioid analgesia. Opioids remain the most effective analgesics for many pain conditions, particularly for chronic pain; however, the adverse effects related to opioid use such as physical dependence, hyperalgesia and tolerance preclude adequate dosing and effective pain control in a large population of pain sufferers. Combination therapy, which combines opioids with another drug that may increase the efficacy of opioids and/or reduce the untoward effects, offers a promising alternative strategy for pain management. Given these unmet challenges associated with opioids in the treatment of pain, the NPFF system represents a promising therapeutic target for developing add-on therapies for pain management.[56]

Several classes of NPFF-like compounds have been reported, most of which were either peptide or peptidomimetics retaining the guanidine functionality. We have conducted a HTS of a GPCR-focused compound library and identified a novel NPFF receptor antagonist hit containing a proline scaffold. The present study explores the potential of these prolines as a promising novel scaffold for the design of NPFF antagonists. The initial SAR investigation focused on the carboxamide region, and revealed substitution at this position influenced

NPFF receptor antagonism and subtype selectivity. Specifically, the carboxamide region prefers substituents such as *n*-pentyl, *n*-hexyl, and phenethyl groups. Several compounds with submicromolar NPFF1 potencies were identified as well. Compounds **16** with the pentyl group and **33** with a 4-nitrophenethyl substituent emerged as the most potent analogs at NPFF1 and showed modest preference over NPFF2. Results from the secondary cAMP assays further confirmed the NPFF antagonistic activities of these compounds. Radioligand binding assays demonstrated that the compounds bind to the NPFF receptors with moderate affinity. Both **16** and **33** possess good solubility and BBB permeability, demonstrating the proline scaffold as a potential potent template with favorable drug-like properties for further investigation of antagonist activity at the NPFF receptors. Finally, both compounds reversed opioid-induced hyperalgesia in rats when i.p. administrated, consistent with previously reported NPFF antagonists. Optimization of other regions of the proline core is in progress and will be reported in the future.

Experimental section

Chemistry.

All solvents and chemicals were reagent grade. Unless otherwise mentioned, all reagents and solvents were purchased from commercial vendors and used as received. Flash column chromatography was carried out on a Teledyne ISCO CombiFlash Rf system using prepacked columns. Solvents used include hexane, ethyl acetate (EtOAc), dichloromethane, methanol, and chloroform/methanol/ammonium hydroxide (80:18:2) (CMA-80). Purity and characterization of compounds were established by a combination of HPLC, TLC, mass spectrometry, and NMR analyses. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-300 (300 MHz) spectrometer and were determined in CDCl$_3$, DMSO-d6, or CD$_3$OD with tetramethylsilane (TMS) (0.00 ppm) or solvent peaks as the internal reference. Chemical shifts are reported in ppm relative to the reference signal, and coupling constant (J) values are reported in hertz (Hz). Thin layer chromatography (TLC) was performed on EMD precoated silica gel 60 F254 plates, and spots were visualized with UV light or iodine staining. Nominal mass spectra were obtained using a Waters Alliance HT/Micromass ZQ system (ESI). High resolution mass spectra were obtained using Agilent 1290 Infinity UHPLC-6230 TOP 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).

Methyl (2S,4R)-1-[(2-chlorophenyl)methyl]-4-hydroxypyrrolidine-2-carboxylate (3).

To a solution of methyl trans-4-hydroxy-L-proline (16.5 mmol, 3.00 g) and 2-chlorobenzaldehyde (21.5 mmol, 2.1 ml) in 1,2-dichloroethane (55 ml) was added acetic acid (0.9 ml) and sodium triacetoxyborohydride (24.8 mmol, 5.26 g). The reaction was stirred at room temperature for 24 h. After quenching with saturated solution of sodium bicarbonate, the reaction mixture was extracted three times with dichloromethane. The combined organic layers were washed sequentially with water and brine, dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified

by column chromatography to give the desired product as yellow liquid (4.00 g, 90%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.44 (dd, J = 1.98, 7.44 Hz, 1H), 7.34 (m, 1H), 7.20 (m, 2H), 4.43 (m, 1H), 3.92 (m, 2H), 3.63 – 3.74 (m, 4H), 3.35 (dd, J = 5.56, 10.08 Hz, 1H), 2.52 (dd, J = 3.58, 10.17 Hz, 1H), 2.24 (m, 1H), 2.12 (m, 1H). MS (ESI) m/z calcl. for C$_{13}$H$_{17}$ClNO$_3$ [M+H]$^+$ 270.1, m/z found 270.2.

Methyl (2S,4R)-1-[(2-chlorophenyl)methyl]-4-[(4-methylbenzenesulfonyl)oxy]pyrrolidine-2-carboxylate (4).

To a solution of **3** (14.8 mmol, 4.00 g) in pyridine (11.4 ml) and anhydrous dichloromethane (11.4 ml) at 0 °C was added dropwise tosyl chloride (17.8 mmol, 3.39 g). The reaction was refluxed for 24 h. After removal of the solvent in vacuo, the residue was dissolved in dichloromethane and washed with saturated copper sulfate, water, and brine. The combined organic layers were dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, ethyl acetate/ hexanes) to provide the desired product as colorless liquid (3.77 g, 60%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.74 – 7.79 (m, 2H), 7.37 – 7.46 (m, 1H), 7.28 – 7.35 (m, 3H), 7.17 – 7.23 (m, 2H), 5.01 (d, J = 5.46 Hz, 1H), 3.74 – 4.04 (m, 2H), 3.69 (s, 1H), 3.66 (s, 3H), 3.29 (dd, J = 6.03, 11.11 Hz, 1H), 2.67 – 2.73 (m, 1H), 2.44 (s, 3H), 2.28 (dd, J = 5.46, 7.54 Hz, 2H). MS (ESI) m/z calcd. for C$_{20}$H$_{23}$ClNO$_5$S [M+H]$^+$ 424.1, m/z found 424.2.

Methyl (2S,4S)-4-azido-1-[(2-chlorophenyl)methyl]pyrrolidine-2-carboxylate (5).

To a solution of **4** (6.87 mmol, 2.91 g) in DMF (40 ml) was added sodium azide (13.74 mmol, 0.89 g). After stirring at 65 °C for 16 h, the reaction mixture was diluted with water, and extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by column chromatography (SiO$_2$, hexanes/ethyl acetate) to give the desired product as yellow liquid (1.48 g, 73%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.54 (dd, J = 1.70, 7.54 Hz, 1H), 7.34 (dd, J = 1.51, 7.72 Hz, 1H), 7.16 – 7.29 (m, 2H), 4.02 – 4.09 (m, 1H), 3.90 – 3.98 (m, 1H), 3.81 – 3.87 (m, 1H), 3.72 (s, 3H), 3.45 (dd, J = 6.03, 9.23 Hz, 1H), 3.13 (dd, J = 1.51, 10.36 Hz, 1H), 2.71 (dd, J = 5.75, 10.27 Hz, 1H), 2.54 (ddd, J = 7.72, 9.23, 14.13 Hz, 1H), 2.12 – 2.21 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.2, 135.7, 133.9, 130.7, 129.4, 128.4, 126.7, 63.7, 59.2, 57.8, 54.2, 51.8, 35.7. MS (ESI) m/z calcd. for C$_{13}$H$_{16}$ClN$_4$O$_2$ [M+H]$^+$ 295.1, m/z found 295.1.

Methyl (2S,4S)-4-amino-1-[(2-chlorophenyl)methyl]pyrrolidine-2-carboxylate (6).

To a solution of azide **5** (4.3 mmol, 1.27 g) in THF (19 ml) under nitrogen was added PPh$_3$ (8.6 mmol, 2.26 g) and water (0.2 ml). The reaction mixture was refluxed with stirring for 6 h. After the solvent was removed, the residue was dissolved in diethyl ether, treated with 0.1 N HCl for 5 min, and then extracted twice with diethyl ether. The aqueous layer was then treated with 1 N NaOH until pH 10, and then extracted with dichloromethane. The combined dichloromethane fractions were dried over anhydrous MgSO$_4$, concentrated in vacuo to afford the desired product as yellow liquid (1.08 g, 93%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.50 (dd, J = 1.9, 7.54 Hz, 1H), 7.33 (m, 1H), 7.16 – 7.20 (m, 2H), 3.95 (d, J = 14.7 Hz, 1H), 3.81 (d, J = 13.9 Hz, 1H), 3.66 (s, 3H), 3.45 (m, 1H), 3.39 (dd, J = 5.5, 9.6 Hz,

1H), 2.85 (m, 1H), 2.68 (dd, J = 5.5, 9.4 Hz, 1H), 2.48 (m, 1H), 1.79 (m, 1H), 1.74 (br, 2H).
^{13}C NMR (75 MHz, CDCl$_3$) δ 174.2, 135.9, 133.6, 130.7, 129.1, 128.1, 126.5, 64.2, 62.2,
54.5, 51.5, 50.6, 39.6. MS (ESI) m/z calcd. for $C_{13}H_{18}ClN_2O_2$ [M+H]$^+$ 269.1, m/z found
269.3.

Methyl (2S,4S)-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxylate (7a).

To a solution of amine **6** (3.72 mmol, 1 g) in 1,2-dichloroethane (12.4 ml) was added 4-
methoxybenzaldehyde (3.72 mmol, 0.45 ml), sodium triacetoxyborohydride (5.58 mmol,
1.18 g) and glacial acetic acid (3.72 mmol, 0.21 ml). After stirring at room temperature for
16 h, the reaction was quenched with saturated sodium bicarbonate, extracted three times
with dichloromethane. The combined organic layers were dried over anhydrous magnesium
sulfate, filtered, and concentrated in vacuo. The residue was purified by column
chromatography (SiO$_2$, dichloromethane/methanol) to give the desired product as colorless
liquid (0.68 g, 47%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.49 (dd, J = 1.79, 7.44 Hz, 1H), 7.33
(m, 1H), 7.16 – 7.25 (m, 4H), 6.84 (m, 2H), 3.95 (d, J = 14.30 Hz, 1H), 3.78 – 3.84 (m, 4H),
3.67 (d, J = 3.20 Hz, 5H), 3.39 (dd, J = 6.03, 9.04 Hz, 1H), 3.28 (m, 1H), 3.03 (dd, J = 2.54,
9.51 Hz, 1H), 2.61 (dd, J = 6.12, 9.51 Hz, 1H), 2.40 (m, 1H), 1.93 (m, 1H). MS (ESI) m/z
calcd. for $C_{21}H_{26}ClN_2O_3$ [M+H]$^+$ 389.2, m/z found: 389.4.

Methyl (2S,4S)-1-[(2-chlorophenyl)methyl]-4-{[(4-(methylsulfanyl)phenyl)methyl]amino} pyrrolidine-2-carboxylate (7b) was synthesized
from **6** and 4-(methylthio)benzylaldehyde according to the procedure for the synthesis of **7a**
as yellow liquid (45% yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.42 (br. s., 1H), 7.32 (d, J =
6.78 Hz, 1H), 7.13 – 7.24 (m, 6H), 3.90 (d, J = 7.91 Hz, 1H), 3.66 (s, 3H), 3.64 (br. s., 1H),
3.22 – 3.30 (m, 1H), 2.87 – 2.97 (m, 1H), 2.47 – 2.50 (m, 2H), 2.46 (s, 3H), 2.02 – 2.12 (m,
1H). MS (ESI) m/z calcd. for $C_{21}H_{26}ClN_2O_2S$ [M+H]$^+$ 405.1, m/z found 405.3.

Methyl (2S,4S)-4-{[(tert-butoxy)carbonyl][(4-methoxyphenyl)methyl]amino}-1-[(2-chlorophenyl)methyl]pyrrolidine-2-carboxylate (8a).

To a solution of amine **7a** (1.75 mmol, 0.68 g) in 1,4-dioxane (10 ml) was added 10% w/v
Na$_2$CO$_3$ solution (2ml) and Boc$_2$O (1.92 mmol, 0.42 g). After stirring at room temperature
after 16 h, 1,4-dioxane was removed under reduced pressure. The remaining aqueous
solution was extracted three times with dichloromethane. The combined organic layers were
dried over anhydrous MgSO$_4$, filtered, and concentrated in vacuo and the crude was used for
the next step without further purification. ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.30 – 7.38 (m,
2H), 7.16 – 7.22 (m, 2H), 7.06 (d, J = 8.67 Hz, 2H), 6.80 (d, J = 8.67 Hz, 2H), 4.47 – 4.63
(m, 1H), 4.26 – 4.44 (m, 2H), 3.83 (d, J = 6.97 Hz, 2H), 3.78 (s, 3H), 3.65 (s, 3H), 3.61 (d, J
= 6.97 Hz, 1H), 3.06 (t, J = 8.29 Hz, 1H), 2.67 (dd, J = 7.44, 8.95 Hz, 1H), 2.13 – 2.25 (m,
2H), 1.53 (s, 9H). MS (ESI) m/z calcd. for $C_{26}H_{34}ClN_2O_5$ [M+H]$^+$ 489.2, m/z found 489.6.

Methyl (2S,4S)-4-{[(tert-butoxy)carbonyl][(4-(methylsulfanyl)phenyl)methyl]amino}-1-[(2-chlorophenyl)methyl]pyrrolidine-2-carboxylate (8b) was synthesized from **7b**
according to the procedure for the synthesis of **8a** as yellow liquid (quant. yield). ^{1}H NMR
(300 MHz, CDCl$_3$) δ 7.34 (d, J = 6.22 Hz, 1H), 7.26 – 7.32 (m, 1H), 7.08 – 7.19 (m, 4H),

7.01 – 7.06 (m, 2H), 4.79 – 5.03 (m, 1H), 4.58 (s, 2H), 3.91 (d, J = 13.75 Hz, 1H), 3.64 (s, 3H), 3.58 (d, J = 13.94 Hz, 1H), 3.27 (t, J = 8.29 Hz, 1H), 2.87 – 2.96 (m, 1H), 2.49 – 2.60 (m, 2H), 2.45 (s, 3H), 1.87 – 1.99 (m, 1H), 1.34 (s, 9H). MS (ESI) m/z calcd. for $C_{26}H_{33}ClN_2O_4S$ [M+H]$^+$ 505.2, m/z found 505.6.

