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Novel pyrrolidine melanin-concentrating hormone receptor 1 antagonists with reduced hERG inhibition

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ABSTRACT

We discovered novel pyrrolidine MCHR1 antagonist **1** possessing moderate potency. Profiling of pyrrolidine **1** demonstrated that it was an inhibitor of the hERG channel. Investigation of the structure–activity relationship of this class of pyrrolidines allowed us to optimize the MCHR1 potency and decrease the hERG inhibition. Increasing the acidity of the amide proton by converting the benzamide in lead **1** to an anilide provided single digit nanomolar MCHR1 antagonists while replacing the dimethoxyphenyl ring of **1** with alkyl groups possessing increased polarity dramatically reduced the hERG inhibition.

Melanin-concentrating hormone (MCH) is a cyclic **19** amino acid peptide in human, rat and mouse that possesses a highly conserved sequence among vertebrates.[1–3] MCH is expressed mainly in neurons in the lateral hypothalamus and zona incerta that project widely into other regions of the brain.[3–8] Several early studies demonstrated the role of MCH in the feeding behavior of rats and mice. Intracerebroventricular (Icv) injection of MCH in rats causes a dose dependent increase in food intake.[9,10] Icv infusion of MCH for 12–24 days causes an increase in food intake and results in a significant increase in body weight in both rats and mice.[10–12] Mice over-expressing MCH displayed similar characteristics including increased food intake and increased body weight and adiposity.[13] In contrast, MCH knockout mice are healthy, hypophagic, have reduced body weight, lower triglyceride levels and increased metabolism.[14]

In 1999, several groups discovered that MCH is the natural ligand for the melanin-concentrating hormone receptor **1** (MCHR1).[15–18] MCHR1 is a 353aa G_i, G_o and G_q coupled GPCR that is highly expressed in brain with highest expression levels in the amygdala, cerebral cortex, hippocampus, hypothalamus and substantia nigra.[19–22] MCHR1 knockout mice are resistant to diet-induced obesity, are hyperphagic, hypermetabolic, have lower fat mass and lower leptin and insulin levels.[23,24] These data suggest that MCHR1 antagonists might be effective for the treatment of obesity.

A high throughput screen of our sample collection resulted in the discovery of **1** as a moderately potent MCHR1 antagonist (Fig. 1). Separation and evaluation of the enantiomers indicated that the (3*S*,4*S*)-isomer **2** is the eutomer with the (3*R*,4*R*)-isomer being 20-fold less potent in the MCHR1 cell based assay. A major liability of pyrrolidine **2** is the potent inhibition of the hERG channel. The pharmacological blockade of outward K^+ currents through the hERG channel can result in QT prolongation, which is associated with an increased risk of torsades de pointes, a ventricular tachyarrhythmia that may degenerate into ventricular fibrillation and sudden death. The successful development of this class of pyrrolidine MCHR1 antagonists for the potential treatment of obesity required that we design compounds with improved MCHR1 potency and decreased inhibition of the hERG channel.

The synthesis of analogs with modifications of the pyrrolidine 3- and 4-positions is shown in Scheme 1. Alkylation of 3,4-dimethoxyphenethylamine **3** with (chloromethyl)trimethylsilane provided the corresponding silylamine that was then treated with formaldehyde followed by methanol in the presence of K_2CO_3 to provide methoxymethylamine **4** in 91% yield over two steps. Subsequent 1,3-dipolar cyclization with (*E*/*Z*)-alkyl and arylnitriles provided pyrrolidines **5** as *cis*/*trans* mixtures in 84–99% yield.[25] The stereochemistry of the alkenes is conserved during this reaction such that *E* alkenes give rise to *trans* products and *Z* alkenes provide *cis* products. Treatment of nitriles **5** with LAH isomerized the pyrrolidines and reduced the nitriles to the corresponding

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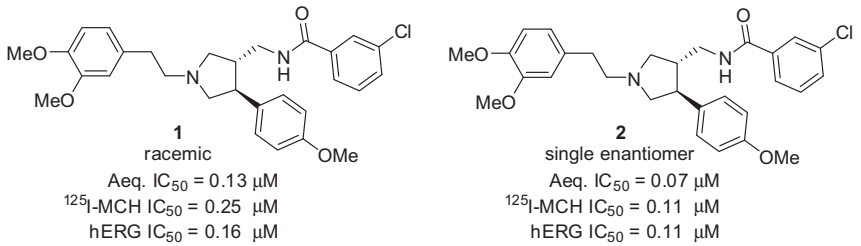
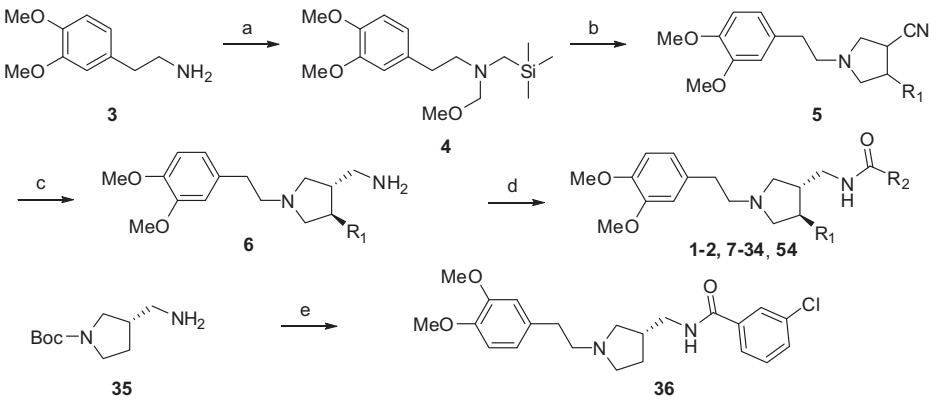


