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Dihydropyrrolopyrazol-6-one MCHR1 antagonists for the treatment of obesity: Insights on in vivo efficacy from a novel FLIPR assay setup

Pratik Devasthale [a,*], Wei Wang [a], Andres S. Hernandez [a], Fang Moore [a], Kishore Renduchintala [b], Radhakrishnan Sridhar [b], Mary Ann Pelleymounter [c], Daniel Longhi [c], Ning Huang [c], Neil Flynn [c], Anthony V. Azzara [c], Kenneth Rohrbach [c], James Devenny [c], Suzanne Rooney [c], Michael Thomas [c], Susan Glick [c], Helen Godonis [c], Susan Harvey [c], Mary Jane Cullen [c], Hongwei Zhang [d], Christian Caporuscio [d], Paul Stetsko [d], Mary Grubb [d], Christine Huang [d], Lisa Zhang [d], Chris Freeden [d], Yi-Xin Li [d], Brian J. Murphy [c], Jeffrey A. Robl [a], William N. Washburn [a,*]

[a] Metabolic Diseases Chemistry, Bristol-Myers Squibb Research and Development, Princeton, NJ 08543-5400, United States
[b] BMS-Biocon Research Center, Biocon, 20th KM, Hosur Road, Electronic City, Bangalore 560 100, India
[c] Metabolic Diseases Biology, Bristol-Myers Squibb Research and Development, Princeton, NJ 08543-5400, United States
[d] Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Research and Development, Princeton, NJ 08543-5400, United States

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ABSTRACT

Our investigation of the structure–activity and structure–liability relationships for dihydropyrrolopyrazol-6-one MCHR1 antagonists revealed that off-rate characteristics, inferred from potencies in a FLIPR assay following a 2 h incubation, can impact in vivo efficacy. The in vitro and exposure profiles of dihydropyrrolopyrazol-6-ones **1b** and **1e** were comparable to that of the thienopyrimidinone counterparts **41** and **43** except for a much faster MCHR1 apparent off-rate. The greatly diminished dihydropyrrolopyrazol-6-one anti-obesity response may be the consequence of this rapid off-rate.

An increasing awareness and recognition of obesity as a serious health hazard across the world[1] has garnered significant attention from the pharmaceutical industry leading to several recently approved treatments, such as Belviq®, Qsymia®, Contrave®, and Saxenda® over a decade after Xenical® became available.[2] Among a number of targets being pursued for the treatment of obesity, antagonism of the MCHR1 receptor was actively sought after by the pharmaceutical industry.[3] MCHR1 and MCHR2 are two class A GPCRs with the latter predominantly expressed in the brain of higher mammals such as humans, monkeys, and dogs but not in rodents such as mice and rats. Antagonism of MCHR1, which is expressed in most species examined, including rodents and humans, results in a reduction in feeding that translates to a reduction in weight gain in rodents. The vast majority of MCHR1 receptor antagonists include a basic functionality that is likely to be associated with the hERG liability that often accompanies them.

A non-basic MCHR1 antagonist from our laboratories, BMS-830216 (a phosphate ester prodrug of BMS-819881), that is devoid of the hERG liability, was advanced into clinical trials (Fig. 1).[4]

A projected human efficacious dose of 400 mg qd and a long half-life led to a follow-up focus toward identifying more potent analogues that would lower the projected human dose and possess a shorter $t_{1/2}$. In an effort to expand upon our discovery that non-basic entities containing a thienopyrimidine core were potent MCHR1 antagonists, multiple non-basic counterparts containing bicyclo[3.3.0] cores were explored.[5] This report details our findings with dihydropyrrolopyrazol-6-one-(**1**), dihydropyrrolopyrazol-4-one-(**2**) and dihydropyrrolotriazolone-based (**3**) antagonists (Fig. 2).

The initial route used for synthesis of dihydropyrrolopyrazol-6-one analogues **1** is described in Scheme 1. Conversion of 4-chloroaniline to its diazonium salt with $NaNO_2$/HCl followed by treatment with benzyl 2-chloro-3-oxobutanoate (**5**), gave hydrazonoyl chloride **6**. Following a known protocol,[6] pyrazole diester **7** was obtained by condensation of (*E*)-ethyl 3-(dimethylamino)acrylate with **6**. Pyrazole diester **7** was converted to acid

* Corresponding authors. Tel.: +1 609 818 4974 (P.D.).
E-mail address: pratik.devasthale@bms.com (P. Devasthale).