(2S,4S)-4-{[(tert-butoxy)carbonyl][(4-methoxyphenyl)methyl]amino}-1-[(2-chlorophenyl)methyl]pyrrolidine-2-carboxylic acid (9a).

To a solution of ester **8a** (1.75 mmol, 0.86 g) in methanol (16 ml) and water (16 ml) was added LiOH (8.75 mmol, 0.21 g). After stirring at room temperature for 3 h, methanol was removed under reduced pressure. The remaining solution was diluted in water and acidified with 1N HCl to pH 5. The mixture was then extracted with ethyl acetate. The combined organic layers were washed with water and brine, dried over anhydrous MgSO$_4$, filtered, and concentrated in vacuo to provide the desired product as white solid (0.83 g, quantitative yield). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.35 (d, J = 8.10 Hz, 2H), 7.17 – 7.24 (m, 2H), 7.02 (d, J = 8.48 Hz, 2H), 6.77 (d, J = 8.48 Hz, 2H), 4.26 – 4.55 (m, 3H), 4.06 – 4.17 (m, 1H), 3.71 – 3.82 (m, 4H), 3.40 – 3.52 (m, 1H), 3.08 (d, J = 6.40 Hz, 1H), 2.70 – 2.82 (m, 1H), 2.44 – 2.58 (m, 1H), 2.07 – 2.17 (m, 1H), 1.40 (s, 9H). MS (ESI) m/z calcd. for $C_{25}H_{32}ClN_2O_5$ [M+H]$^+$ 473.2, m/z found 473.5.

(2S,4S)-4-{[(tert-butoxy)carbonyl][(4-(methylsulfanyl)phenyl)methyl]amino}-1-[(2-chlorophenyl)methyl]pyrrolidine-2-carboxylic acid (9b) was synthesized from 8b according to the procedure for the synthesis of 9a as white solid (quant. yield).

^{1}H NMR (300 MHz, CDCl$_3$) δ 7.42 (br. s., 1H), 7.34 (d, J = 7.72 Hz, 1H), 7.16 – 7.26 (m, 2H), 7.13 (d, J = 8.10 Hz, 2H), 7.02 (d, J = 8.10 Hz, 2H), 4.47 – 4.61 (m, 1H), 4.31 – 4.46 (m, 2H), 4.23 (d, J = 10.93 Hz, 1H), 3.77 – 3.88 (m, 1H), 3.58 (d, J = 10.74 Hz, 1H), 3.02 – 3.13 (m, 1H), 2.81 (d, J = 10.36 Hz, 1H), 2.52 – 2.66 (m, 1H), 2.44 (s, 3H), 2.06 – 2.17 (m, 1H), 1.38 (s, 8H). MS (ESI) m/z calcd. for $C_{25}H_{32}ClN_2O_4S$ [M-H]$^-$ 489.2, m/z found 489.5.

General procedure A:

To a solution of acid **9** (0.2 mmol, 1 eq) in DMF (0.6 ml, 0.3 M) was added HBTU (0.22 mmol, 1.1 eq), corresponding amine (0.22 mmol, 1.1 eq), DIEA (0.66 mmol, 3 eq). After stirring at room temperature for 16 h, the reaction mixture was diluted with water, extracted three times with ethyl acetate. The combined organic fractions were dried over anhydrous magnesium sulfate and concentrated in vacuo. The residue was dissolved in 20% v/v trifluoroacetic acid (1 ml) in dichloromethane and stirred at room temperature for 16 h. The reaction mixture was concentrated under reduced pressure and the residue was purified on column chromatography (SiO$_2$, methanol/dichloromethane) to afford the desired product.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-methylpyrrolidine-2-carboxamide (10) was prepared according to the general procedure A

as yellow oil (9%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.43 (dd, J = 1.79, 7.44 Hz, 1H), 7.28 – 7.34 (m, 2H), 7.12 – 7.25 (m, 3H), 6.87 (s, 2H), 3.90 – 3.96 (m, 2H), 3.75 (s, 3H), 3.58 – 3.69 (m, 2H), 3.28 (s, 2H), 3.09 (dd, J = 4.14, 7.54 Hz, 1H), 2.68 (d, J = 4.90 Hz, 3H), 2.43 – 2.59 (m, 1H), 2.03 – 2.14 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.3, 160.5, 134.4,

133.9, 131.4, 131.0, 129.6, 129.0, 127.1, 114.6, 66.2, 55.6, 55.2, 54.9, 54.8, 53.6, 42.0, 33.5, 11.8. HRMS (ESI) *m/z* calcd. for $C_{21}H_{27}ClN_3O_2$ [M+H]$^+$ 388.1791, *m/z* found 388.1789.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-ethyl-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (11) was prepared according to the general procedure A as yellow oil (32%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.41 (br. s., 1H), 7.30 – 7.38 (m, 2H), 7.14 – 7.25 (m, 4H), 6.84 (d, *J* = 8.67 Hz, 1H), 3.83 – 3.91 (m, 1H), 3.79 (s, 3H), 3.58 – 3.73 (m, 3H), 3.33 (td, *J* = 2.80, 5.89 Hz, 1H), 3.19 – 3.28 (m, 1H), 3.10 – 3.19 (m, 2H), 3.00 (d, *J* = 9.98 Hz, 1H), 2.62 (dd, *J* = 5.56, 10.08 Hz, 1H), 2.49 (ddd, *J* = 6.40, 9.84, 13.70 Hz, 1H), 1.87 – 1.97 (m, 1H), 1.02 (t, *J* = 14.70 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.9, 158.9, 135.7, 134.3, 131.1, 129.7, 129.5, 128.9, 126.9, 113.9, 67.1, 59.5, 57.4, 56.2, 55.3, 51.1, 37.0, 33.7, 14.6. HRMS (ESI) *m/z* calcd. for $C_{22}H_{29}ClN_3O_2$ [M+H]$^+$ 402.1948, *m/z* found 402.1930.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-propylpyrrolidine-2-carboxamide (12) was prepared according to the general procedure A as yellow oil (48%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.82 (t, *J* = 5.27 Hz, 1H), 7.29 – 7.50 (m, 4H), 7.24 (d, *J* = 8.67 Hz, 2H), 6.85 (d, *J* = 8.67 Hz, 2H), 4.26 – 4.46 (m, 3H), 4.14 (d, *J* = 13.00 Hz, 2H), 3.94 – 4.03 (m, 1H), 3.78 (s, 3H), 3.46 (d, *J* = 5.09 Hz, 2H), 3.07 – 3.22 (m, 2H), 2.88 – 3.02 (m, 1H), 2.27 – 2.40 (m, 1H), 1.42 – 1.54 (m, 2H), 0.87 (t, *J* = 7.44 Hz, 3H). ^{13}C NMR (75 MHz, CDCl3) δ 174.0, 158.9, 135.7, 134.1, 131.0, 129.7, 129.6, 128.8, 126.9, 113.9, 67.1, 59.2, 57.2, 56.1, 55.3, 51.0, 40.7, 36.8, 22.7, 11.4. HRMS (ESI) *m/z* calcd. for $C_{23}H_{31}ClN_3O_2$ [M+H]$^+$ 416.2105, *m/z* found 416.2091.

(2S,4S)-N-Butyl-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (13) was prepared according to the general procedure A as yellow oil (12%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.74 (br. s., 1H), 7.47 (d, *J* = 7.72 Hz, 1H), 7.39 – 7.45 (m, 2H), 7.34 (dd, *J* = 2.07, 7.16 Hz, 1H), 7.24 (d, *J* = 8.67 Hz, 2H), 6.87 (d, *J* = 8.29 Hz, 2H), 4.34 – 4.56 (m, 3H), 4.19 (d, *J* = 13.37 Hz, 2H), 3.99 – 4.07 (m, 1H), 3.78 (s, 3H), 3.56 (d, *J* = 3.20 Hz, 2H), 3.13 – 3.27 (m, 2H), 2.94 – 3.08 (m, 1H), 2.33 – 2.46 (m, 1H), 1.38 – 1.50 (m, 2H), 1.29 (dd, *J* = 6.97, 14.88 Hz, 2H), 0.90 (t, *J* = 7.25 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.2, 159.8, 135.1, 133.9, 130.8, 130.6, 129.6, 128.8, 127.0, 114.3, 66.7, 57.2, 55.8, 55.5, 55.2, 49.6, 39.0, 34.8, 31.4, 20.0, 13.6. HRMS (ESI) *m/z* calcd. for $C_{24}H_{33}ClN_3O_2$ [M+H]$^+$ 430.2261, *m/z* found 430.2253.

(2S,4S)-N-(Butan-2-yl)-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (14) was prepared according to the general procedure A as colorless oil (74%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.29 – 7.39 (m, 3H), 7.16 – 7.25 (m, 4H), 6.83 (d, *J* = 8.67 Hz, 1H), 3.87 – 3.94 (m, 1H), 3.74 – 3.84 (m, 5H), 3.61 – 3.72 (m, 3H), 3.20 – 3.33 (m, 2H), 2.97 (td, *J* = 1.62, 10.13 Hz, 1H), 2.60 (ddd, *J* = 3.01, 5.60, 10.03 Hz, 1H), 2.45 – 2.54 (m, 1H), 1.87 – 1.96 (m, 1H), 1.76 (br. s., 1H), 1.29 – 1.39 (m, 2H), 1.02 (d, *J* = 6.59 Hz, 2H), 0.94 (d, *J* = 6.59 Hz, 2H), 0.74 – 0.85 (m, 5H). ^{13}C NMR (75 MHz, CDCl3) δ 173.4, 173.4, 158.7, 158.7, 135.9, 134.3, 134.2, 131.9, 131.2, 130.9, 129.8, 129.8, 129.4, 128.9, 128.8, 126.9, 126.9, 113.8, 113.8, 67.3, 67.2, 59.7, 59.6, 57.7, 57.5, 56.2, 56.1, 55.3, 51.3, 46.0, 45.8, 37.4, 37.2, 29.6, 20.1, 10.5, 10.4. HRMS (ESI) *m/z* calcd. for $C_{24}H_{33}ClN_3O_2$ [M+H]$^+$ 430.2261, *m/z* found 430.2277.