Figure 1. Structures and biochemical potencies of **1** and **2**.



Scheme 1. Reagents and conditions: (a) (i) CH_3CN, $TMSCH_2Cl$, reflux; (ii) 37% aq H_2CO, 0 °C, then K_2CO_3, MeOH, 0 °C; (b) $R_1CH{=}CHCN$, TFA, toluene, 0 °C to rt; (c) LAH, THF, 0 °C to rt; (d) R_2COCl, NEt_3, THF, reflux; (e) (i) 3-chlorobenzoylchloride, NEt_3, DCM, rt; (ii) TFA, DCM, rt; (iii) 2-(3,4-dimethoxyphenyl)acetaldehyde, $NaBH(OAc)_3$, DCM, rt.

amines to provide *trans*-pyrrolidineamines **6** as the exclusive products in 85–99% yield. Elaboration of amines **6** to the amides (**1**–**2**, **7**–**34** and **54**) was accomplished in 80–94% yield using standard amide coupling techniques.

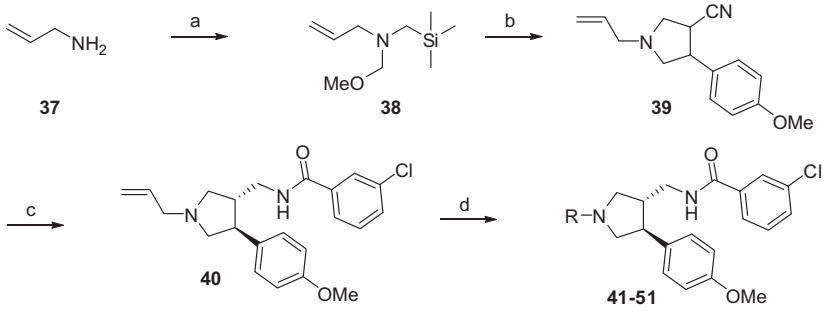
Des-phenyl analog **36** was prepared from commercially available (*S*)-*tert*-butyl 3-(aminomethyl)pyrrolidine-1-carboxylate (**35**) as shown in Scheme 1. Acylation using 3-chlorobenzoyl chloride in the presence of triethylamine provided the amide in 92% yield. Deprotection of the Boc group using TFA followed by reductive amination with 2-(3,4-dimethoxyphenyl)acetaldehyde provided **36** in 75% yield over two steps.

Analogs with variations of the 3,4-dimethoxyphenethyl group were prepared as shown in Scheme 2. Using the standard procedure described in Scheme 1 but employing allylamine **37** instead of **3** provided amide **40** in excellent yields. Removal of the allyl group of amide **40** by heating in the presence of Wilkinson's catalyst provided the secondary amine in 85% yield.[26] Subsequent reductive amination or alkylation provided products **41**–**51**.

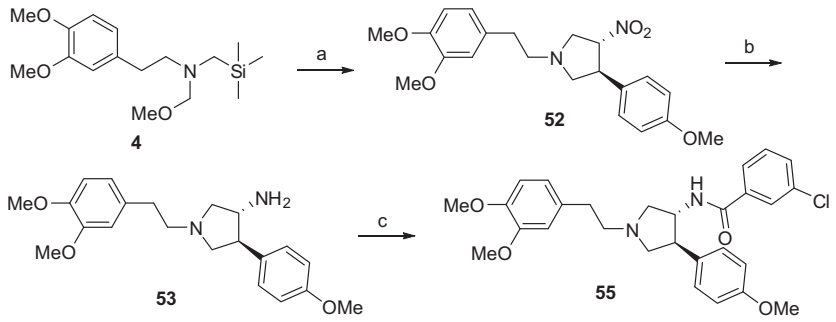
Analogs having varied length and arrangement of the amide linker were prepared as shown in Schemes 3–6. Reaction of amine **4** with (*E*)-4-methoxy-β-nitrostyrene in the presence of TFA provided pyrrolidine **52** in 72% yield (Scheme 3). Reduction of the nitro group with $SnCl_2$ yielded amine **53** in 50% yield. Elaboration of amine **53** provided amide **55** in 61% yield.