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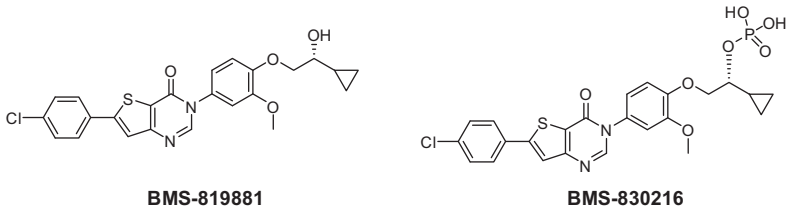


Figure 1. Parent (BMS-819881) and prodrug (BMS-830216) structures of the BMS clinical lead.

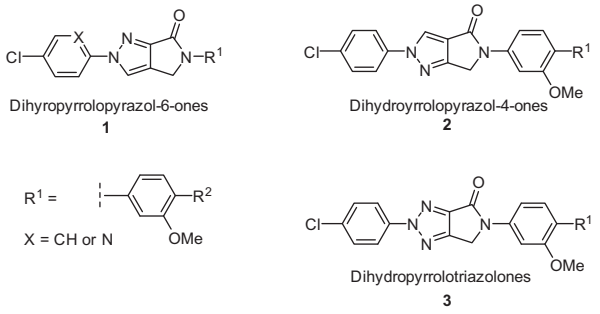
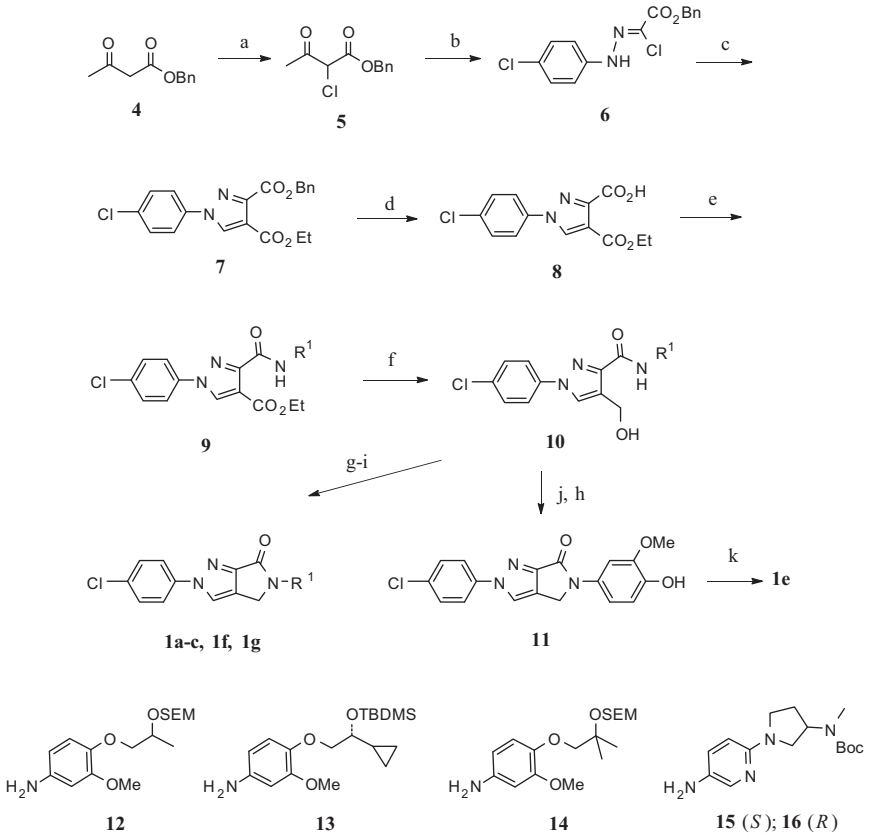


Figure 2. Non-basic dihydroazolopyrrolidinone-containing MCHR1 antagonists.