(2S,4S)-N-tert-Butyl-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (15) was prepared according to the general procedure A as colorless oil (88%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.30 – 7.39 (m, 2H), 7.15 – 7.25 (m, 5H), 6.82 – 6.87 (m, 2H), 3.76 – 3.82 (m, 5H), 3.60 – 3.75 (m, 2H), 3.28 – 3.36 (m, 1H), 3.14 (dd, J = 5.27, 9.80 Hz, 1H), 3.06 (d, J = 10.17 Hz, 1H), 2.64 (dd, J = 5.65, 9.98 Hz, 1H), 2.47 (ddd, J = 6.40, 9.80, 13.56 Hz, 1H), 1.89 (td, J = 3.67, 13.56 Hz, 1H), 1.18 (s, 9H). ^{13}C NMR (75 MHz, CDCl3) δ 173.5, 158.8, 136.0, 134.4, 131.0, 129.7, 129.4, 128.9, 127.0, 113.9, 67.8, 59.9, 57.6, 56.2, 55.3, 51.2, 50.2, 37.2, 28.5. HRMS (ESI) *m/z* calcd. for C$_{24}$H$_{33}$ClN$_3$O$_2$ [M+H]$^+$ 430.2261, *m/z* found 430.2242.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-pentylpyrrolidine-2-carboxamide (16) was prepared according to the general procedure A as yellow oil (25%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.32 – 7.43 (m, 3H), 7.17 – 7.24 (m, 4H), 6.81 – 6.86 (m, 2H), 3.84 – 3.91 (m, 1H), 3.78 (s, 3H), 3.64 – 3.74 (m, 3H), 3.31 – 3.38 (m, 1H), 3.25 (dd, J = 5.65, 9.80 Hz, 1H), 2.99 – 3.16 (m, 3H), 2.61 (dd, J = 5.56, 10.27 Hz, 1H), 2.48 (dd, J = 2.83, 6.78 Hz, 1H), 1.92 (d, J = 18.08 Hz, 1H), 1.31 – 1.43 (m, 2H), 1.16 – 1.30 (m, 4H), 0.84 (t, J = 6.97 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.9, 158.9, 135.7, 134.1, 130.9, 129.7, 129.6, 128.8, 126.9, 113.9, 67.1, 59.2, 57.2, 56.1, 55.3, 51.0, 38.9, 36.8, 29.1, 29.1, 22.3, 13.9. HRMS (ESI) *m/z* calcd. for C$_{25}$H$_{35}$ClN$_3$O$_2$ [M+H]$^+$ 444.2418, *m/z* found 444.2411.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-(3-methylbutyl)pyrrolidine-2-carboxamide (17) was prepared according to the general procedure A as colorless oil (71%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.23 – 7.34 (m, 3H), 7.10 – 7.18 (m, 4H), 6.76 (d, J = 8.67 Hz, 2H), 3.77 – 3.83 (m, 1H), 3.71 (s, 3H), 3.57 – 3.67 (m, 3H), 3.27 (td, J = 2.87, 5.93 Hz, 1H), 3.17 (dd, J = 5.46, 9.80 Hz, 1H), 3.05 (dt, J = 7.72, 12.90 Hz, 2H), 2.92 – 2.98 (m, 1H), 2.54 (dd, J = 5.65, 10.17 Hz, 1H), 2.42 (ddd, J = 6.59, 9.80, 13.75 Hz, 1H), 1.80 – 1.89 (m, 1H), 1.44 (td, J = 6.69, 13.37 Hz, 1H), 1.16 – 1.23 (m, 2H), 0.77 (dd, J = 3.39, 6.59 Hz, 6H). ^{13}C NMR (75 MHz, CDCl3) δ 172.9, 157.9, 134.7, 133.1, 130.0, 128.7, 128.6, 127.8, 125.9, 112.9, 66.1, 58.2, 56.2, 55.1, 54.2, 49.9, 37.3, 36.2, 35.8, 24.8, 21.4. HRMS (ESI) *m/z* calcd. for C$_{25}$H$_{35}$ClN$_3$O$_2$ [M+H]$^+$ 444.2418, *m/z* found 444.2419.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-hexylpyrrolidine-2-carboxamide (18) was prepared according to the general procedure A as colorless oil (49%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.45 (t, J = 5.65 Hz, 1H), 7.31 – 7.38 (m, 2H), 7.16 – 7.24 (m, 4H), 6.81 – 6.85 (m, 2H), 3.84 – 3.91 (m, 1H), 3.79 (s, 3H), 3.58 – 3.72 (m, 3H), 3.31 (td, J = 2.76, 5.98 Hz, 1H), 3.25 (dd, J = 5.56, 9.89 Hz, 1H), 3.11 (dt, J = 7.06, 12.86 Hz, 2H), 3.00 (d, J = 10.17 Hz, 1H), 2.60 (dd, J = 5.56, 10.08 Hz, 1H), 2.49 (ddd, J = 6.50, 9.84, 13.61 Hz, 1H), 1.90 (td, J = 3.84, 13.61 Hz, 2H), 1.31 – 1.41 (m, 2H), 1.17 – 1.29 (m, 6H), 0.85 (t, J = 13.40 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.0, 158.8, 135.8, 134.2, 131.8, 131.0, 129.7, 129.4, 128.8, 126.9, 113.9, 67.2, 59.6, 57.4, 56.2, 55.2, 51.2, 38.9, 37.2, 31.5, 29.4, 26.6, 22.5, 14.0. HRMS (ESI) *m/z* calcd. for C$_{26}$H$_{37}$ClN$_3$O$_2$ [M +H]$^+$ 458.2574, *m/z* found 458.2583.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-decyl-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (19) was prepared according to the general procedure A as colorless oil (58%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.42 (t, J = 5.75 Hz, 1H), 7.33 – 7.37 (m, 2H), 7.16 – 7.25 (m, 5H), 6.83 (d, J = 8.67 Hz, 1H), 3.84 – 3.91 (m, 1H), 3.78 (s, 3H), 3.62 – 3.75 (m, 4H), 3.33 – 3.40 (m, 1H), 3.25 (dd, J = 5.65, 9.61 Hz, 1H), 2.90 – 3.16 (m, 6H), 2.61 (dd, J = 5.65, 10.17 Hz, 1H), 2.49 (ddd, J = 6.59, 9.61, 13.75 Hz, 1H), 1.89 – 1.97 (m, 1H), 1.16 – 1.42 (m, 18H), 0.85 – 0.90 (m, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.9, 159.0, 135.7, 134.1, 130.9, 129.7, 129.7, 128.8, 127.0, 114.0, 67.1, 59.1, 57.1, 56.1, 55.3, 50.9, 39.0, 36.7, 31.9, 29.6, 29.5, 29.5, 29.3, 27.0, 22.7, 14.1. HRMS (ESI) m/z calcd. for C$_{30}$H$_{45}$ClN$_3$O$_2$ [M+H]$^+$ 514.3200, m/z found 514.3190.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-[2-(morpholin-4-yl)ethyl]pyrrolidine-2-carboxamide (20) was prepared according to the general procedure A as colorless oil (18%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.52 (t, J = 5.27 Hz, 1H), 7.34 – 7.38 (m, 2H), 7.18 – 7.25 (m, 4H), 6.84 (d, J = 8.67 Hz, 2H), 3.84 – 3.91 (m, 1H), 3.74 – 3.78 (m, 4H), 3.68 – 3.73 (m, 2H), 3.59 – 3.65 (m, 4H), 3.42 (t, J = 7.72 Hz, 1H), 3.22 – 3.37 (m, 4H), 2.35 – 2.45 (m, 7H), 2.13 – 2.21 (m, J = 1.00, 1.00 Hz, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.5, 159.2, 135.6, 134.2, 130.9, 129.8, 129.7, 128.9, 126.9, 114.0, 66.8, 66.4, 57.4, 57.3, 55.4, 55.2, 53.4, 51.4, 36.7, 35.3, 30.9. HRMS (ESI) m/z calcd. for C$_{26}$H$_{36}$ClN$_4$O$_3$ [M+H]$^+$ 487.2476, m/z found 487.2460.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-(2-cyclohexylethyl)-4-{[(4-methoxyphenyl)methyl] amino}pyrrolidine-2-carboxamide (21) was prepared according to the general procedure A as colorless oil (53%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.32 – 7.41 (m, 3H), 7.15 – 7.25 (m, 4H), 6.84 (d, J = 8.67 Hz, 2H), 3.83 – 3.90 (m, 1H), 3.79 (s, 3H), 3.59 – 3.73 (m, 3H), 3.32 (td, J = 2.80, 5.89 Hz, 1H), 3.25 (dd, J = 5.56, 9.70 Hz, 1H), 3.08 – 3.19 (m, 2H), 3.02 (d, J = 10.17 Hz, 1H), 2.60 (dd, J = 5.65, 9.98 Hz, 1H), 2.49 (ddd, J = 6.50, 9.80, 13.66 Hz, 1H), 1.86 – 1.95 (m, 1H), 1.56 – 1.70 (m, 5H), 1.08 – 1.30 (m, 6H), 0.76 – 0.93 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.9, 158.8, 135.8, 134.1, 130.9, 129.7, 129.5, 128.8, 126.9, 113.9, 67.2, 59.4, 57.3, 56.2, 55.2, 51.1, 37.0, 36.8, 36.7, 35.3, 33.1, 33.1, 30.9, 26.5, 26.2. HRMS (ESI) m/z calcd. for C$_{28}$H$_{39}$ClN$_3$O$_2$ [M+H]$^+$ 484.2731, m/z found 484.2731.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N,N-diethyl-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (22) was prepared according to the general procedure A as colorless oil (11%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.78 (br. s., 1H), 7.46 (dd, J = 2.26, 6.97 Hz, 1H), 7.33 – 7.38 (m, 1H), 7.16 – 7.25 (m, 4H), 6.80 – 6.88 (m, 2H), 3.83 – 3.91 (m, 1H), 3.79 (s, 3H), 3.64 – 3.76 (m, 3H), 3.16 – 3.36 (m, 4H), 3.03 (d, J = 9.61 Hz, 1H), 2.43 – 2.61 (m, 7H), 1.79 – 2.05 (m, 3H), 0.96 (t, J = 7.06 Hz, 6H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.2, 158.8, 135.8, 134.0, 130.8, 129.5, 129.4, 128.6, 126.9, 113.9, 67.2, 56.8, 56.1, 55.3, 51.5, 51.2, 46.6, 37.3, 36.2, 11.1. HRMS (ESI) m/z calcd. for C$_{26}$H$_{38}$ClN$_4$O$_2$ [M+H]$^+$ 473.2683, m/z found 473.2687.

(2S,4S)-N-Benzyl-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino} pyrrolidine-2-carboxamide (23) was prepared according to the general procedure A as yellow oil (44%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.82 (t, J = 5.75 Hz, 1H), 7.22 – 7.27 (m,

5H), 7.10 – 7.21 (m, 6H), 6.77 – 6.83 (m, 2H), 4.32 (dd, J = 5.93, 8.95 Hz, 2H), 3.87 (d, J = 13.19 Hz, 1H), 3.77 (s, 3H), 3.58 – 3.71 (m, 3H), 3.34 – 3.41 (m, 1H), 3.31 (dd, J = 5.56, 9.70 Hz, 1H), 2.98 (d, J = 10.36 Hz, 1H), 2.60 (dd, J = 5.46, 10.36 Hz, 1H), 2.49 (ddd, J = 6.78, 9.70, 13.85 Hz, 1H), 1.92 – 2.02 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.9, 159.1, 138.3, 135.4, 134.1, 131.0, 129.7, 129.7, 128.8, 128.6, 127.8, 127.3, 126.9, 114.0, 67.0, 58.7, 56.9, 56.0, 55.3, 50.7, 43.1, 36.3. HRMS (ESI) m/z calcd. for C$_{27}$H$_{31}$ClN$_3$O$_2$ [M+H]$^+$ 464.2105, m/z found 464.2104.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-(2-phenylethyl)pyrrolidine-2-carboxamide (24) was prepared according to the general procedure A as yellow oil (59%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.52 (t, J = 5.84 Hz, 1H), 7.32 – 7.37 (m, 1H), 7.09 – 7.24 (m, 11H), 6.83 (d, J = 8.67 Hz, 1H), 3.73 – 3.81 (m, 4H), 3.63 – 3.67 (m, 1H), 3.56 – 3.62 (m, 2H), 3.43 – 3.55 (m, 1H), 3.30 – 3.42 (m, 1H), 3.19 – 3.28 (m, 2H), 2.92 (d, J = 10.17 Hz, 1H), 2.67 – 2.76 (m, 2H), 2.42 – 2.55 (m, 2H), 1.96 (br. s., 1H), 1.81 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.1, 158.7, 138.9, 135.8, 134.1, 132.0, 130.7, 129.6, 129.4, 128.7, 128.6, 128.5, 126.9, 126.4, 113.9, 67.3, 59.5, 57.2, 56.0, 55.3, 51.2, 39.8, 37.3, 35.5. HRMS (ESI) m/z calcd. for C$_{28}$H$_{33}$ClN$_3$O$_2$ [M+H]$^+$ 478.2261, m/z found 478.2264.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-(3-phenylpropyl)pyrrolidine-2-carboxamide (25) was prepared according to the general procedure A as colorless oil (70%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.44 (t, J = 5.37 Hz, 1H), 7.13 – 7.36 (m, 10H), 7.09 (d, J = 6.78 Hz, 1H), 6.80 (d, J = 8.67 Hz, 2H), 3.83 – 3.90 (m, 1H), 3.75 (s, 3H), 3.66 – 3.73 (m, 3H), 3.34 – 3.41 (m, 1H), 3.25 (dd, J = 5.56, 9.70 Hz, 1H), 3.11 – 3.20 (m, 2H), 3.05 (d, J = 10.55 Hz, 1H), 2.43 – 2.64 (m, 4H), 1.96 (d, J = 3.20 Hz, 1H), 1.65 – 1.77 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.9, 159.1, 141.5, 135.6, 134.1, 130.9, 129.8, 129.7, 128.9, 128.4, 128.3, 127.0, 125.9, 114.0, 67.0, 58.9, 56.9, 56.0, 55.2, 50.8, 38.6, 36.5, 33.2, 31.1. HRMS (ESI) m/z calcd. for C$_{29}$H$_{35}$ClN$_3$O$_2$ [M+H]$^+$ 492.2418, m/z found 492.2411.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-methoxyphenyl)ethyl]-4-{[(4-methoxyphenyl) methyl]amino}pyrrolidine-2-carboxamide (26) was prepared according to the general procedure A as colorless oil (20%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.40 (t, J = 5.65 Hz, 1H), 7.32 – 7.36 (m, 1H), 7.15 – 7.22 (m, 4H), 7.10 – 7.14 (m, 1H), 7.05 (d, J = 8.67 Hz, 2H), 6.85 (d, J = 8.67 Hz, 2H), 6.77 (d, J = 8.48 Hz, 2H), 3.78 (s, 3H), 3.75 (s, 3H), 3.70 – 3.73 (m, 1H), 3.65 (d, J = 9.04 Hz, 2H), 3.42 – 3.52 (m, 1H), 3.37 (dd, J = 5.65, 9.61 Hz, 1H), 3.23 – 3.32 (m, 1H), 3.10 – 3.22 (m, 2H), 2.67 (dt, J = 3.30, 7.02 Hz, 2H), 2.25 (t, J = 8.01 Hz, 1H), 2.01 – 2.16 (m, 2H), 1.40 – 1.68 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.8, 158.8, 158.2, 135.7, 134.2, 132.1, 131.0, 130.9, 129.6, 129.6, 129.2, 128.8, 126.9, 114.0, 113.9, 66.7, 60.1, 57.8, 56.1, 55.3, 55.2, 52.1, 40.0, 37.9, 34.8, 30.9. HRMS (ESI) m/z calcd. for C$_{29}$H$_{35}$ClN$_3$O$_3$ [M+H]$^+$ 508.2367, m/z found 508.2357.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(3,4-dimethoxyphenyl)ethyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (27) was prepared according to the general procedure A as white solid (61%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.29 – 7.35 (m, 1H), 7.18 – 7.25 (m, 3H), 7.09 – 7.15 (m, 2H), 6.82 (d, J = 8.67 Hz, 2H), 6.68 (s, 3H),