1,3-Dipolar cyclization of 4 with (*E*)-ethyl 4-methoxycinnamate provided ester **56** in 93% yield (Scheme 4). LAH reduction followed by mesylation using methanesulfonic anhydride and cyanide displacement yielded nitrile **57** in quantitative yield over three steps. Hydrolysis of **57** with NaOH provided acid **58** in quantitative yield. Conversion to the acid chloride and reaction with various amines resulted in amides **59**–**61** in 19–24% yield over two steps. Nitrile **57** was also elaborated to amide **62** in 15% yield over two steps.

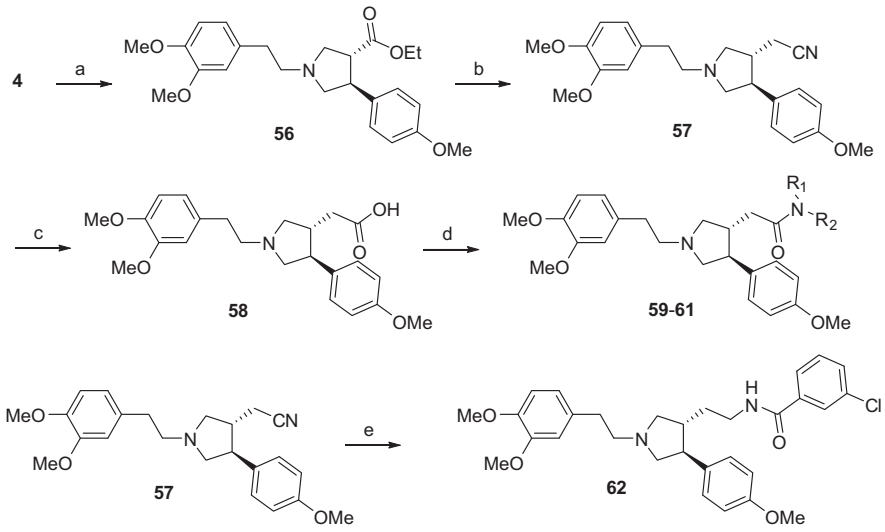
DIBAL-H reduction of ester **56** followed by conversion to the α,β-unsaturated nitrile provided **64** in quantitative yield over two steps (Scheme 5). Hydrogenation of **64** followed by LAH reduction and benzoylation resulted in the isolation of **65** in 11% yield over three steps.



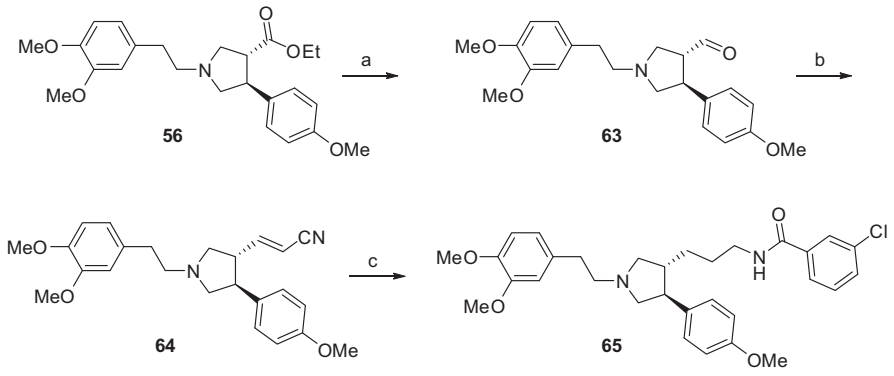
Scheme 2. Reagents and conditions: (a) (i) CH_3CN, $TMSCH_2Cl$, reflux; (ii) 37% aq H_2CO, 0 °C, then K_2CO_3, MeOH, 0 °C; (b) 4-methoxycinnamonitrile, TFA, toluene, 0 °C to rt; (c) (i) LAH, THF, 0 °C to rt; (ii) 3-chlorobenzoyl chloride, NEt_3, THF, reflux; (d) (i) $(Ph_3P)_3RhCl$, 84% aq CH_3CN, reflux; (ii) RCHO, $NaBH(OAc)_3$, 1,2-dichloroethane or ROMs, NEt_3, THF, rt.



Scheme 3. Reagents and conditions: (a) (*E*)-4-methoxy-β-nitrostyrene, TFA, toluene, rt; (b) $SnCl_2{\cdot}2H_2O$, EtOH, 70 °C; (c) 3-chlorobenzoyl chloride, NEt_3, THF, rt.