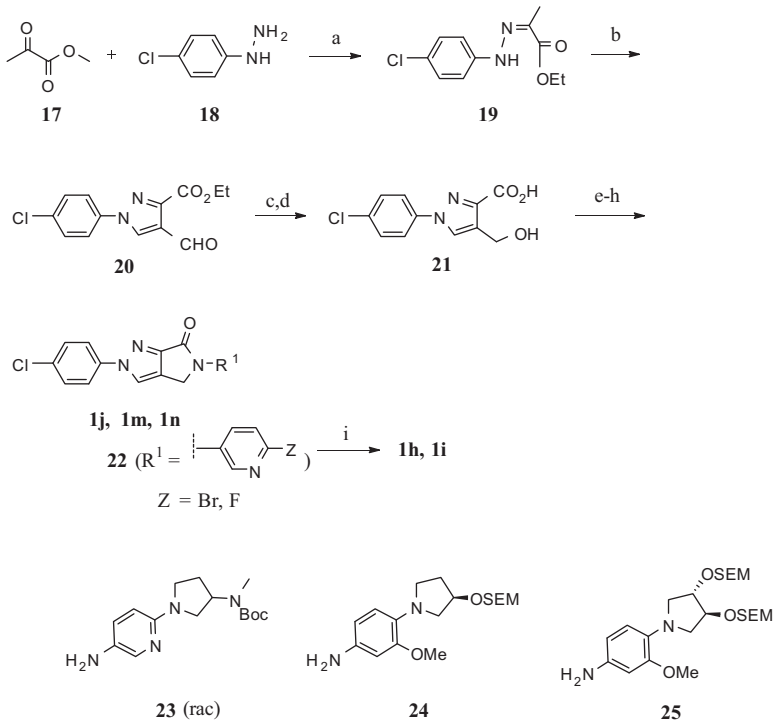
8 by hydrogenation, and was coupled with the appropriate anilines to afford ester **9**. Conversion of ester **9** to chloride **10** followed by subsequent cyclization and deprotection yielded **1a–c**, **1f**, and **1g**. In the case of **11**, the chlorinating conditions (MsCl/Et_3N/DCM) also removed the SEM protecting group to reveal the phenol which was cyclized in the presence of a base to yield **11**. Alkylation of **11** with 1-(2-chloroethyl)pyrrolidine yielded **1e**.

An alternative method to access dihydropyrrolopyrazol-6-one analogues **1** is shown in Scheme 2. Starting from **17** and **18**, pyrazole aldehyde **20** was prepared using known procedures.[7] Subsequent reduction and hydrolysis of **20** yielded acid **21**. Acid **21** was coupled with appropriate anilines under standard conditions. The resulting alcohol was converted to chloride and then cyclized to yield **1j**, **1m**, **1n**, and **22**. Substitution of **22** with suitable amines yielded **1h** and **1i**.

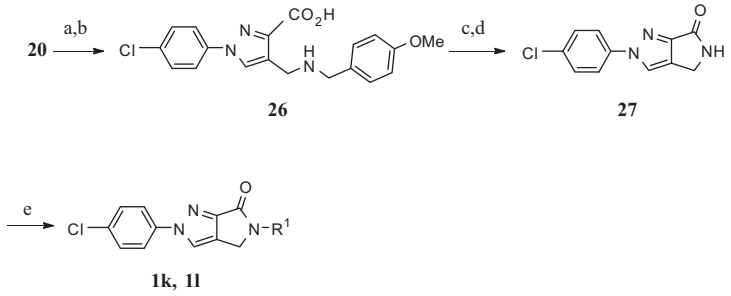
Dihydropyrrolopyrazolon-6-ones **1k** and **1l** were prepared as outlined in Scheme 3. Reductive amination of ester **20** with 4-methoxy benzylamine, followed by hydrolysis of the ester yielded acid **26** (Scheme 3). Cyclization using standard amide coupling conditions followed by deprotection afforded lactam **27**. Lactam



Scheme 1. Reagents and conditions: (a) $SOCl_2$, chloroform, 0 °C–rt, 4 h, 99%; (b) 4-chloroaniline, $NaNO_2$, 12 N HCl, H_2O, NaOAc, 0 °C–rt, 3 d, 87%; (c) (*E*)-ethyl 3-(dimethylamino)acrylate, TEA, *i*-PrOH, 80 °C, 2 h, 46%; (d) 10% Pd/C, H_2, EtOAc, rt, 5 h, 47%; (e) **12**/**13**/**14**/**15**/**16** or 3-methoxy-4-((2-(trimethylsilyl)ethoxy)methoxy)aniline, BOP, DIPEA, CH_2Cl_2, rt, 4.5 h, 80–100%; (f) $LiBH_4$, THF, rt, 2 h, 82%; (g) MsCl, DIPEA or Et_3N, CH_2Cl_2, rt, 16 h, 51–80%; (h) K_2CO_3, DMF, rt, 16 h, 42–95%; (i) deprotection, **1a**, **1b**, and **11**, –OSEM to –OH: TFA, CH_2Cl_2, 0 °C, 45 min–2.5 h, 21-62%; **1c**, –OTBS to –OH: TBAF, AcOH, THF, rt, 3 h, 31%; **1f** and **1g**, –NBocMe to –NHMe: 4 M HCl in dioxane, rt, 2 h, 90–100%; (j) MsCl, Et_3N, CH_2Cl_2, rt, 1.5 h, 44%; SEM group removed under these conditions (k) 1-(2-chloroethyl)pyrrolidine, Cs_2CO_3, MeCN, 80 °C, 5 h, 11%.