3.63 – 3.88 (m, 13H), 3.45 – 3.60 (m, 3H), 3.28 – 3.41 (m, 1H), 3.12 – 3.20 (m, 2H), 2.70 (qd, J = 7.21, 14.93 Hz, 2H), 2.43 – 2.58 (m, 2H), 1.99 (ddd, J = 3.49, 6.26, 13.99 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 172.6, 160.3, 148.9, 147.6, 134.6, 133.6, 131.2, 131.1, 130.4, 129.5, 128.7, 126.9, 120.6, 114.5, 111.8, 111.1, 66.8, 56.0, 55.8, 55.2, 55.0, 54.9, 48.9, 40.1, 34.8, 33.8. HRMS (ESI) m/z calcd. for C$_{30}$H$_{37}$ClN$_3$O$_4$ [M+H]$^+$ 538.2473, m/z found 538.2457.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-{2-[4-(dimethylamino)phenyl]ethyl}-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (28) was prepared according to the general procedure A as yellow oil (25%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.30 – 7.34 (m, 1H), 7.15 – 7.25 (m, 5H), 6.98 (d, J = 8.67 Hz, 2H), 6.84 (d, J = 8.67 Hz, 2H), 6.59 (d, J = 8.48 Hz, 2H), 3.62 – 3.81 (m, 7H), 3.33 – 3.45 (m, 3H), 3.20 (dd, J = 5.46, 9.42 Hz, 1H), 3.05 (d, J = 10.36 Hz, 1H), 2.86 (s, 6H), 2.53 – 2.69 (m, 3H), 2.40 – 2.51 (m, 1H), 1.91 (d, J = 13.56 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.7, 159.3, 149.4, 135.4, 133.9, 130.8, 130.1, 129.5, 129.2, 128.6, 126.9, 126.5, 114.1, 112.9, 66.8, 58.2, 56.1, 55.8, 55.3, 50.3, 40.7, 40.3, 35.8, 34.4. HRMS (ESI) m/z calcd. for C$_{30}$H$_{38}$ClN$_4$O$_2$ [M+H]$^+$ 521.2683, m/z found 521.2661.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-acetamidophenyl)ethyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (29) was prepared according to the general procedure A as colorless liquid (50%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.44 (br. s., 1H), 7.67 (t, J = 5.75 Hz, 1H), 7.37 (dd, J = 1.60, 7.44 Hz, 1H), 7.23 – 7.31 (m, 6H), 7.11 – 7.21 (m, 2H), 7.03 (d, J = 8.29 Hz, 2H), 6.86 (d, J = 8.67 Hz, 2H), 3.84 (s, 2H), 3.76 (s, 3H), 3.62 – 3.74 (m, 2H), 3.39 – 3.48 (m, 2H), 3.34 (td, J = 6.31, 12.62 Hz, 1H), 3.11 – 3.23 (m, 2H), 2.66 – 2.75 (m, 2H), 2.54 (dd, J = 5.56, 11.02 Hz, 1H), 2.28 – 2.41 (m, 1H), 2.07 (s, 3H), 1.76 (d, J = 14.32 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.1, 168.9, 160.1, 136.1, 134.5, 134.5, 133.5, 130.9, 130.5, 129.2, 129.0, 128.5, 126.8, 120.6, 114.3, 65.6, 55.0, 54.8, 54.4, 53.5, 48.7, 39.8, 34.4, 33.7, 23.9. HRMS (ESI) m/z calcd. for C$_{30}$H$_{36}$ClN$_4$O$_3$ [M+H]$^+$ 535.2476, m/z found 535.2470.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-{2-[4-(methylcarbamoyl)phenyl]ethyl}pyrrolidine-2-carboxamide (30) was prepared according to the general procedure A as colorless oil (28%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.75 (t, J = 5.84 Hz, 1H), 7.53 (d, J = 8.10 Hz, 2H), 7.37 (dd, J = 2.17, 6.88 Hz, 1H), 7.22 (d, J = 8.67 Hz, 2H), 7.09 – 7.18 (m, 4H), 6.82 (d, J = 8.48 Hz, 3H), 3.86 (d, J = 2.45 Hz, 2H), 3.71 – 3.77 (m, 3H), 3.57 – 3.68 (m, 2H), 3.33 – 3.54 (m, 3H), 3.12 – 3.23 (m, 2H), 3.06 (q, J = 7.41 Hz, 1H), 2.89 (d, J = 4.71 Hz, 3H), 2.74 – 2.83 (m, 2H), 2.38 – 2.55 (m, 2H), 1.94 (dd, J = 3.58, 14.13 Hz, 1H), 1.34 – 1.45 (m, 5H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.0, 168.3, 160.4, 142.5, 134.7, 133.6, 132.7, 131.3, 130.5, 129.5, 128.8, 128.7, 127.1, 114.5, 66.5, 55.2, 54.9, 54.7, 53.7, 48.9, 42.0, 39.8, 35.0, 33.8, 26.7, 11.8. HRMS (ESI) m/z calcd. for C$_{30}$H$_{35}$ClN$_4$O$_3$ [M+H]$^+$ 535.2476, m/z found 535.2458.

(2S,4S)-N-[2-(4-tert-Butylphenyl)ethyl]-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (31) was prepared according to the general procedure A as colorless oil (36%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.51 (br. s., 1H), 7.31 – 7.36 (m, 1H), 7.14 – 7.25 (m, 8H), 7.05 (d, J = 8.29 Hz, 2H), 6.83 (d, J = 8.67

Hz, 2H), 3.75 – 3.83 (m, 5H), 3.57 – 3.67 (m, 3H), 3.40 – 3.50 (m, 1H), 3.30 – 3.39 (m, 1H), 3.20 – 3.26 (m, 2H), 2.92 (d, J = 10.17 Hz, 1H), 2.67 (q, J = 7.03 Hz, 2H), 2.41 – 2.56 (m, 2H), 1.80 – 1.89 (m, 1H), 1.26 (s, 9H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.1, 158.7, 149.2, 135.8, 135.8, 134.1, 132.1, 130.9, 129.6, 129.3, 128.7, 128.3, 126.9, 125.4, 113.8, 67.2, 59.6, 57.2, 56.1, 55.2, 51.2, 39.9, 37.3, 35.1, 34.3, 31.3. HRMS (ESI) m/z calcd. for C$_{32}$H$_{41}$ClN$_3$O$_2$ [M+H]$^+$ 534.2887, m/z found 534.2887.

(2S,4S)-N-[2-(4-Chlorophenyl)ethyl]-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (32) was prepared according to the general procedure A as colorless oil (55%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.46 (t, J = 5.93 Hz, 1H), 7.27 – 7.31 (m, 1H), 7.08 – 7.25 (m, 7H), 7.00 – 7.04 (m, 2H), 6.81 – 6.86 (m, 2H), 3.75 – 3.80 (m, 0H), 3.73 (s, 3H), 3.68 (d, J = 13.19 Hz, 2H), 3.53 – 3.60 (m, 1H), 3.31 – 3.50 (m, 3H), 3.18 (dd, J = 6.78, 9.04 Hz, 1H), 3.08 (d, J = 10.93 Hz, 1H), 2.69 (q, J = 7.03 Hz, 2H), 2.42 – 2.55 (m, 2H), 1.92 (dt, J = 3.20, 7.06 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.1, 159.8, 137.2, 134.9, 133.7, 132.1, 130.6, 130.2, 130.0, 129.6, 128.7, 128.5, 126.9, 114.3, 67.1, 57.3, 55.8, 55.2, 55.2, 49.7, 39.7, 34.9, 34.7. HRMS (ESI) m/z for C$_{28}$H$_{32}$Cl$_2$N$_3$O$_2$ [M+H]$^+$ 512.1872, m/z found 512.1868.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-methoxyphenyl)ethyl]-4-{[(4-nitrophenyl)methyl]amino}pyrrolidine-2-carboxamide (33) was prepared according to the general procedure A as yellow oil (22%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.06 (d, J = 8.67 Hz, 2H), 7.45 – 7.51 (m, 1H), 7.31 – 7.36 (m, 1H), 7.27 (s, 1H), 7.13 – 7.26 (m, 6H), 6.82 – 6.89 (m, 2H), 3.79 (s, 3H), 3.62 – 3.74 (m, 4H), 3.34 – 3.53 (m, 3H), 3.11 – 3.22 (m, 2H), 2.83 (dt, J = 2.92, 6.92 Hz, 2H), 2.22 – 2.33 (m, 1H), 2.08 (t, J = 7.54 Hz, 2H), 1.46 – 1.72 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.1, 158.9, 146.7, 146.7, 135.5, 134.1, 130.9, 129.7, 129.5, 129.2, 129.0, 128.8, 127.0, 123.7, 113.9, 66.6, 60.1, 58.1, 55.9, 55.3, 52.0, 39.2, 37.8, 35.6. HRMS (ESI) m/z calcd. for C$_{28}$H$_{32}$ClN$_4$O$_4$ [M+H]$^+$ 523.2112, m/z found 523.2103.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(3,4-difluorophenyl)ethyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (34) was prepared according to the general procedure A as white solid (62%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.55 (t, J = 6.03 Hz, 1H), 7.29 (d, J = 2.26 Hz, 1H), 7.11 – 7.25 (m, 5H), 6.88 – 6.96 (m, 2H), 6.75 – 6.86 (m, 3H), 3.72 – 3.79 (m, 4H), 3.54 – 3.71 (m, 3H), 3.30 – 3.47 (m, 3H), 3.19 (dd, J = 6.69, 9.14 Hz, 1H), 3.07 (d, J = 10.93 Hz, 1H), 2.67 (q, J = 6.91 Hz, 2H), 2.43 – 2.56 (m, 2H), 1.94 (dd, J = 3.11, 6.50 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.2, 159.7, 135.8, 135.0, 133.7, 130.5, 130.2, 129.6, 128.7, 126.9, 124.5, 124.5, 124.4, 124.4, 117.5, 117.2, 117.1, 116.9, 114.2, 67.2, 57.6, 56.0, 55.3, 55.2, 49.8, 39.6, 38.6, 35.1, 34.5. HRMS (ESI) m/z calcd. for C$_{28}$H$_{30}$ClF$_2$N$_3$O$_2$ [M+H]$^+$ 514.2073, m/z found 514.2065.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-fluorophenyl)ethyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (35) was prepared according to the general procedure A as colorless oil (74%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.38 (t, J = 5.84 Hz, 1H), 7.16 – 7.26 (m, 4H), 7.01 – 7.12 (m, 4H), 6.77 – 6.86 (m, 4H), 3.73 – 3.87 (m, 2H), 3.70 (s, 3H), 3.60 – 3.67 (m, 1H), 3.47 – 3.58 (m, 3H), 3.24 – 3.37 (m, 1H), 3.10 – 3.21 (m, 2H), 2.63 – 2.81 (m, 2H), 2.44 – 2.56 (m, 2H), 1.93 – 2.04 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 172.3, 160.5, 134.4, 134.3, 134.3, 133.5, 131.4, 130.0, 129.9, 129.5, 128.6, 126.9,

121.8, 115.3, 115.0, 114.5, 67.1, 55.8, 55.2, 54.9, 54.6, 48.7, 40.1, 34.4, 33.4. HRMS (ESI) *m/z* calcd. for $C_{28}H_{31}ClFN_3O_2$ [M+H]$^+$ 496.2167; *m/z* found 496.2168.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-{2-[4-(trifluoromethyl)phenyl]ethyl}pyrrolidine-2-carboxamide (36) was prepared according to the general procedure A as yellow oil (69%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.54 (t, *J* = 5.84 Hz, 1H), 7.43 (d, *J* = 8.10 Hz, 2H), 7.32 (dd, *J* = 1.88, 7.35 Hz, 1H), 7.14 – 7.24 (m, 7H), 6.81 – 6.86 (m, 2H), 3.73 – 3.80 (m, 4H), 3.57 – 3.70 (m, 3H), 3.38 – 3.48 (m, 2H), 3.29 – 3.36 (m, 1H), 3.23 (dd, *J* = 5.84, 9.80 Hz, 1H), 3.00 (s, 1H), 2.71 – 2.81 (m, 2H), 2.42 – 2.58 (m, 2H), 1.80 – 1.90 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.0, 159.0, 143.0, 135.5, 134.0, 130.6, 129.6, 129.0, 128.8, 126.9, 125.4, 125.3, 114.0, 67.1, 59.0, 57.0, 56.0, 55.2, 50.9, 39.5, 36.7, 35.4. HRMS (ESI) *m/z* calcd. for $C_{29}H_{32}ClF_3N_3O_2$ [M+H]$^+$ 546.2135, *m/z* found 546.2140.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N-[2-(pyridin-4-yl)ethyl]pyrrolidine-2-carboxamide (37) was prepared according to the general procedure A as white solid (73%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 8.34 (d, *J* = 5.84 Hz, 2H), 7.65 (t, *J* = 5.93 Hz, 1H), 7.24 – 7.33 (m, 3H), 7.20 (d, *J* = 8.67 Hz, 2H), 7.10 – 7.15 (m, 2H), 7.05 (d, *J* = 6.03 Hz, 2H), 6.82 (d, *J* = 8.67 Hz, 2H), 3.75 – 3.86 (m, 2H), 3.67 (br. s., 5H), 3.50 (quin, *J* = 6.83 Hz, 2H), 3.35 – 3.44 (m, 1H), 3.10 – 3.20 (m, 2H), 2.76 (dt, *J* = 2.83, 7.06 Hz, 2H), 2.44 – 2.57 (m, 2H), 1.93 (ddd, *J* = 2.73, 7.16, 14.22 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 172.7, 160.3, 149.4, 148.3, 134.5, 133.6, 131.2, 130.2, 129.6, 128.7, 126.9, 124.2, 114.5, 67.1, 56.2, 55.2, 54.8, 49.1, 38.9, 34.6, 34.0. HRMS (ESI) *m/z* calcd. for $C_{27}H_{32}ClN_4O_2$ [M+H]$^+$ 479.2214, *m/z* found 479.2212.