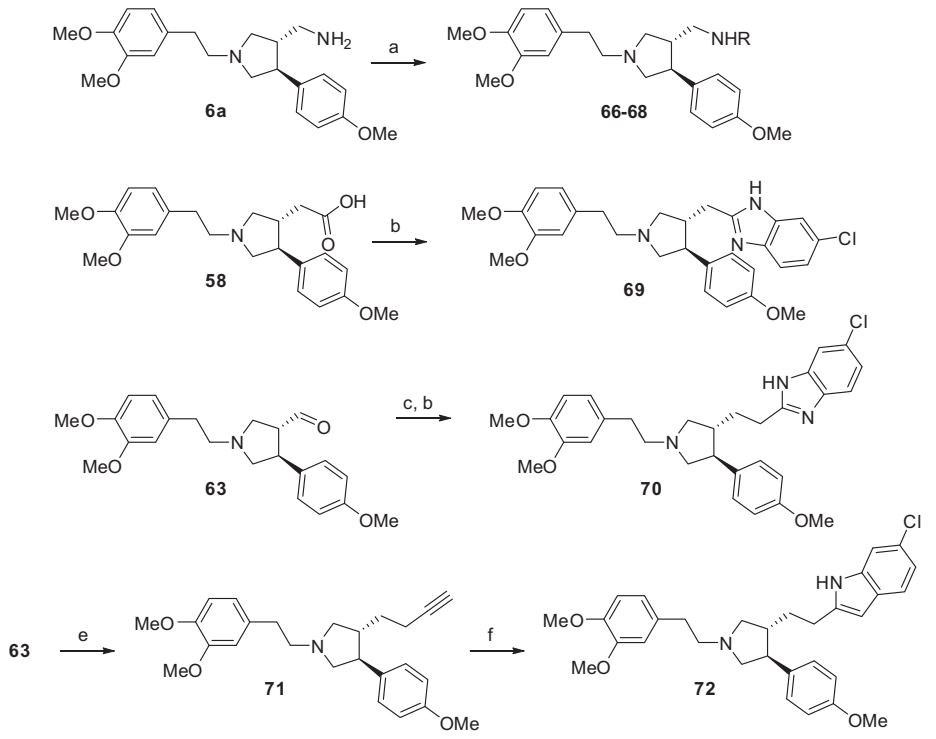
Scheme 4. Reagents and conditions: (a) (*E*)-ethyl 4-methoxycinnamate, TFA, toluene, 0 °C to rt; (b) (i) LAH, THF, 0 °C to rt; (ii) Ms_2O, NEt_3, CH_2Cl_2, rt; (iii) NaCN, DMSO, 90 °C; (c) (i) NaOH, MeOH, reflux; (d) (i) $(COCl_2)$, DMF (cat), CH_2Cl_2, rt; (ii) R_1R_2NH, NEt_3, THF, rt; (e) (i) LAH, THF, 0 °C to rt; (ii) 3-chlorobenzoyl chloride, NEt_3, THF, rt.



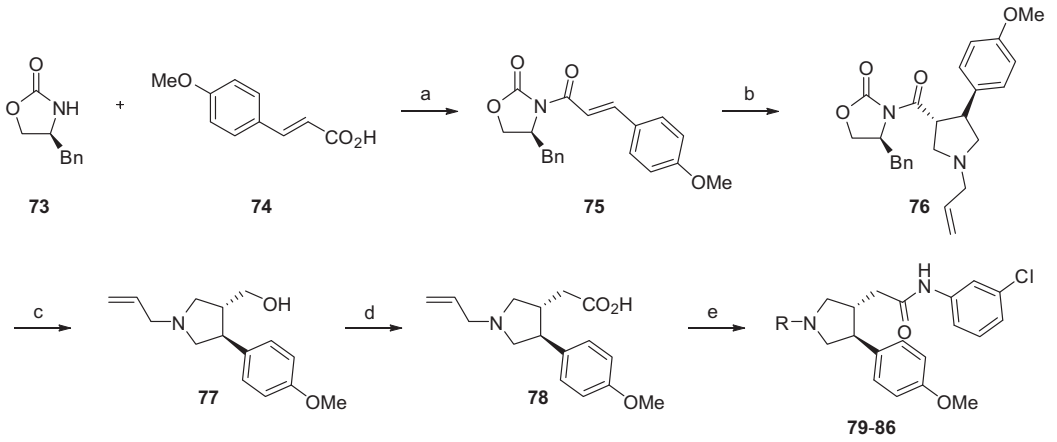
Scheme 5. Reagents and conditions: (a) DIBAL-H, CH_2Cl_2, −78 °C; (b) $(EtO)_2P(O)CH_2CN$, NaH, THF, 0 °C to rt; (c) (i) 10% Pd/C, H_2, EtOH, rt; (ii) LAH, THF, 0 °C to rt; (iii) 3-chlorobenzoylchloride, NEt_3, THF, rt.