Scheme 2. Reagents and conditions: (a) NaOAc, H_2O, rt, 0.5 h, 80%; (b) $POCl_3$, DMF, 0 °C–rt, 17 h, 66%; (c) $NaBH_4$, THF, 0 °C, 2 h, 77%; (d) NaOH, H_2O, reflux, 2 h, 91%; (e) **23**/**24**/**25**, HOBt, EDC, rt, 16 h, 56–100%; (f) MsCl, DIEA, CH_2Cl_2, rt, 16 h, 23–99%; (g) K_2CO_3, DMF, rt, 12–40 h, 25–100%; (h) deprotection, **1j**, –NBocMe to –NHMe: 4 M HCl in dioxane, rt, 2 h, 100%; **1m** and **1n**, –OSEM to –OH: TFA, CH_2Cl_2, 0 °C, 3 h, 43%; (i) **1h**, from **23** (Z = Br), (*S*)-N,N-dimethylpyrrolidin-3-amine, DMSO, microwave reactor, 120 °C, 1 h, 100%; **1i**, from **23** (Z = F), (1) (*S*)-*tert*-butyl pyrrolidin-3-ylcarbamate, DMSO, microwave reactor, 120 °C, 0.5 h; (2) TFA, CH_2Cl_2, 2 h, 96% for two steps.



Scheme 3. Reagents and conditions: (a) 4-methoxy benzyl amine, $NaBH(OAc)_3$, CH_2Cl_2, rt, 1.5 h, 88%; (b) NaOH, THF/EtOH/H_2O, rt, 12 h, 75%; (c) EDCI, HOBt, TEA, CH_2Cl_2, 5 °C–rt, 12 h, 60%; (d) ceric ammonium nitrite, MeCN/H_2O (3:1), rt, 3 h, 50%; (e) aryl bromide intermediate, N^1,N^2-dimethylethane-1,2-diamine, K_3PO_4, CuI, dioxane, 115 °C, 12 h, ~10%.

27 was coupled with appropriate bromides using Buchwald conditions to yield **1k** and **1l**.

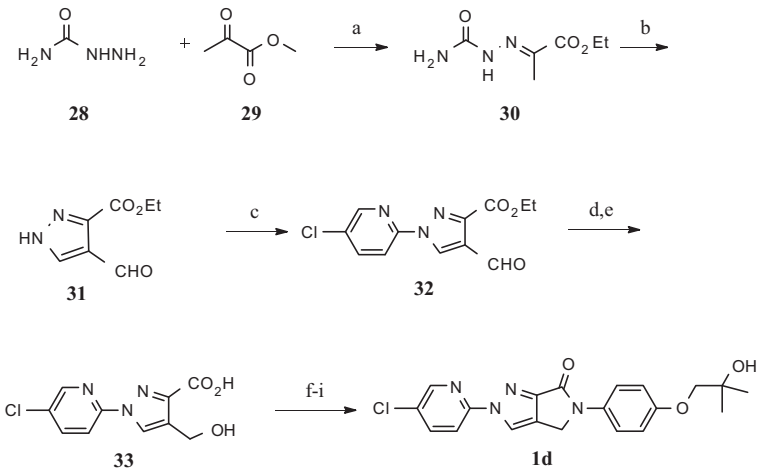
Arylation of **31**[8] with 2-bromo-5-chloropyridine yielded pyrazole **32**, which was converted to acid **33** via reduction and hydrolysis (Scheme 4). Acid **33** was coupled with the corresponding SEM-protected substituted aniline (**14**) under standard conditions. The resulting alcohol was converted to chloride. Subsequent cyclization and SEM-deprotection yielded **1d**.

The synthesis of regioisomeric dihydropyrrolopyrazol-4-one analogues **2** is described in Scheme 5. Selective reduction of **7** followed by hydrolysis afforded acid **34**, which was coupled with appropriate anilines **12**, **13**, or **14** under standard conditions. Transformation of the resulting alcohol to the corresponding chloride followed by subsequent cyclization and deprotection gave dihydropyrrolopyrazol-4-one **2a**, **2b**, and **2c**.

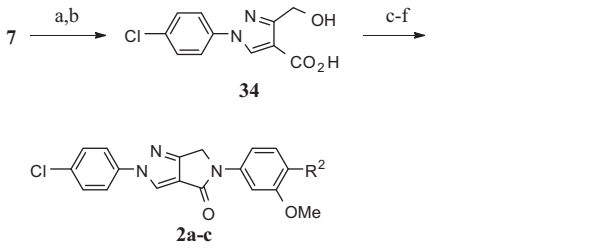
Scheme 6 describes the synthesis of dihydropyrrolotriazinones **3**. The copper-mediated amidation of bromide **36** with 4-methoxy-1*H*-pyrrol-2(5*H*)-one followed by demethylation yielded arylpyrrolidinedione **37**. Conversion of **37** to oxime with $NaNO_2$/H_2SO_4/NaOH followed by reaction with (4-chlorophenyl) hydrazine yielded **38**. Subsequent cyclization with PCl_5 and deprotection afforded phenol **39**. Alkylation of **39** with 2,2-dimethyloxirane yielded **3a**. Compound **3b** was obtained by alkylation of **39** followed by reduction of the resulting ketone and **3c** by alkylation with the corresponding alkyl chloride.