(2S,4S)-N-[2-(4-Acetylphenyl)ethyl]-1-[(2-chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (38) was prepared according to the general procedure A as colorless liquid (35%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.65 – 7.72 (m, 2H), 7.35 – 7.42 (m, 1H), 7.18 – 7.24 (m, 3H), 7.05 – 7.16 (m, 4H), 6.83 (d, *J* = 8.67 Hz, 2H), 3.78 – 3.89 (m, 2H), 3.66 – 3.74 (m, 4H), 3.48 – 3.61 (m, 3H), 3.37 (d, *J* = 5.65 Hz, 1H), 3.18 (d, *J* = 8.10 Hz, 2H), 2.74 – 2.87 (m, 2H), 2.43 – 2.60 (m, 2H), 2.18 (d, *J* = 5.84 Hz, 3H), 1.93 – 2.06 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 172.4, 160.5, 157.6, 140.4, 136.6, 134.4, 133.7, 131.5, 130.5, 129.5, 128.7, 128.6, 127.0, 126.7, 121.7, 114.6, 66.9, 55.5, 55.2, 54.7, 54.2, 48.6, 40.0, 35.1, 33.4, 14.8. HRMS (ESI) *m/z* calcd. for $C_{30}H_{35}ClN_3O_3$ [M+H]$^+$ 520.2367, *m/z* found 520.2407.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-methanesulfonylphenyl)ethyl]-4-{[(4-methoxyphenyl)methyl]amino}pyrrolidine-2-carboxamide (39) was prepared according to the general procedure A as colorless oil (55%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.75 (d, *J* = 8.29 Hz, 2H), 7.64 (t, *J* = 5.93 Hz, 1H), 7.25 – 7.35 (m, 5H), 7.16 – 7.22 (m, 4H), 6.81 – 6.86 (m, 2H), 3.76 (s, 3H), 3.59 – 3.74 (m, 4H), 3.42 (q, *J* = 6.97 Hz, 2H), 3.32 – 3.37 (m, 1H), 3.23 (dd, *J* = 5.84, 9.61 Hz, 1H), 3.01 (d, *J* = 1.70 Hz, 1H), 2.98 (s, 3H), 2.79 (qd, *J* = 6.99, 14.46 Hz, 2H), 2.55 (dd, *J* = 5.65, 10.55 Hz, 1H), 2.41 – 2.50 (m, 1H), 1.81 – 1.91 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.0, 159.2, 145.6, 138.7, 135.4, 133.9, 130.7, 129.9, 129.7, 129.6, 128.9, 127.5, 127.0, 114.1, 67.0, 58.6, 56.7, 55.9, 55.3, 50.7, 44.5, 39.5, 36.3, 35.5. HRMS (ESI) *m/z* calcd. for $C_{29}H_{35}ClN_3O_4S$ [M+H]$^+$ 556.2037, *m/z* found 556.2035.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N,N-diethyl-4-{[(4-methoxyphenyl)methyl]amino} **pyrrolidine-2-carboxamide (40)** was prepared according to the general procedure A as yellow oil (54%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.37 – 7.44 (m, 3H), 7.31 – 7.35 (m, 1H), 7.18 – 7.23 (m, 2H), 6.87 – 6.93 (m, 2H), 4.09 (d, J = 2.07 Hz, 2H), 3.91 – 4.00 (m, 3H), 3.82 – 3.86 (m, 1H), 3.81 (s, 3H), 3.39 – 3.51 (m, 2H), 3.18 – 3.29 (m, 1H), 2.88 – 3.08 (m, 3H), 2.36 – 2.48 (m, 1H), 2.14 (d, J = 14.69 Hz, 1H), 1.04 (t, J = 7.25 Hz, 3H), 0.97 (t, J = 7.16 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.0, 160.3, 135.1, 133.6, 131.3, 130.7, 129.5, 128.8, 127.1, 123.1, 114.6, 59.0, 56.7, 56.4, 55.3, 54.3, 48.5, 42.4, 41.6, 33.7, 15.0, 12.8. HRMS (ESI) m/z calcd. for C$_{24}$H$_{33}$ClN$_3$O$_2$ [M+H]$^+$ 430.2261, m/z found 430.2239.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-methoxyphenyl)methyl]amino}-N,N- **dipropylpyrrolidine-2-carboxamide (41)** was prepared according to the general procedure A as colorless oil (63%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.55 (dd, J = 1.70, 7.54 Hz, 1H), 7.13 – 7.34 (m, 6H), 6.85 (d, J = 8.48 Hz, 2H), 3.73 – 3.92 (m, 7H), 3.70 (dd, J = 4.52, 8.85 Hz, 1H), 3.44 (d, J = 5.84 Hz, 1H), 2.99 – 3.35 (m, 6H), 2.81 (dd, J = 6.03, 9.61 Hz, 1H), 2.40 (td, J = 8.34, 13.28 Hz, 1H), 1.80 – 1.89 (m, 1H), 1.43 – 1.57 (m, 4H), 0.86 (dt, J = 4.52, 7.44 Hz, 6H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.4, 158.9, 136.4, 133.6, 130.8, 129.7, 129.2, 128.1, 126.8, 113.9, 60.9, 58.3, 56.3, 55.3, 53.9, 50.7, 49.4, 48.3, 36.3, 22.8, 20.9, 11.4, 11.2. HRMS (ESI) m/z calcd. for C$_{26}$H$_{37}$ClN$_3$O$_2$ [M+H]$^+$ 458.2574, m/z found 458.2563.

(3S,5S)-1-[(2-Chlorophenyl)methyl]-N-[(4-methoxyphenyl)methyl]-5-(4- **phenylpiperazine-1-carbonyl)pyrrolidin-3-amine (42)** was prepared according to the general procedure A as colorless oil (23%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.45 (dd, J = 1.79, 7.44 Hz, 1H), 7.27 – 7.39 (m, 5H), 7.14 – 7.25 (m, 2H), 6.84 – 6.95 (m, 5H), 3.90 – 3.99 (m, 4H), 3.85 (dd, J = 3.49, 9.14 Hz, 1H), 3.80 (s, 3H), 3.77 (s, 1H), 3.54 – 3.71 (m, 3H), 3.46 (br. s., 1H), 3.35 (d, J = 10.55 Hz, 1H), 2.88 – 3.10 (m, 5H), 2.37 – 2.49 (m, 1H), 2.06 (d, J = 14.13 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 172.6, 159.7, 150.7, 135.4, 133.7, 130.9, 130.6, 129.6, 129.3, 128.8, 127.0, 120.7, 116.6, 114.3, 57.5, 56.4, 55.3, 54.6, 49.7, 49.6, 49.3, 45.6, 42.2, 34.7. HRMS (ESI) m/z calcd. for C$_{30}$H$_{36}$ClN$_4$O$_2$ [M+H]$^+$ 519.2527, m/z found 519.2511.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-ethyl-4-{[(4-(methylthio)phenyl)methyl]amino} **pyrrolidine-2-carboxamide (1)** was prepared according to the general procedure A as colorless oil (52%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.45 (br. s., 1H), 7.37 (dd, J = 3.67, 5.56 Hz, 1H), 7.29 – 7.33 (m, 1H), 7.23 (dd, J = 3.58, 5.84 Hz, 2H), 7.16 – 7.21 (m, 4H), 3.83 – 3.90 (m, 1H), 3.63 – 3.73 (m, 3H), 3.29 (d, J = 5.84 Hz, 1H), 3.24 (dd, J = 5.46, 9.98 Hz, 1H), 3.10 – 3.19 (m, 2H), 2.98 (d, J = 9.98 Hz, 1H), 2.61 (dd, J = 5.56, 10.08 Hz, 1H), 2.49 – 2.54 (m, 1H), 2.46 (s, 3H), 1.91 (dd, J = 2.73, 8.76 Hz, 3H), 1.02 (t, J = 7.35 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.5, 136.6, 136.3, 135.3, 133.8, 130.7, 129.3, 128.5, 128.3, 126.5, 126.5, 66.6, 59.2, 57.1, 55.8, 50.8, 36.7, 33.2, 15.6, 14.1. HRMS (ESI) m/z calcd. for C$_{22}$H$_{29}$ClN$_3$OS [M+H]$^+$ 418.1720, m/z found 418.1707.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-(methylthio)phenyl)methyl]amino}-N- **pentylpyrrolidine-2-carboxamide (43)** was prepared according to the general procedure A as colorless oil (62%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.47 (br. s., 1H), 7.31 – 7.39 (m, 2H),

7.21 – 7.26 (m, 2H), 7.17 – 7.20 (m, 4H), 3.84 – 3.91 (m, 1H), 3.59 – 3.75 (m, 3H), 3.30 (br. s., 1H), 3.25 (dd, J = 5.46, 9.80 Hz, 1H), 3.10 (dt, J = 6.97, 12.72 Hz, 2H), 2.99 (d, J = 10.17 Hz, 1H), 2.60 (dd, J = 5.46, 9.98 Hz, 1H), 2.48 – 2.54 (m, 1H), 2.46 (s, 3H), 1.91 (d, J = 10.36 Hz, 2H), 1.32 – 1.43 (m, 2H), 1.17 – 1.29 (m, 4H), 0.84 (t, J = 6.78 Hz, 3H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.0, 137.1, 136.7, 135.8, 134.2, 131.0, 129.7, 128.9, 128.7, 126.9, 67.1, 59.6, 57.5, 56.3, 51.3, 38.9, 37.1, 29.2, 29.1, 22.3, 16.1, 13.9. HRMS (ESI) *m/z* calcd. for C$_{25}$H$_{35}$ClN$_3$OS [M+H]$^+$ 460.2189, *m/z* found 460.2172.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-4-{[(4-(methylthio)phenyl)methyl]amino}-N-(2-phenylethyl)pyrrolidine-2-carboxamide (44) was prepared according to the general procedure A as colorless oil (46%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.40 (br. s., 1H), 7.33 (dd, J = 2.92, 4.43 Hz, 1H), 7.09 – 7.24 (m, 12H), 3.60 – 3.79 (m, 4H), 3.37 – 3.50 (m, 2H), 3.34 (d, J = 6.03 Hz, 1H), 3.21 (dd, J = 5.75, 9.51 Hz, 1H), 2.98 (d, J = 10.36 Hz, 1H), 2.67 – 2.76 (m, 2H), 2.47 – 2.58 (m, 2H), 2.45 (s, 3H), 1.86 (d, J = 12.62 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.8, 138.8, 137.8, 135.5, 134.7, 134.0, 130.7, 129.6, 129.1, 128.7, 128.6, 128.5, 126.9, 126.8, 126.4, 67.0, 58.6, 56.6, 55.9, 50.7, 39.9, 36.4, 35.4, 15.9. HRMS (ESI) *m/z* calcd. for C$_{28}$H$_{33}$ClN$_3$OS [M+H]$^+$ 494.2033, *m/z* found 494.2022.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-(methylthio)phenyl)ethyl]-4-{[(4-methoxyphenyl) methyl]amino}pyrrolidine-2-carboxamide (45) was prepared according to the general procedure A as colorless oil (50%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.47 (br. s., 1H), 7.32 – 7.38 (m, 1H), 7.15 – 7.24 (m, 7H), 7.02 (d, J = 8.48 Hz, 2H), 6.73 (d, J = 8.67 Hz, 2H), 3.71 – 3.80 (m, 4H), 3.59 – 3.69 (m, 3H), 3.42 (dd, J = 6.69, 13.28 Hz, 1H), 3.32 – 3.38 (m, 1H), 3.26 (d, J = 4.14 Hz, 1H), 3.19 – 3.24 (m, 1H), 2.93 (d, J = 9.98 Hz, 1H), 2.61 – 2.71 (m, 2H), 2.51 (dd, J = 5.37, 10.08 Hz, 2H), 2.45 (s, 3H), 2.04 (br. s., 2H), 1.78 – 1.88 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.0, 158.2, 137.2, 136.5, 135.7, 134.0, 130.8, 130.7, 129.6, 129.5, 128.8, 128.7, 126.9, 113.9, 67.2, 59.3, 57.1, 56.1, 55.2, 51.2, 40.0, 37.1, 34.6, 16.0. HRMS (ESI) *m/z* calcd. for C$_{29}$H$_{35}$ClN$_3$O$_2$S [M+H]$^+$ 524.2138, *m/z* found 524.2128.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(4-(methylthio)phenyl)ethyl]-4-{[(4-nitrophenyl) methyl]amino}pyrrolidine-2-carboxamide (46) was prepared according to the general procedure A as colorless oil (44%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.93 (d, J = 8.67 Hz, 2H), 7.53 (t, J = 6.03 Hz, 1H), 7.23 – 7.28 (m, 1H), 7.06 – 7.18 (m, 8H), 3.51 – 3.70 (m, 4H), 3.37 (q, J = 6.78 Hz, 2H), 3.22 (d, J = 3.20 Hz, 1H), 3.17 (dd, J = 5.65, 9.80 Hz, 1H), 2.88 (d, J = 10.17 Hz, 1H), 2.72 (qd, J = 7.10, 14.13 Hz, 2H), 2.40 – 2.49 (m, 2H), 2.39 (s, 3H), 1.85 (br. s., 1H), 1.70 – 1.79 (m, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.3, 145.7, 145.6, 136.4, 135.4, 134.6, 132.9, 129.4, 128.7, 128.4, 127.8, 127.7, 125.9, 125.8, 122.6, 66.1, 58.4, 56.3, 55.3, 50.3, 38.2, 36.1, 34.5, 14.9. HRMS (ESI) *m/z* calcd. for C$_{28}$H$_{32}$ClN$_4$O$_3$S [M+H]$^+$ 539.1884, *m/z* found 539.1858.

(2S,4S)-1-[(2-Chlorophenyl)methyl]-N-[2-(3,4-difluorophenyl)ethyl]-4-{[(4-(methylthio)phenyl)methyl]amino}pyrrolidine-2-carboxamide (47) was prepared according to the general procedure A as colorless liquid (56%). ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.58 (br. s., 1H), 7.34 (d, J = 7.35 Hz, 1H), 7.15 – 7.25 (m, 7H), 6.88 – 7.00 (m, 2H), 6.75 – 6.83 (m, 1H), 3.70 – 3.80 (m, 1H), 3.59 – 3.69 (m, 3H), 3.35 – 3.45 (m, 1H), 3.33 (d, J =

6.97 Hz, 2H), 3.24 (dd, J = 5.65, 9.61 Hz, 1H), 2.96 (d, J = 9.80 Hz, 2H), 2.60 – 2.70 (m, 2H), 2.54 (dd, J = 5.56, 10.46 Hz, 2H), 2.41 – 2.49 (m, 4H), 1.85 (d, J = 13.00 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 174.1, 137.5, 135.9, 135.6, 134.0, 130.6, 129.7, 128.8, 128.8, 126.9, 126.9, 124.5, 124.5, 124.4, 124.4, 117.5, 117.3, 117.2, 117.0, 67.1, 59.2, 57.1, 56.1, 51.2, 39.6, 36.9, 34.7, 15.9. HRMS (ESI) m/z calcd. for $C_{28}H_{31}ClF_2N_3OS$ [M+H][+] 530.1844, m/z found 530.1824.

96-well calcium mobilization assay.