Amide replacements **66**–**68** were prepared from common amine **6a** as shown in Scheme 6. Conversion of carboxylate **58** to the amide followed by heating in acetic acid provided benzimidazole **69** in 86% yield over two steps. Aldehyde **63** underwent Horner–Wadsworth–Emmons (HWE) conditions followed by hydrogenation of the double bond to provide the saturated ester in 85% yield over two steps. Hydrolysis of the ester followed by amide coupling conditions and heating the resulting amide at 65 °C in acetic acid provided **70** in 66% yield over three steps. Aldehyde **63** was converted to alkyne **71** in 15% yield over four steps consisting of HWE, hydrogenation, DIBAL-H reduction and treatment with Ohira's reagent.[27,28] Sonagashira coupling of alkyne **71** followed by palladium catalyzed ring closure provided indole **72** in 54% yield.

Synthesis of enantiomerically pure analogs of **59** is shown in Scheme 7. Acylation of chiral oxazolidinone **73** was accomplished in 87% yield from the mixed anhydride of 4-methoxycinnamic acid **74**.[29] 1,3-Dipolar cyclization of **75** and **38** resulted in a 1:1 mixture of diastereomers that provided desired isomer **76** in 41% yield after column chromatography. The absolute configuration of the active



Scheme 6. Reagents and conditions: (a) (i) 3-chlorobenzaldehyde, $Na(OAc)_3BH$, dichloroethane, rt provided **66** in 42% yield; (ii) 3-chlorophenyl isocyanate, CH_3CN, rt provided **67** in 61% yield; (iii) 3-chlorobenzenesulfonyl chloride, pyridine, CH_3CN, reflux provided **68** in 43% yield; (b) (i) 4-chloro-1,2-diaminobenzene, HBTU, HOBT, DIPEA, CH_3CN, 55 °C; (ii) AcOH, 65 °C; (c) (i) trimethyl phosphonoacetate, NaH, THF, 0 °C to rt; (ii) H_2, 10% Pd/C, MeOH, rt; (iii) LiOH, MeOH, H_2O, rt; (e) (i) trimethyl phosphonoacetate, NaH, THF, 0 °C to rt; (ii) H_2, 10% Pd/C; iii. DIBAL-H, toluene −78 °C; (iv) dimethyl 1-diazo-2-oxopropylphosphonate, K_2CO_3, MeOH, 0 °C to rt; (f) (i) 4-chloro-2-iodoaniline, $Pd(Ph_3P)_4$, CuI, pyrrolidine, rt; (ii) $PdCl_2$, Bu_4NCl, 2 M HCl, CH_2Cl_2, rt.



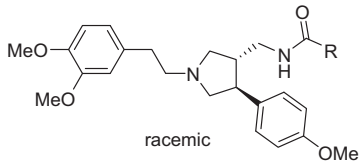
Scheme 7. Reagents and conditions: (a) (i) pivaloyl chloride, NEt_3, THF, 0 °C to rt; (ii) BuLi, THF, −78 °C; (b) 38, TFA, toluene, 0 °C to rt; (c) LAH, THF, −78 °C to rt; (d) (i) Ms_2O, NEt_3, THF, 0 °C; (ii) NaCN, DMSO, 90 °C; (iii). NaOH, MeOH, H_2O, reflux; (e) (i) 3-chloroaniline, DCC, CH_2Cl_2, 0 °C to rt; (ii) $(Ph_3P)_3RhCl$, 84% aq CH_3CN, reflux; (iii) RCHO, $Na(OAc)_3BH$, 1,2-dichloroethane, rt.

isomer was assigned by obtaining a single crystal X-ray structure of **76**. Reduction of **76** using LAH yielded alcohol **77** in quantitative yield. Mesylation of **77** followed by displacement with NaCN in DMSO and subsequent hydrolysis provided acid **78** in 71% yield over three steps. DCC coupling with 3-chloroaniline in dichloromethane followed by removal of the allyl protecting group provided the penultimate intermediate in 63% yield. Reductive amination with the corresponding aldehydes provided **79**–**86** as single enantiomers.

As shown in Table 1 no improvements in MCHR1 potency were achieved by replacing the *m*-chlorophenyl ring of compound **1**. *meta*-Substituted analogs **7**–**11** failed to provide an increase in MCHR1 activity with trifluoromethyl analog **7** being essentially equipotent with **1**. *para*-Substituted analogs **12**–**16** lost a significant amount of potency with the exception of fluoride **14**. All analogs prepared with *ortho*-substituted phenyl rings were significantly less potent except for the *o*-fluorophenyl analog **18** that retained weak MCHR1 potency. Replacement of the phenyl amide with the corresponding 2-, 3-, or 4-pyridyl amides (**21**–**23**) also resulted in complete loss of MCHR1 potency. Taken together, these data suggests that to maintain MCHR1 inhibition, electron poor, *meta*-substituted hydrophobic aryl rings are preferred in the phenyl amide portion of this molecule.

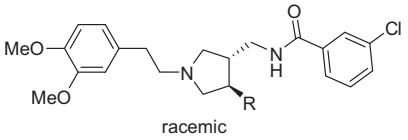
Investigation of the SAR around the *p*-methoxyphenyl ring also resulted in no improvement in MCHR1 potency (Table 2). Placing the methoxy substituent in the *meta*-position (**27**) resulted in a

Table 1
MCHR1 activity for racemic benzamide analogs.