A comparative analysis of equivalent compounds from each of the three cores (X = CH), **1**, **2** and **3**, shown in Table 1 suggested series **1** to be most promising from an MCHR1 potency perspective. For example, tertiary alcohol **1b** was the most potent analogue among **1b**, **2b**, and **3a**. Conversion of the chlorophenyl moiety of **1b** to a more polar 5-chloro-2-pyridyl (X = N) **1d** diminished MCHR1 affinity 6-fold.

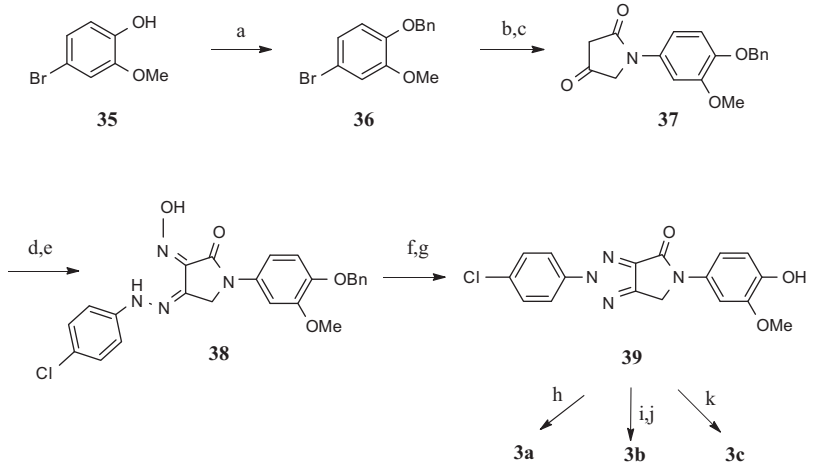
Among all alcohol-containing analogs from this chemotype (all data not shown), dosed as prodrugs, only compound **1b** (rat K_i = 3.8 nM; rat plasma free fraction = 1%), dosed at 30 mpk as a glycine prodrug, exhibited promising brain exposures of 1060 nM



Scheme 4. Reagents and conditions: (a) NaOAc, H_2O, rt, 12 h, 90%; (b) $POCl_3$, DMF, 0 °C–rt, 17 h, 55%; (c) 2-bromo-5-chloropyridine, K_2CO_3, DMF, 120 °C, 2 h, 90%; (d) $NaBH_4$, THF, 0 °C, 1.5 h, 70%; (e) 1 N NaOH, MeOH, rt, 2 h, 100%; (f) 3-methoxy-4-(2-methyl-2-((2-(trimethylsilyl)ethoxy)methoxy)propoxy)aniline, EDC, HOBt, CH_2Cl_2, rt, 2 h, 100%; (g) MsCl, $(i\text{-Pr})_2NEt$, CH_2Cl_2, rt, 4 h, 24%; (h) K_2CO_3, DMF, 95 °C, 2 h; (i) TFA, CH_2Cl_2, 0 °C, 1 h, 24% two steps.



Scheme 5. Reagents and conditions: (a) $LiBH_4$, THF, rt, 5 h, 58%; (b) NaOH, H_2O, reflux, 2 h, 90%; (c) **12**, **13**, or **14**, HOBt, EDC, CH_2Cl_2, rt, 12 h, 61–72%; (d) MsCl, $(i\text{-Pr})_2NH$, CH_2Cl_2, rt, 12 h–3 d, 52–76%; (e) K_2CO_3, DMF, 95 °C, 2-3.5 h, 48–70%; (f) **2a** and **2b**: TFA, CH_2Cl_2, 0 °C, 45 min–2.5 h, 32–62%; **2c**: TBAF, AcOH, THF, rt–63 °C, 3 h, 31%.