Two individual stable cell lines were created by over-expressing human NPFFR1 and NPFFR2 receptors in CHO-RD-HGA16 (Molecular Devices) cells. The day before the assay, cells were plated into 96-well black-walled assay plates at 30,000 cells/well (100 μL volume) in Ham's F12 supplemented with 10% fetal bovine serum, 100 units of penicillin/ streptomycin, and 100 μg/mL normocin™. The cells were incubated overnight at 37°C, 5% CO$_2$. Prior to the assay, Calcium 5 dye (Molecular Devices) was reconstituted according to the manufacturer's instructions. The reconstituted dye was diluted 1:40 in warm 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 warm assay buffer. The cells were incubated for 45 minutes at 37°C, 5% CO$_2$ in 200 μL of the diluted Calcium 5 dye. A single concentration of each test compound was prepared at 10x the desired final concentration in 2.25% BSA/8% DMSO/assay buffer. Serial dilutions of NPFF were prepared at 10x the desired final concentration in 0.25% BSA/1% DMSO/assay buffer, aliquoted into 96-well polypropylene plates, and warmed to 37°C. After the dye-loading incubation period, the cells were pretreated with 25 μL of the test compounds and incubated for 15 min at 37°C. After the pretreatment incubation period, the plate was read with a FlexStation II (Molecular Devices). Calcium-mediated changes in fluorescence were monitored every 1.52 seconds over a 60 second time period, with the FlexStation II adding 25 μL of the NPFF serial dilutions at the 19 second time point (excitation at 485 nm, detection at 525 nm). Peak kinetic reduction (SoftMax, Molecular Devices) relative fluorescent units (RFU) were plotted against the log of compound concentration. Nonlinear regression analysis (3-parameter) was used to generate EC$_{50}$ values (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). Apparent K$_e$ values were calculated using the equation $K_e = [L]/((EC_{50}^+/EC_{50}^-) - 1)$ where [L] is the concentration of test compound, EC_{50}^+ is the EC$_{50}$ of NPFF with test compound, and EC_{50}^- is the EC$_{50}$ of NPFF alone. K$_e$ values were considered valid when the EC_{50}^+/EC_{50}^- ratio was at least 4.

384-well HTS.

Stable human NPFFR1 CHO-RD-HGA16 cells were plated in 30 μL/well volume at 5,000 cells/well in Ham's F12 medium supplemented with 1% FBS and 100 units penicillin/ streptomycin in 384-well Greiner μClear® black walled microplates using a MicroFlo™ Select dispenser fitted with a 5 μL cassette (BioTek). The plated cells were incubated overnight at 37 °C, 5% CO2, 95% relative humidity. The next day, compound test plates were prepared by diluting previously replicated library daughter plates with assay buffer to achieve a 100 μM (10X desired final concentration) working solution and filling columns 1, 2, 23, and 24 with positive and negative controls. An additional compound test plate containing the NPFF EC$_{60}$ concentration (250 nM prepared at 10x the desired final

concentration in 1% DMSO/assay buffer) was prepared for the antagonist portion of the screen. Calcium 5 dye (Bulk Kit, Molecular Devices), reconstituted according to the manufacturer's instructions, was diluted 1:20 in pre-warmed (37°C) assay buffer (1X HBSS, 20 mM HEPES, 2.5 mM probenecid, pH 7.4 at 37 °C) and 30 μL was added to the plate with the Biomek NX, which was then incubated for 45 minutes at 37 °C, 5% CO_2, 95% relative humidity. Using the Biomek NX, the dye loaded plate was pretreated with 8.5 μL of 8% DMSO/assay buffer and incubated for 15 minutes at 37 °C, 5% CO_2, 95% relative humidity. After this incubation period, the plate was read with the Tetra to evaluate agonist activity. Calcium-mediated changes in fluorescence were monitored every 1 second over a 60 second time period, with the Tetra adding 8.5 μL from the compound plate at the 10 second time point (excitation at 470–495 nm, detection at 515–575 nm). The cell plate was then incubated for another 15 minutes at 37 °C, 5% CO_2, 95% relative humidity after which it was read with the Tetra to evaluate antagonist activity. Calcium-mediated changes in fluorescence were monitored every 1 second over a 60 second time period, with the Tetra adding 8.5 μL from the NPFF EC_{60} plate at the 10 second time point (excitation at 470–495 nm, detection at 515–575 nm). Data was exported from ScreenWorks (Molecular Devices) using the response over baseline (ROB) statistic which presents data as a fold-response compared with the baseline sample. Percent inhibition was calculated using the equation (1 – (cmpd ROB / NPFF EC_{60} ROB)) × 100.

cAMP assay.

Stable human NPFFR1-CHO (ES-491-C) and NPFFR2-CHO (ES-490-C) cell lines were purchased from PerkinElmer and used with the Lance *Ultra* kit (TRF0262) to detect cAMP accumulation in low volume 96-well plates. Stimulation buffer containing 1X HBSS, 5 mM HEPES (pH 7.4), 0.1% BSA stabilizer, and 0.5 mM IBMX was prepared. Serial dilutions of the agonist control NPFF were prepared at 8X the desired final concentration in 2% DMSO/ stimulation buffer and 2.5 μL was added to the assay plate. A single concentration of each test compound was prepared at 4X the desired final concentration in 2% DMSO/stimulation buffer and 5 μL was added to the assay plate. The EC_{80} concentration of forskolin (1 μM) was prepared at 8X in 2% DMSO/stimulation buffer and 2.5 μL was added to the assay plate. Cells were lifted from flasks with versene and spun at 270g for 10 minutes. The cell pellet was resuspended in stimulation buffer and 4,000 cells (10 μL) were added to each well. After incubating for 30 min at RT, Eu-cAMP tracer and u*LIGHT*-anti-cAMP working solutions were added per the manufacturer's instructions. After 1 hour, the TR-FRET signal (ex 337 nm) was read on a CLARIOstar multimode plate reader (BMG Biotech, Cary NC). Fluorescence values at 665 nm were plotted against the log of compound concentration and nonlinear regression analysis (3-parameter) was used to generate EC_{50} values (GraphPad Prism, GraphPad Software, Inc., San Diego CA). K_e values were calculated using the same equation as described in the 96-well calcium mobilization methods.

Radioligand binding assay.

Binding assays were performed according to PerkinElmer's protocol in a final volume of 500 μL of assay buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 60 mM NaCl, 0.5% BSA, pH 7.4). For NPFF1, the assay mixture contained 25 μL of 0.065 nM [^{125}I]NPFF (PerkinElmer, K_D = 0.11 nM, NEX381), 25 μL of test compounds (prepared at 8X the final desired concentration

in 8% DMSO/assay buffer), and 150 μL of CHO-hNPFFR1 membranes (1 μg protein/well, PerkinElmer, RBHNF1M400UA). Specific binding was defined as the difference between [^{125}I]NPFF binding in the absence and presence of 100 nM final nonradiolabeled NPFF. After incubating at 27°C for 120 minutes, the binding assay was terminated by vacuum filtration onto Unifilter GF/C glass-fiber filters (pre-soaked in 0.1% PEI) using a Brandel (Gaithersburg, MD, USA) 96-well harvester, followed by three washes with ice-cold wash buffer (50 mM Tris-HCl, 0.1% BSA, pH 7.4). The filter plate was dried for 1 hr at 55°C. Microscint 20 (50 μL) was added to each well and filter-bound radioactivity was counted on a Packard TopCount NXT microplate scintillation and luminescence counter. Percentage of specific [^{125}I]NPFF binding was plotted against the log of compound concentration. Data were fit to a one site (fit Ki) competitive binding model, logEC50=log(10^logKi*(1+RadioligandNM/HotKdNM)), to generate Ki values for the test compounds using GraphPad Prism (GraphPad Software, Inc., San Diego CA). NPFF2 binding assays were conducted with the same protocol, except that 0.1 nM [^{125}I]NPFF (PerkinElmer, $K_D = 0.15$ nM, NEX381) and CHO-hNPFFR2 membranes (1 μg protein/well, PerkinElmer, RBHNF2M400UA) were used.

Kinetic solubility assay.

10 μL of test compound stock solution (10 mM DMSO) was combined with 490 μL of PBS (potassium phosphate monobasic 1 mM, sodium phosphate dibasic 3 mM and sodium chloride 155 mM buffer). 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 200-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.

Bidirectional MDCK-MDR1 permeability assay.

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 °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 °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$_2$O (H$_2$O 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:

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

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

In vivo Pharmacology

Animals.

Adult male (n = 18) Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 225–300 g were individually housed on a 12/12-hour light/dark cycle with behavioral experiments conducted during the light period. Rats had free access to food and water except during test sessions, and were maintained and experiments were conducted in accordance with guidelines of the International Association for the Study of Pain [57] 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), and were approved by the Institutional Animal Care and Use Committee, University at Buffalo, the State University of New York (Buffalo, NY).

Drugs.

Fentanyl was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in 0.9% saline, and injected subcutaneously in a volume of 1 ml/kg. Compounds **16** and **33** were dissolved in a vehicle of 20% dimethyl sulfoxide in saline and injected intraperitoneally in a volume of 1 ml/kg.

Fentanyl-induced hyperalgesia.

Nociceptive thresholds were measured using calibrated von Frey filaments (1.4–26 g; North Coast Medical, Morgan Hill, CA). Rats (n = 6 per group) were placed in elevated plastic chambers with a wire mesh floor (IITC Life Science Inc., Woodland Hills, CA) and allowed to habituate prior to testing. Filaments were applied perpendicularly to the medial plantar surface of the hind paw from below the mesh floor in an ascending order of filament force, beginning with the lowest filament. Filaments were applied until buckling occurred for approximately two seconds. Mechanical paw withdrawal thresholds (PWTs) correspond to the lowest force that elicited a withdrawal of the hind paw in at least two out of three

applications. Forces larger than 26 g would physically elevate the non-CFA-treated paw and did not reflect pain-like behavior.

After a nociceptive baseline was established for each rat on day prior to and the day of fentanyl treatment (D_{-1} and D_0), four subcutaneous injections of 0.06 mg/kg fentanyl each were injected at 15 min intervals for a total dose of 0.24 mg/kg.[58, 59] In one group of rats, PWT measurements were taken on days 1–5 to monitor the onset of and recovery from the fentanyl-induced hyperalgesia. In the other two groups of rats, antinociceptive dose-effect curves for compounds **16** and **33** were established on day 1 using a multi-cycle cumulative dosing procedure, in which measurements were taken immediately prior to drug administration then 30 min after drug administration before the next injection, and continued for doses ranging from 3.2–32 mg/kg.

Data analysis.

PWTs were averaged within each group and plotted as a function of dose. Repeated measures one-way ANOVAs, with time or treatment entered as the within-subject factor, followed by Bonferroni's post-hoc test were used to determine the statistical significances. $P < 0.05$ was considered statistically significant for all tests.

Acknowledgments

ABBREVIATIONS

|	
DCM	dichloromethane
1,2-DCE	1,2-dichloroethane
DME	1,2-dimethoxyethane
FLIPR	fluorometric imaging plate reader
GPCR	G-protein-coupled receptor
HPLC	high performance liquid chromatography
EC$_{50}$	half-maximum effective concentration
IC$_{50}$	half-maximum inhibitory concentration
MS	mass spectrometry
NPFF	neuropeptide FF
SAR	structure−activity relationship
TLC	thin-layer chromatography

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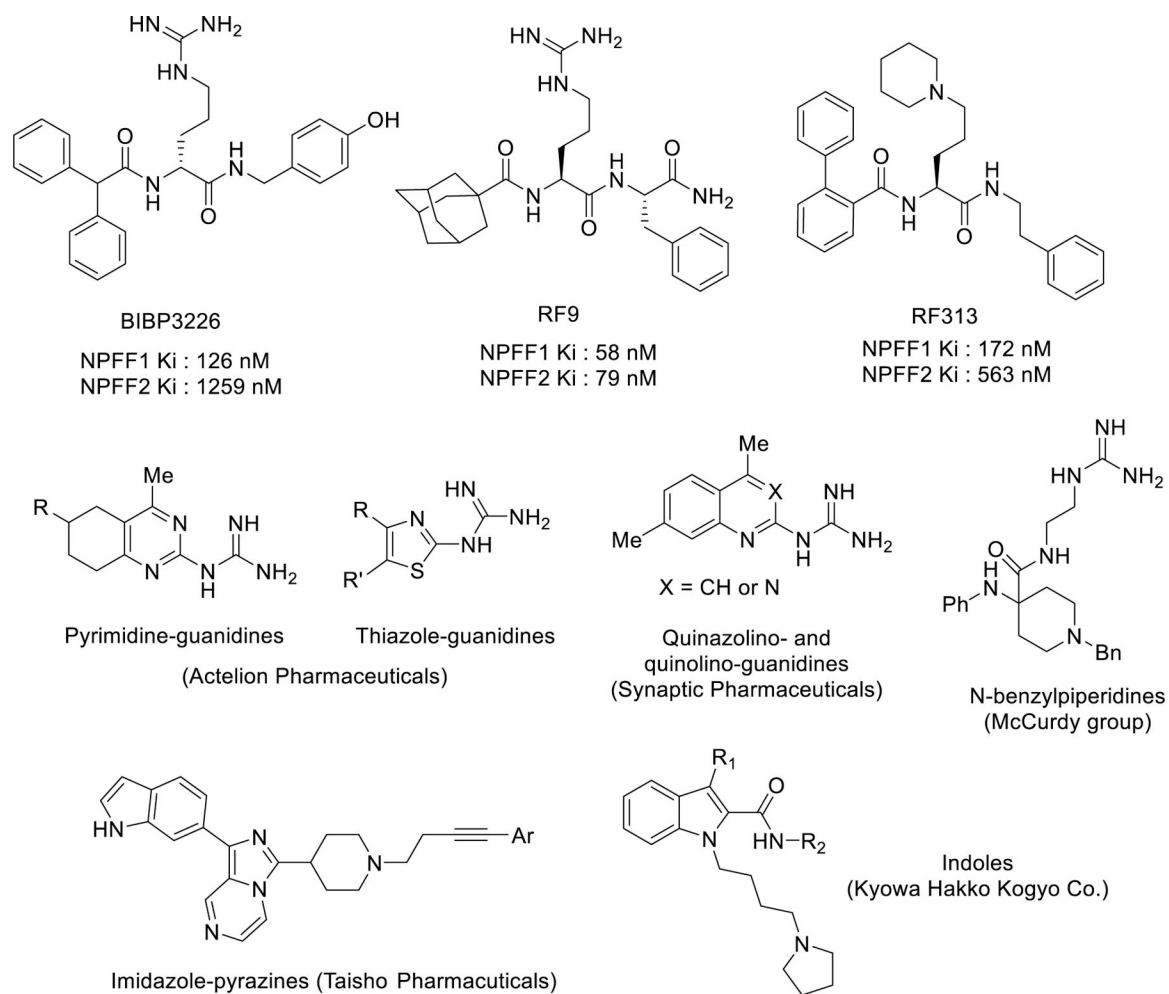
Figure 1.
Reported peptidic, peptidomimetics and small molecule NPFF ligands.
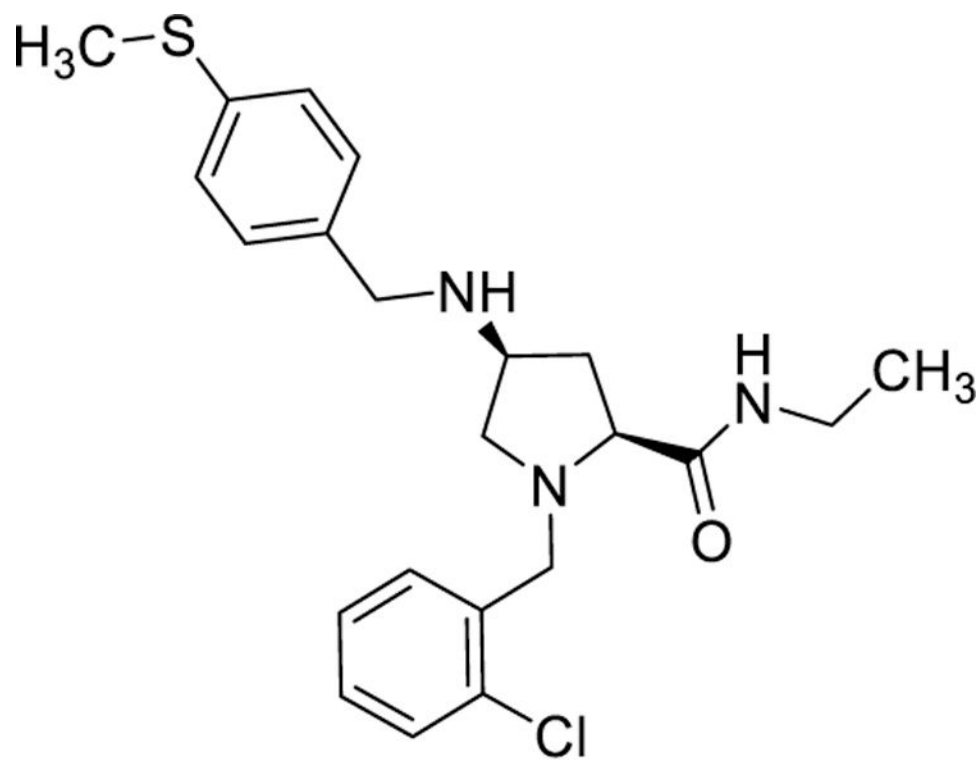
Compound 1