Cmpd	R	Aeq. IC_{50} [30] (μM)
1	3-Cl-phenyl	0.13
7	3-CF_3-phenyl	0.16
8	3-F-phenyl	0.53
9	3-CN-phenyl	0.67
10	3-OMe-phenyl	1.13
11	3-SO_2CH_3-phenyl	>10.0
12	4-Cl-phenyl	1.02
13	4-CF_3-phenyl	>10.0
14	4-F-phenyl	0.1
15	4-CN-phenyl	>10.0
16	4-OMe-phenyl	>10.0
17	Phenyl	0.41
18	2-F-phenyl	3.8
19	2-Cl-phenyl	>10.0
20	2-CF_3-phenyl	>10.0
21	2-Pyridyl	>10.0
22	3-Pyridyl	>10.0
23	4-Pyridyl	>10.0

Table 2
MCHR1 activity for racemic analogs of the 4-OMe-phenyl ring

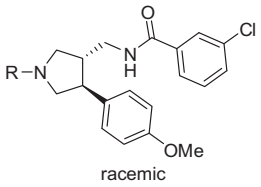


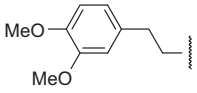
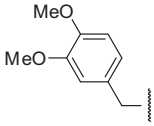
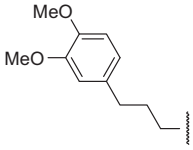
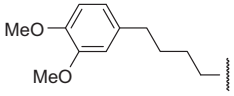
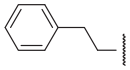
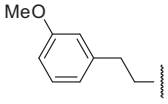
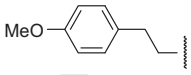
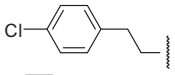
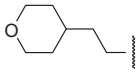
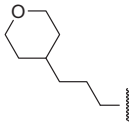
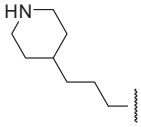
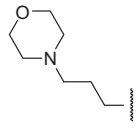
Compd	R	Aeq. IC_{50} (μM)
1	4-OMe-phenyl	0.13
24	4-$N(CH_3)_2$-phenyl	0.14
25	Phenyl	0.17
26	4-Cl-phenyl	0.17
27	3-OMe-phenyl	0.36
28	4-OCF_3-phenyl	2.6
29	2-OMe-phenyl	3.18
30	2-Thiophenyl	3.2
31	3-Thiophenyl	4.8
32	3-Furanyl	2.3
33	2-Furanyl	>10.0
34	*t*-Butyl	>10.0
36	Hydrogen	>10.0

moderate loss in potency whereas *o*-methoxy analog **29** was significantly less potent than **1**. Deletion of the methoxy group (**25**) as well as replacement of the methoxy group with a dimethyl amine moiety (**24**) or chlorine (**26**) provided compounds that were equipotent to **1**. Attempts to replace the phenyl ring with either thiophene or furan provided compounds **30**–**33**, all significantly less potent than **1**. Finally, both *t*-butyl analog **34** and des-phenyl compound **36** failed to register any MCHR1 activity.

Investigation of the SAR of the dimethoxyphenethyl group resulted in analogs with increased MCHR1 potency and analogs with decreased hERG inhibition (Table 3). Altering the linker length, as exemplified by analogs **41**–**43**, indicated that a three carbon linker was the optimal tether length for MCHR1 potency. The importance of the 3-OMe group was demonstrated by analogs **44**–**47** as all three analogs lacking the 3-OMe group had decreased MCHR1 potency. Unfortunately, no decrease in hERG inhibition was observed with analog **42** or **45**.

Table 3
MCHR1 and hERG activity for analogs of the pyrrolidine nitrogen substituent



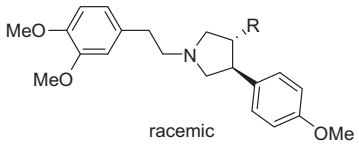
Compd	R	Aeq. IC_{50} (μM)	hERG IC_{50} (μM)
1		0.13	0.16
41		1.52	0.6
42		0.031	0.20
43		0.25	
44		2.05	
45		0.17	0.047
46		0.84	0.028
47		0.94	
48		0.35	1.96
49		0.31	1.03
50		1.52	20.3
51		2.62	