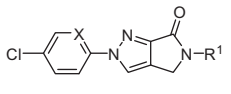


Scheme 6. Reagents and conditions: (a) BnBr, K_2CO_3, MeCN, rt–70 °C, 14 h, 100%; (b) 4-methoxy-1*H*-pyrrol-2(5*H*)-one, CuI, K_2CO_3, *N*,*N*′-dimethylethylene-diamine, toluene, 18 h, 105 °C, 76%; (c) 5 M HCl, dioxane, 38 °C, 24 h, 94%; (d) $NaNO_2$, H_2SO_4, NaOH, THF/H_2O, 0 °C, 30 min; (e) (4-chlorophenyl)hydrazine, THF/H_2O, rt, 12 h; (f) PCl_5, DME, 0 °C–rt, 3.5 h, 20% three steps; (g) thioanisole, TFA, CH_2Cl_2, rt, 17 h, 76%; (h) 2,2-dimethyloxirane, K_2CO_3, MeCN/H_2O, microwave 120 °C, 1.5 h, 67%; (i) 2-cyclopropyl-2-oxoethyl 4-methylbenzenesulfonate, Cs_2CO_3, MeCN, 80 °C, 5 h, 77%; (j) $NaBH_4$, MeOH/THF, rt, 12 h, 86%; (k) 1-(2-chloroethyl)pyrrolidine, Cs_2CO_3, MeCN, 78 °C, 12 h, 62%.

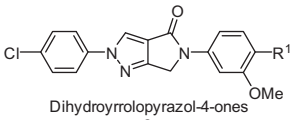
at 20 h (C_{trough}), corresponding to a free fraction coverage of 2.8-fold over K_i (Table 4). Oral administration at 30 mg/kg qd of **1b** as the glycine pro-drug **40** for four days to young growing SD rats produced a statistically insignificant 1.8% reduction in weight gain whereas the structurally similar thienopyrimidinone standard **41** (rat K_i = 7.2 nM) (compound **19** in Ref. 4) caused a significant 6.2% decrease when administered as the glycine pro-drug **42** at 10 mg/kg (Fig. 3).

This lack of efficacy obtained with **1b** was unexpected since the free brain concentrations at trough, assuming free brain and free plasma fractions are equivalent, for both **1b** (at 30 mpk; B/P = 0.42 at 20 h post-dose) and **41** (at 10 mpk; B/P = 1.7 at 20 h post-dose) were ~2–3-fold greater than K_i. To probe whether the weak in vivo activity of **1b** was an inherent property of this bicyclo[3.3.0] chemotype, we prepared and compared pyrrolidine-containing dihydropyrrolopyrazolone **1e** and

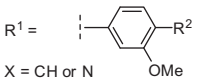
Table 1
MCHR antagonist activities of dihydroazolopyrrolidinones

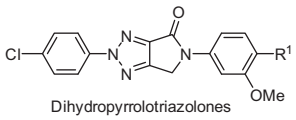


Dihyropyrrolopyrazol-6-ones
1

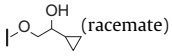


Dihydroyrrolopyrazol-4-ones
2





Dihydropyrrolotriazolones
3

Compound	X	R^1	K_i, nM[a]
1a	H	$-OCH_2CH(OH)CH_3$	13
1b	H	$-OCH_2C(OH)(CH_3)_2$	8
1c	H		9
1d	N	$-OCH_2C(OH)(CH_3)_2$	44
1e	H		0.7
2a	H	$-OCH_2CH(OH)CH_3$	334
2b	H	$-OCH_2C(OH)(CH_3)_2$	107
2c	H		41
3a	H	$-OCH_2C(OH)(CH_3)_2$	34
3b	H	 (racemate)	112
3c	H		2.3

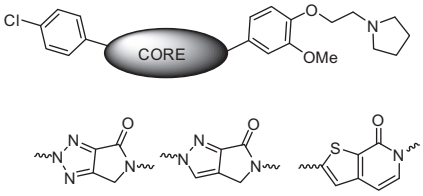
[a] $n = 3–5$; versus human MCHR1.[12b]

dihydropyrrolotriazolone **3c** to the thienopyrimidinone counterpart **43** (Table 2) as previous SAR within the group had suggested that the pyrrolidine moiety would confer a significant boost in potency.[9]

Since the dihydropyrrolopyrazolone **1e** exhibited a three-fold affinity differential over the pyrrolotriazolone **3c** as had been previously observed for the alcohol counterparts, all subsequent work focused on the dihydropyrrolopyrazolone series. Before embarking on in vivo studies, we sought unsuccessfully to reduce the potent hERG activity of **1e**. A number of variations of basic functionalities were prepared and assessed for MCHR1 antagonist activity and hERG inhibition (Table 3). Initially *N*-pyridyl-3-amino pyrrolidines **1f–1i** were explored as a replacement for the *N*-(2-aryloxyethyl)pyrrolidine moiety of **1e**.[10]

Several SAR observations emerge from Table 3. There was minimal difference in MCHR K_i and hERG inhibition between

Table 2
Comparative profiles of dihydroazolopyrrolidinones



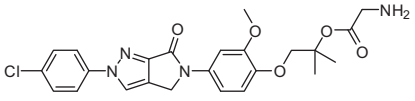
Compound	**3c**	**1e**	**43**
K_i, nM[a]	2.3	0.7	1
HPLC log *P*	3.3	2.8	3.5
hERG% inhib @ 0.3 μM	68	58	77