$$NPFF1\ K_e = 1.62 \pm 0.38\ \mu M$$
$$NPFF2\ K_e = 7.25 \pm 1.73\ \mu M$$

Figure 2.
Hit compound **1** in calcium mobilization assays
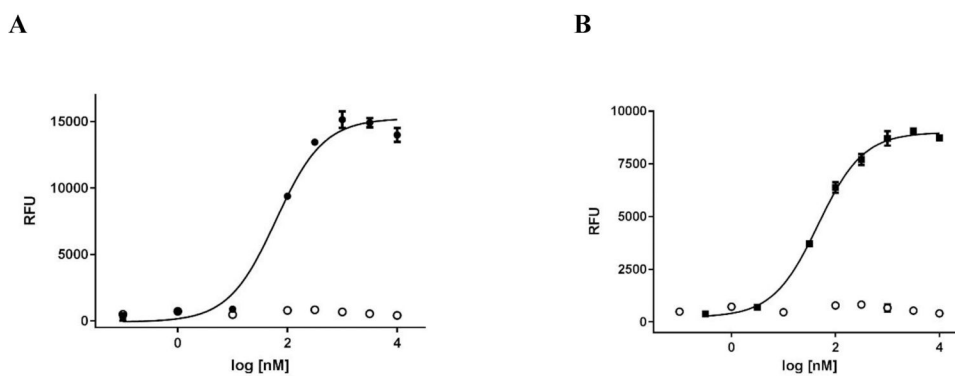
Figure 3.
NPFF EC_{50} in functional calcium mobilization assays. (A) NPFF response in stable NPFF1-RD-HGA16 cells (●) and parental RD-HGA16 CHO cells (○). (B) NPFF response in stable NPFF2-RD-HGA16 cells (■) and parental RD-HGA16 CHO cells (○). Representative data from one experiment is shown and each data point is the mean ± SD of duplicate determinations.

A

B
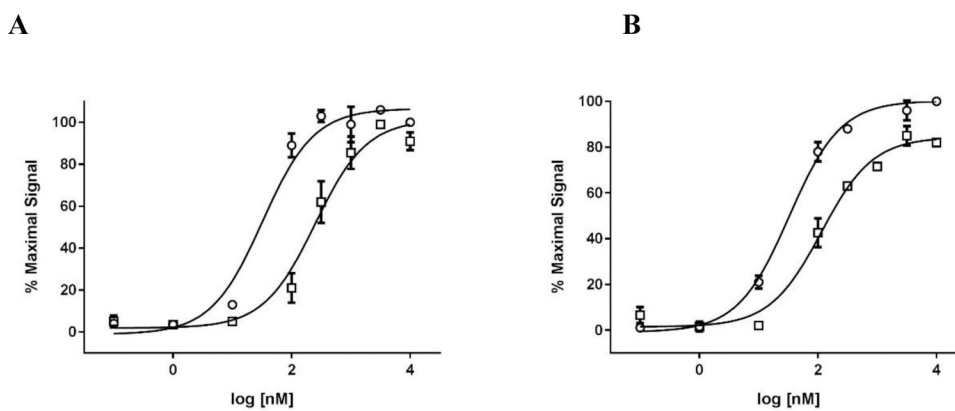
Figure 4.

Antagonist activity of compound **16** in NPFF1 (A) and NPFF2 (B) calcium mobilization functional K_e assays. (A) Concentration-response curves of NPFF alone (○) and NPFF + 5 μM final **16** (□) in stable NPFF1-RD-HGA16 cells. (B) Concentration-response curves of NPFF alone (○) and NPFF + 10 μM final **16** (□) in stable NPFF2-RD-HGA16 cells. The right shift of the NPFF curve in the presence of test compound was used to calculate K_e values as described in the Methods. Representative data from one experiment are shown and each data point is mean ± SD of duplicate determinations.
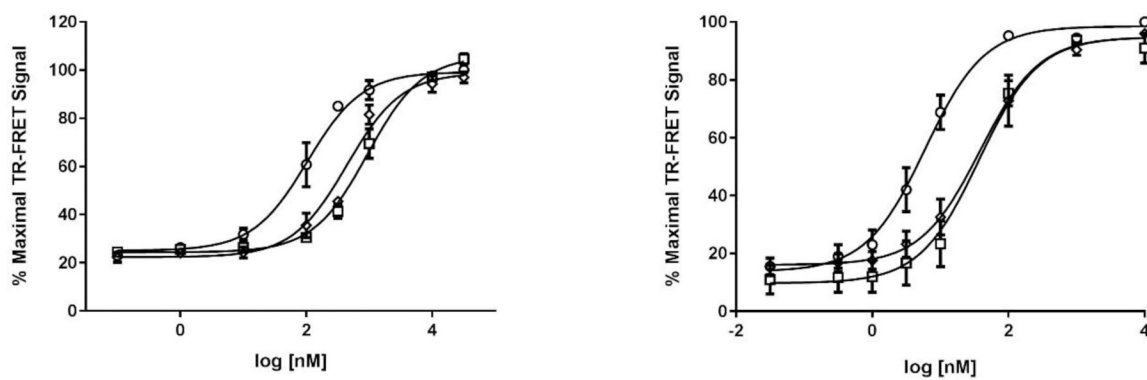
Figure 5.
Antagonist activity of compounds **16** and **33** in NPFF1 (A) and FF2 (B) cAMP functional K_e assays. (A) Concentration-response curves of NPFF alone (O), NPFF + 4 μM final **16** (□), and NPFF + 2 μM final **33** ( ) in stable NPFF1-CHO cells. (B) Concentration-response curves of NPFF alone (O), NPFF + 10 μM final **16** (□), and NPFF + 10 μM final **33** ( ) in stable NPFF2-CHO cells. The right shift of the NPFF curve in the presence of test compound was used to calculcate K_e values as described in the Methods. Each data point is mean ± SEM of at least N=3 conducted in duplicate.
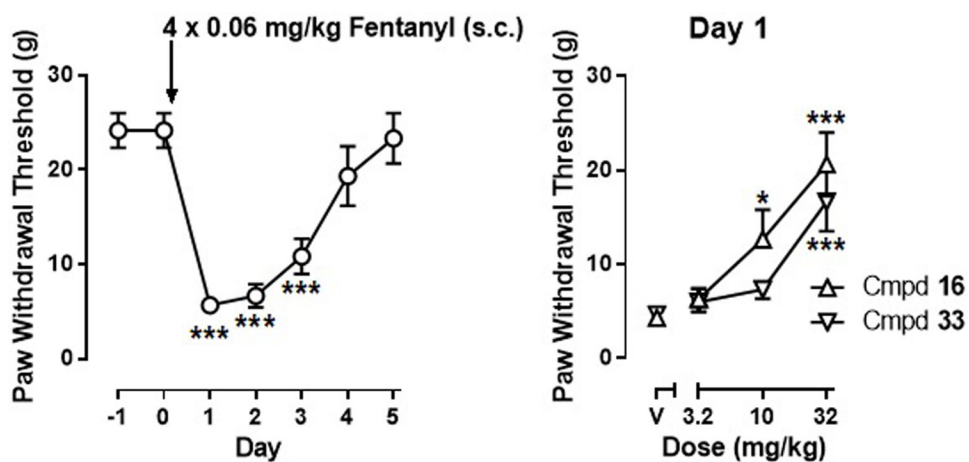
Figure 6.
(A) Fentanyl-induced mechanical hyperalgesia; (B): anti-hyperalgesic effects of compounds **16** and **33**. (N=6 per group). Abscissa: time. Ordinate: paw withdrawal threshold (gram). P < 0.05 compared to pre-fentanyl treatment (Day 0) (left) or compared to "V" (vehicle) treatment.
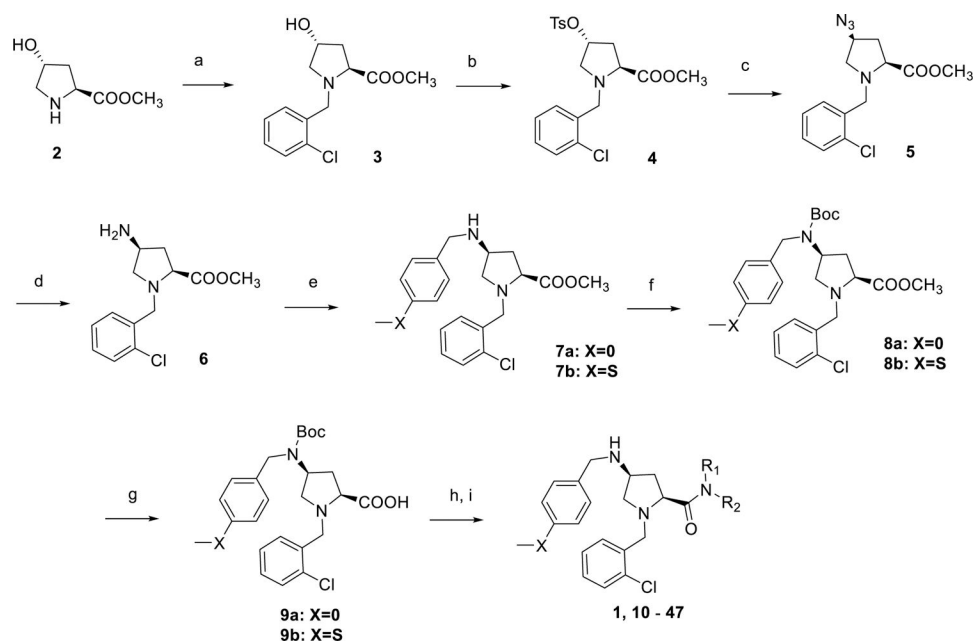
Scheme 1.

Synthesis of compounds **10** – **47**.

Reagents and conditions: (a) 2-chlorobenzaldehyde, Na(OAc)$_3$BH, 1,2-DCE, rt, 24 h (b) TsCl, pyridine, DCM, rt, 24 h (c) NaN$_3$, DMF, 70 °C, 16 h (d) PPh$_3$, THF, H$_2$O, reflux, 16 h (e) 4-MeOPhCHO or 4-MeSPhCHO, Na(OAc)$_3$BH, 1,2-DCE, rt, 24 h (f) Boc$_2$O, aq. Na$_2$CO$_3$, 1,4-dioxane, rt, 16 h (g) aq. LiOH, MeOH, rt, 16 h (h) corresponding amine, HBTU, DIEA, DMF, rt, 24 h (i) TFA, DCM, rt, 1 h.
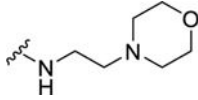
Table 1.
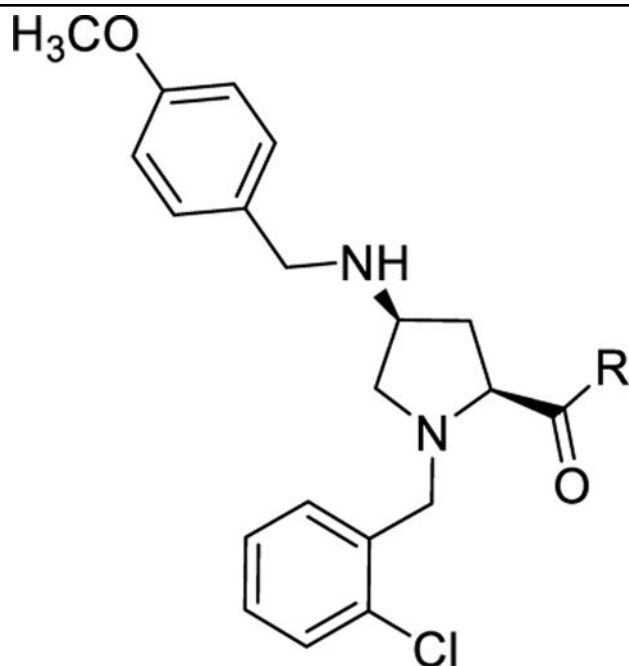
SAR at the carboxamide region.