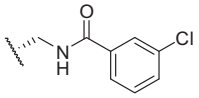
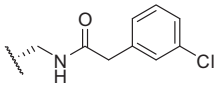
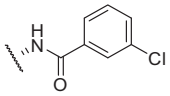
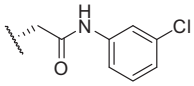
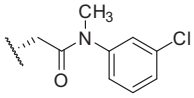
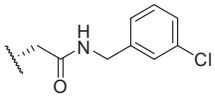
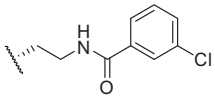
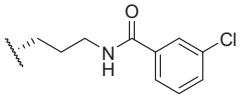
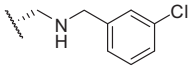
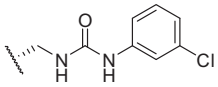
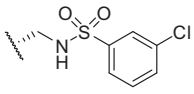
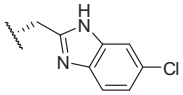
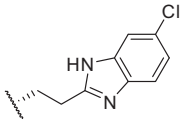
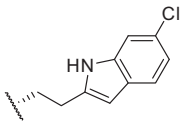
Based on a proprietary hERG binding model we hypothesized that replacing the phenethyl ring with less hydrophobic alkyl groups would result in reduced hERG inhibition. Investigation of a few alkyl replacements resulted in the discovery that tetrahydropyran analogs **48** and **49** maintained moderate MCHR1 potency while dramatically reducing hERG inhibition. Unfortunately, further increasing the polarity by incorporating a basic amine into cycloalkyl analogs **50** and **51** resulted in a significant loss in MCHR1 potency. Having found a way to drastically decrease the

hERG inhibition with alkyl analogs we sought to increase the MCHR1 potency by investigating the SAR of the amide linker.

We explored the effect of shortening and lengthening the linker length between the pyrrolidine ring and the amide nitrogen as well as the distance between the amide carbonyl and the phenyl ring with analogs **54**–**55**, **59**–**62** and **65** (Table 4). The data indicated that optimal MCHR1 potency was achieved when the nitrogen of the amide was three atoms from the pyrrolidine ring. Increasing the acidity of the amide proton as in anilide **59** resulted in a significant increase in MCHR1 potency while methylated amide **60** and

Table 4
MCHR1 and hERG activity for amide replacements



Compd	R	Aeq. IC_{50} (μM)	^{125}I-MCH IC_{50} μM[31]	hERG IC_{50} [32] (μM)
1		0.13	0.25	0.16
54		>10.0		
55		>10.0		
59		0.011	0.040	0.066
60		1.6		0.029
61		0.64		0.034
62		0.041	0.077	0.056
65		4.60		
66		2.20		
67		>10.0		
68		>10.0		
69		0.15		0.028
70		0.091	0.10	0.016
72		3.12		0.045

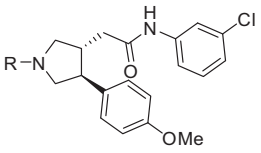
amine **66** were both significantly less potent. The decrease in potency could indicate the necessity of an acidic proton but could also be explained by changing the angle between the amide and the phenyl ring as analogs **54**, **67** and **68** all have acidic protons at the right length but are completely devoid of MCHR1 potency. Finally, our attempts to replace the amide functionality all together led to benzimidazoles **69** and **70** which retained MCHR1 potency, whereas indole **72** lost a significant amount of MCHR1 activity. Unfortunately, neither the alteration of linker length, increasing the acidity of the amide proton nor benzimidazole replacement of the amide succeeded in reducing hERG inhibition.

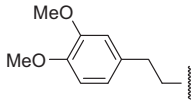
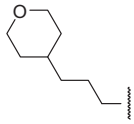
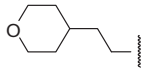
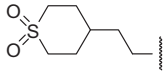
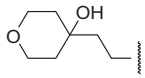
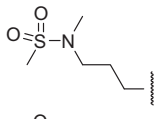
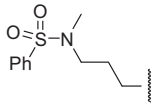
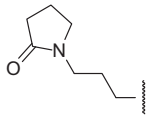
Finally, we synthesized molecules that combined the knowledge gained from the SAR studies described above. We prepared single enantiomer analogs of anilide **59** with various alkyl groups extending from the pyrrolidine nitrogen with the aim of decreasing hERG inhibition (Table 5). Synthesis of the eutomer of **59** provided **79** possessing 7 nM MCHR1 antagonist activity and hERG IC_{50} = 2 nM.

Introduction of polarity into this part of the molecule as with sulfone **82** resulted in no loss in MCHR1 potency compared to **79** but a 145-fold decrease in hERG inhibition. Incorporation of a tetrahydropyran group that so dramatically affected hERG inhibition in the lead series resulted in compounds **80** and **81** possessing good MCHR1 potency. The hERG inhibition of **80** was reduced over 180-fold compared to **79** by replacing the dimethoxyphenyl ring with the three carbon linked tetrahydropyran; however, the hERG IC_{50} value (0.37 μM) was only 10-fold higher than the MCHR1 IC_{50} value (0.037 μM). Increasing the polarity of the tetrahydropyran analogs further provided tertiary alcohol **83** possessing MCHR1 IC_{50} = 57 nM and hERG IC_{50} = 8.24 μM. This is a dramatic example of the effect of polarity on hERG inhibition as the incorporation of the tertiary alcohol decreased hERG inhibition almost 70-fold compared to **81** while maintaining MCHR1 inhibition. Sulfonamide analogs **84** and **85** also demonstrate the profound effect of polarity in this part of the molecule on hERG inhibition. Methyl sulfonamide **84** possessed a 33-fold lower hERG IC_{50} value compared to the more hydrophobic phenyl sulfonamide **85** despite their similar MCHR1 potencies. Moderate success was also achieved with pyrrolidinone **86** albeit with reduced differentiation between MCHR1 and hERG inhibition.