[a] $n = 3–5$; versus human MCHR1.[12b]

enantiomers **1f** and **1g**. Nor was there any significant difference between primary (**1i**), secondary (**1f/1g**) and tertiary (**1h**) amine analogues. Pyridine (**1f/1g**) and benzene (**1j**) analogues also appeared to be equivalent. Adding steric bulk on the amine (**1l**) worsened the hERG liability. Although reduction of the basicity of the amine (**1k**) produced a favorable impact on hERG inhibition, it was accompanied by a 6-fold decrease in MCHR potency. Addition of polar hydrogen-bonding functionalities is known to generally mitigate the hERG liability.[11] Hydroxylation or bishydroxylation to generate **1m** and **1n** respectively reduced hERG activity such that for **1n** the hERG electrophysiology Q-patch IC_{50} was >30 μM. However this approach was abandoned due to the accompanying decrease in MCHR1 affinity.

Given the inability to improve significantly upon the profile of amines such as **1e** and **1f** and before launching into a more extensive effort for optimizing the MCHR1/hERG balance, a 7 day dose-response study with **1f** dosed orally qd at 1, 3 and 10 mg/kg was conducted using young SD rats.[12] One cohort was administered 10 mg/kg of **42** (prodrug of **41**) as a positive control. Results are summarized in Table 4 along with historical results for **43** in a similar study. For the amine **1f** to cause weight loss the free drug coverage in the brain at 20 h (C_{trough}) must be markedly higher than its K_i value than that required for the thienopyrimidinones **41** and **43** just as was observed for the alcohol **1b**. These findings suggest that this weak efficacy is due to the dihydropyrrolopyrazolone core and not the polar group.

We hypothesized that the weak in vivo potency of dihydropyrrolopyrazolones **1b** and **1f** may be due to a faster apparent off-rate relative to the thienopyrimidinone counterparts, **41** and **43**. A qualitative estimate of relative off-rates was gauged by measuring IC_{50} values in a novel FLIPR assay setup after incubation of test compounds for 120 min (Table 5) followed by measurement of Ca^{2+}



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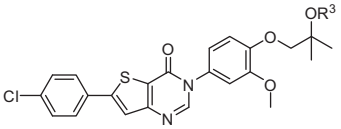
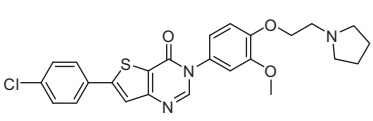


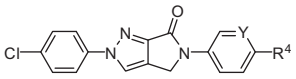
41, R^3 = H
42, R^3 = -$COCH_2NH_2$

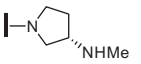
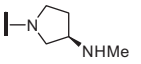
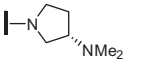
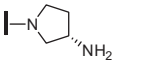
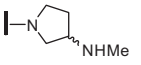
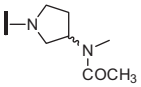
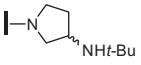
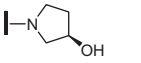
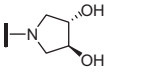


43 (GSK)

Figure 3. Thienopyrimidinone MCHR1 antagonists.

Table 3
3-Aminopyrrolidines



Compound	R^4	Y	K_i, nM[a]	hERG@ 1 μM[b]
1f		N	1.0	66%
1g		N	1.3	50%
1h		N	0.7	50%
1i		N	2.1	58%
1j		CH	0.8	51%[c]
1k		N	6.5	23%
1l		N	2.7	71%
1m		C-OMe	7.1	NA[d]
1n		C-OMe	11	<5%

[a] n = 3–5; versus human MCHR1.
[b] An automated whole-cell patch clamp electrophysiology system (Q-patch) was used to directly measure hERG currents in CHO cells stably expressing the cloned hERG potassium channel.
[c] Percent inhibition @ 0.3 μM.
[d] Not available; hERG flux IC_{50} >80 μM.

Table 4
Summary of findings from 7-day weight loss studies with **1f**, **41** (dosed as **42**) and **43** using SD rats[12]

Compd	Rat K_i (nM)	Dose (mg/kg)	Wt. loss versus vehicle control[b] (%)	Plasma free fraction (%)	C_{plasma} at 20 h post-dose, nM	C_{brain} at 20 h post-dose, nM	CNS free drug[c]/K_i
40[a]	3.8	30	1.8	1.0	2557	1060	2.8
1f	0.8	1	2.2	4.2	131	<LLQ	—
1f	0.8	3	3.8	4.2	574	739	39
1f	0.8	10	7.5*	4.2	1577	2767	145
42	7.2 (for **41**)	10	8.8*	0.7 (for **41**)	546	1610	1.6
43	0.34	15	8.7*	1.2	378	654	23

* Statistically significant weight loss versus vehicle.
[a] 4-Day weight loss study.
[b] Vehicle control: 0.5% methocel/0.1% tween 80/99.4% distilled H_2O.
[c] Assumption: plasma free fraction = brain free fraction.

influx upon stimulation with MCH. Faster off-rates were inferred from higher IC_{50} values following a 120-min incubation.