Compound	R	Ca²⁺		cAMP	
		NPFF1 K_e (μM)^a	NPFF2 K_e (μM)^a	NPFF1 K_e (μM)^a	NPFF2 K_e (μM)^a
10	NHMe	>10^b	>10^b	N.D.	N.D.
11	NHEt	>10^b	>10^b	N.D.	N.D.
12	NH(n-Pr)	2.60 ± 0.15	>10^b	N.D.	N.D.
13	NH(n-Bu)	1.24 ± 0.14	d	N.D.	N.D.
14	NH(s-Bu)	1.50 ± 0.06	3.68 ± 0.35^b	N.D.	N.D.
15	NH(t-Bu)	1.88 ± 0.21	1.26 ± 0.8^c	N.D.	N.D.
16	NH(n-Pentyl)	0.72 ± 0.01	3.09 ± 0.58^b	0.36 ± 0.08	2.15 ± 0.36
17	NH(i-Pentyl)	1.32 ± 0.09	1.54 ± 0.13^c	N.D.	N.D.
18	NH(n-hexyl)	0.82 ± 0.08	1.49 ± 0.20^c	1.98 ± 0.28	2.48 ± 0.27
19	NH(n-decyl)	>10^{b,c}	d	N.D.	N.D.
20		1.96 ± 0.07	d	N.D.	N.D.
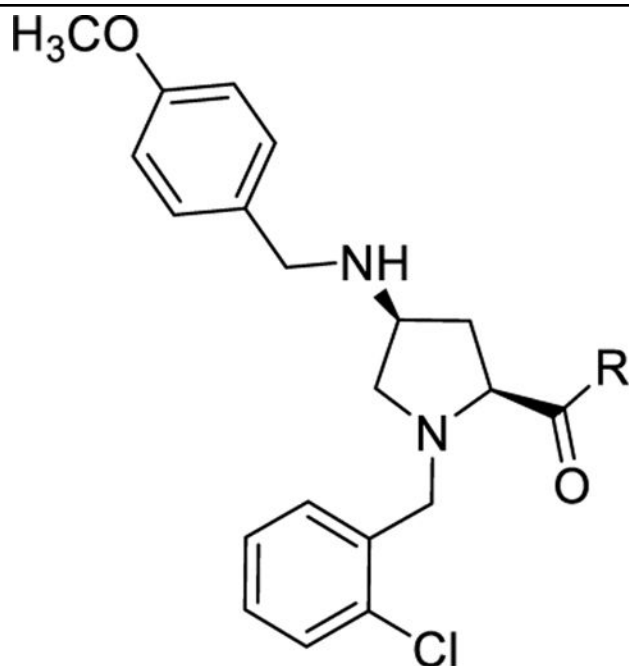
Compound	R	Ca²⁺		cAMP	
		NPFF1 K_e (μM)[a]	NPFF2 K_e (μM)[a]	NPFF1 K_e (μM)[a]	NPFF2 K_e (μM)[a]
21		1.12 ± 0.23	1.22 ± 0.36[c]	N.D.	N.D.
22		>10[b]	d	N.D.	N.D.
23		0.78 ± 0.08[c]	2.01 ± 0.44[c]	1.72 ± 0.32	3.90 ± 1.1
24		0.85 ± 0.14	2.34 ± 0.31[c]	0.77 ± 0.12	3.79 ± 0.45
25		>10[f]	2.60 ± 0.28[c]	N.D.	N.D.
26		0.67 ± 0.06	1.75 ± 0.11[c]	0.57 ± 0.15[c]	2.56 ± 0.20

Compound	R	Ca²⁺		cAMP	
		NPFF1 K_e (μM)[a]	NPFF2 K_e (μM)[a]	NPFF1 K_e (μM)[a]	NPFF2 K_e (μM)[a]
27	OCH₃ / OCH₃	1.03 ± 0.05	d	1.52 ± 0.11	> 10[b]
28		1.20 ± 0.25	2.08 ± 0.38[c]	N.D.	N.D.
29		2.51 ± 0.08	>10[b]	N.D.	N.D.
30		>10[b]	>10[b]	N.D.	N.D.
31	tBu	e	2.42 ± 0.26[c]	N.D.	N.D.
32	Cl	0.99 ± 0.22[c]	1.83 ± 0.09[c]	1.49 ± 0.10	2.53 ± 0.36
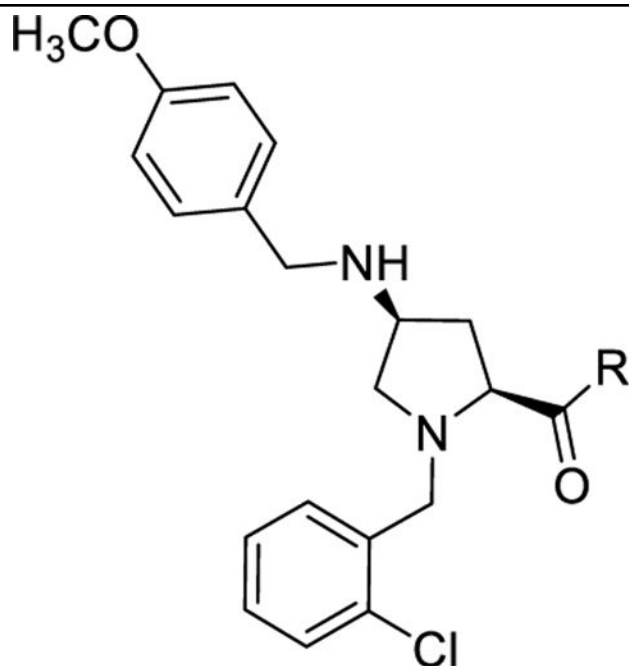
Compound	R	Ca²⁺		cAMP	
		NPFF1 K_e (µM)^a	NPFF2 K_e (µM)^a	NPFF1 K_e (µM)^a	NPFF2 K_e (µM)^a
33		0.25 ± 0.06^c	0.69 ± 0.14^c	0.49 ± 0.09	1.17 ± 0.36
34		0.61 ± 0.13	$3.49 \pm 1.4^{b,c}$	0.57 ± 0.17	2.16 ± 0.30
35		1.13 ± 0.11	$>10^b$	1.41 ± 0.18	$>10^b$
36		1.67 ± 0.22^c	d	N.D.	N.D.
37		1.82 ± 0.20	d	N.D.	N.D.
38		$>10^b$	$>10^b$	N.D.	N.D.

Compound	R	Ca^{2+}		cAMP	
		NPFF1 K$_e$ (μM)^a	NPFF2 K$_e$ (μM)^a	NPFF1 K$_e$ (μM)^a	NPFF2 K$_e$ (μM)^a
39	(structure with SO$_2$CH$_3$)	1.88 ± 0.25	d	N.D.	N.D.
40	NEt$_2$	2.90 ± 0.15	4.93 ± 0.02^b	N.D.	N.D.
41	N(n-Pr)$_2$	0.88 ± 0.12^c	1.73 ± 0.27^c	2.31 ± 0.42	>10^b
42	(phenylpiperazine structure)	1.33 ± 0.29^c	2.08 ± 0.30^c	N.D.	N.D.

^{a}Values are the mean ± SEM of at least three independent experiments in duplicate

^{b}Values are the mean ± SEM of two independent experiments in duplicate

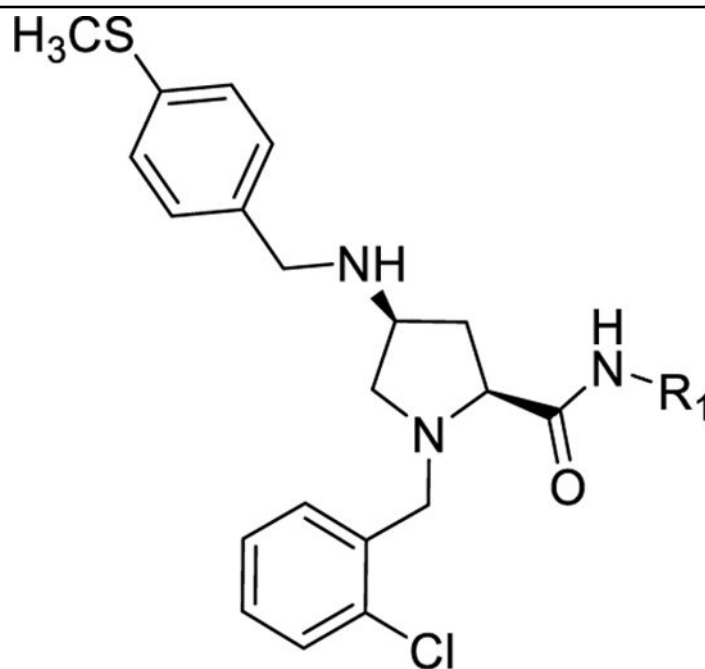^{c}Pre-incubation of antagonist and test compound was 45 min or 1 hr

^{d}Compound was inactive in antagonist screen at 10 μM final (N=2).

^{e}Compound appeared cytotoxic in the assay and potency was not determined.

^{f}Value is IC$_{50}$ because compound was noncompetitive in K$_e$ assay. N.D., Not determined.

Table 2.

SAR at the carboxamide region of the 4-(4-methylthio)benzylamino series.

Compound	R₁	Ca²⁺		cAMP	
		NPFF1 K_e (µM)^a	NPFF2 K_e (µM)^a	NPFF1 K_e (µM)^a	NPFF2 K_e (µM)^a
43	n-Pentyl	0.36 ± 0.05	0.97 ± 0.03^c	1.07 ± 0.20	2.74 ± 0.21
44		0.53 ± 0.08	3.70 ± 0.94	1.30 ± 0.14	>10^b
45	OCH₃	0.87 ± 0.10	2.30 ± 0.28^c	1.15 ± 0.12	>10^b
46	NO₂	0.37 ± 0.07^c	1.35 ± 0.20	0.89 ± 0.16	0.75 ± 0.12
47	F F	0.47 ± 0.06	0.88 ± 0.16^c	1.07 ± 0.27	>10^b

^{a}Values are the mean ± SEM of at least three independent experiments in duplicate

^{b}Values are the mean ± SEM of two independent experiments in duplicate

^{c}Pre-incubation of antagonist and test compound was 1 hr.

Table 3.

Results from the cAMP, radioligand binding and calcium mobilization assays of representative compounds
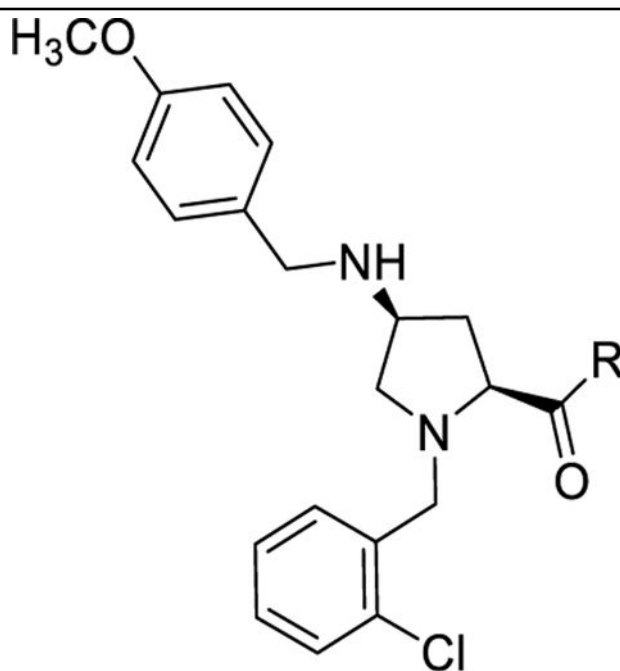
#	R$_1$	Radioligand binding[a]		cAMP assay[b]		Calcium mobilization assay[b]	
		K$_i$ NPFF1 (µM)	K$_i$ NPFF2 (µM)	K$_e$ NPFF1 (nM)	K$_e$ NPFF2 (nM)	K$_e$ NPFF1 (nM)	K$_e$ NPFF2 (nM)
16	NH(*n*-Pentyl)	0.89 ± 0.05	1.44 ± 0.13	0.36 ± 0.08	2.15 ± 0.36	0.72 ± 0.01	3.09 ± 0.58
24		0.71 ± 0.02	1.23 ± 0.27	0.77 ± 0.12	3.79 ± 0.45	0.85 ± 0.14	2.34 ± 0.31
26		0.92 ± 0.03	1.46 ± 0.05	0.57 ± 0.15	2.56 ± 0.20	0.67 ± 0.06	1.75 ± 0.11
33		0.61 ± 0.03	1.67 ± 0.06	0.49 ± 0.09	1.17 ± 0.36	0.25 ± 0.06[c]	0.69 ± 0.14
34		0.56 ± 0.04	1.49 ± 0.27	0.57 ± 0.17	2.16 ± 0.30	0.61 ± 0.13	3.49 ± 1.4

[a] Values are the mean ± SEM of at least two independent experiments in duplicate

[b] Values are the mean ± SEM of at least three independent experiments in duplicate.

Table 4.

Physicochemical and Preliminary ADME Properties of Compounds **16** and **33**

	Desired value	1	16	33
MW	< 500	418	444	523
ClogP	1 – 4	3.57	4.59	4.37
PSA	< 70	69. 7	53.6	54.4
pKa	< 8	8.6, 5.0	9.1, 4.8	9.1, 4.8
HBD	< 3	2	2	2
HBA	< 7	4	5	8
Solubility (δM)	> 60	N.D.	146.8 ± 6.9	45.9 ± 7.7
P_{app} (10^{-6} cm/sec) A-to-B	> 2	N.D.	7.6	2.7
P_{app} (10^{-6} cm/sec) B-to-A		N.D.	6.7	3.6
Efflux ratio	< 2.5	N.D.	0.9	1.3

HBD: H-bond donor; HBA: H-bond acceptor. N.D.: Not determined.