In summary, a novel class of MCHR1 antagonists was discovered and investigation of the SAR resulted in the optimization of MCHR1 potency from the hundred nanomolar range to antagonists with single digit nanomolar MCHR1 inhibitory activity. We were able to improve MCHR1 activity by altering the groups attached to the pyrrolidine nitrogen, by optimizing the length of the amide linker and by increasing the acidity of the amide proton. More importantly, we were able to differentiate the SAR of hERG inhibition from MCHR1 inhibition by removing the aryl ring and incorporating polarity into the part of the molecule attached to the pyrrolidine nitrogen. This resulted in the discovery of compounds like alcohol **83** and methyl sulfonamide **84** possessing

Table 5
MCHR1 and hERG activity for single enantiomers 79–86



Compd	R	Aeq. IC_{50} (μM)	I^{125}-MCH IC_{50} μM	hERG IC_{50} (μM)
79		0.007	0.013	0.002
80		0.014	0.037	0.37
81		0.024	0.021	0.12
82		0.009	0.012	0.29
83		0.025	0.057	8.24
84		0.044	0.030	4.62
85		0.012	0.014	0.14
86		0.027	0.046	1.26

double digit nanomolar MCHR1 IC_{50} values and micromolar hERG inhibition.

Acknowledgments

The authors thank Dr. Mario Cardozo for information obtained from the hERG pharmacophore model.

References and notes

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30. The mean and standard deviation of a common standard included in each run was acceptable. A description of the aequorin assay can be found in the following references. (a) An, S.; Thieu, B.; Yuhua, Z.; Goetzl, E. *J. Mol. Pharm.* **1998**, *54*, 88; (b) Bandoh, K.; Aoki, J.; Hosono, H.; Kobayashi, S.; Kobayashi, T.; Murakami-Murofushi, K.; Tsujimoto, M.; Arai, H.; Inoue, K. J. *Biol. Chem.* **1999**, *274*, 27776.
31. The mean and standard deviation of a common standard included in each run was acceptable. Material and solutions: Binding-buffer: 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA; 0.2% BSA (w/v). Washing-buffer: 25 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% BSA (w/v), 0.5 M NaCl. Mulitiscreen®-FB plates (Cat# MAFBN0B50, Millipore USA) were, prior to use, treated with 100 μl of 0.5% BSA (w/v)/H_20 solution. After 2 h at room temperature the solution was removed by vacuum filtration and the wells were washed 2 times with 100 μl of binding-buffer. *Methods*: Fifty microliters of compound-dilution, made in binding-buffer, was added to 50 μl of binding buffer containing 1 unit MCHR1 membrane preparation (Cat# ES-370 M, Euroscreen; Belgium) and 0.02 μCi ^{125}I-MCH (2200 Ci (81.4TBq)/mmol; 100 μCi/mL, PerkinElmer, USA). The 100 μl reaction mixture was incubated in a Corning 96-well plate (Cat# 3363, Corning USA) at 37 °C for 2 h on a shaking platform. Bound and unbound ligand was separated by vacuum filtration by transferring 80 μl of the reaction mixture into a corresponding well of the Mulitiscreen®-FB plates. After filtration the wells were washed 4 times with 100 μl of washing-buffer before 100 μl of Scintillation cocktail (PerkinElmer, USA) was added. Plates were read on a MicroBeta® Trilux (PerkinElmer, USA).
32. The mean and standard deviation of a common standard included in each run was acceptable. Finlayson, K.; Turnbull, L.; January, C. T.;Sharkey, J.; Kelly, J. S. *Eur. J. Pharm.*2001, *430*, 147. A stable HEK293 cell line expressing the hERG channel was established in house. Compounds were tested in the [^{3}H] dofetilide binding assay with cell membranes prepared from this cell line using method of Finlayson et al. (2001) with some modifications. Briefly, filtration assays were carried out in 194 μL of binding buffer (10 mM HEPES, pH 7.4, 60 mM KCl, 70 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) with 10 μg/well membrane (based on membrane protein) and [^{3}H]-dofetilide (8 nM), 6 μL of compound dissolved in 100% DMSO. Non-specific binding was determined by using 10 μM cold dofetilide (~1000-fold molar excess over hot ligand). The entire assay was conducted in 96-well Whatman® Unifilter plates at room temperature for 90 min. The binding assay was terminated by washing the plates four times on a Millipore® Vacuum filtration manifold with 100 μL/well of ice cold wash buffer (130 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4). The bound radioisotope was quantified using a Packard TopCount® NTS liquid scintillation counter with scintillation fluid.