Compound **1b** (Table 5) exhibited a significantly higher T_{120} IC_{50} than **32** which may account for the absence of in vivo efficacy with **1b** despite free brain coverage over MCHR antagonist K_i similar to that of **41**. As in the case of **1b** in Table 2, a significantly higher T_{120} IC_{50} for **1e** relative to **43** suggested a faster off-rate and lower intrinsic potency for the dihydropyrrolopyrazolone core. For **1f** itself, a high IC_{50} (241 nM) was observed which is consistent with a lack of efficacy when free fraction exposure multiples in the brain over K_i were less than 40.[13]

Given the lower intrinsic potency of dihydropyrrolopyrazolones such as **1b** and **1f** despite achieving excellent trough free drug levels in the CNS well above K_i, the difficulty in achieving a desirable MCHR1/hERG balance, and the inability to modulate the apparent off-rate of this chemotype, further work on dihydropyrrolopyrazolones was discontinued to pursue more promising opportunities in other chemotypes within our laboratories.

Table 5
K_i and T_{120}[a] values

Compound	hMCHR1 K_i nM ± SEM	T_{120}, IC_{50}, nM
41	7.9 ± 1.8	13
43	2.2 ± 1.1	16
1b	8.7 ± 5.3	1173
1e	0.42 ± 0.32	3057
1f	0.96 ± 0.19	241

[a] See note 13.

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12. Experimental protocols:
(a) *Chemistry:* Experimental procedures and analytical data for dihydroazolopyrrolidones **1**, **2**, and **3** are described in Devasthale, P. V.; Washburn, W. N.; Wang, W.; Hernandez, A.; Ahmad, S.; Zhao, G. U S patent 8,415,386, 2013; *Chem. Abstr.* **2010**, *152*, 501369.
(b) K_i *determination:* Membranes from stably transfected HEK-293 cells expressing a mutated (E4Q, A5T) hMCHR1 receptor were prepared by dounce homogenization and differential centrifugation. Binding experiments were carried out with 0.5–1.0 μg of membrane protein incubated in a total of 0.2 mL in 25 mM HEPES (pH 7.4) with 10 mM $MgCl_2$, 2 mM EGTA, and 0.1% BSA (Binding Buffer) for 90 min. For competition binding assays, reactions were carried out in the presence of with 0.06–0.1 nM [Phe^{13}, [^{125}I]Tyr^{19}]-MCH and increasing concentrations of unlabeled test molecules. Reactions were terminated by rapid vacuum filtration over 96 well-GFC Unifilter plates pre-coated with 0.075 mL binding buffer containing 1% BSA, and washed 3 times with 0.4 mL of Phospho-buffered Saline (pH 7.4) containing 0.01% TX-100. Filters were dried, 0.05 mL microscint 20 was added to each well and radioactivity was subsequently quantified by scintillation counting on a TopCount microplate scintillation counter (Packard). Inhibitory constants were determined by nonlinear least squares analysis using a four parameter logistic equation;
(c) *In vivo rat efficacy studies:* Male Sprague Dawley rats were dosed orally with vehicle (0.5% methocel/0.1% tween 80/99.4% distilled H_2O) or compounds dissolved in vehicle, at a volume of 5 mL/kg. Test compounds were administered orally one hour before the onset of the dark cycle. Body weight and food consumption were measured daily at the time of drug administration. Upon completion of the study total caloric consumption, diet preference, and change in body weight were tabulated. Drug effects on body weight were analyzed using body weights converted to percent change from baseline and evaluated across treatment groups using a one-way ANOVA.
13. *FLIPR assay protocol:*
Inhibition of MCH-mediated Ca^{2+} was measured in a FLIPR in a hemiequilibria based-format. Stable HEK-293 cells expressing human MCHR1 receptor were plated at a density of 50,000 cells / well in 96 well poly-lysine coated plates (BD #35-6640) and cultured overnight in DMEM (high glucose (4.5 g/mL), 25 mM HEPES, pH 7.4, 10% Fetal Bovine Serum, 1 mM Na Cl) at 37 °C, 5% CO_2 conditions. For assay, media was replaced with 90 mL per well dye solution consisting of 3.8 mM Fluo4 AM (Invitrogen #F14201), 0.04% Pluronic F-127 (Invitrogen #P3000MP), 2.5 mM Probencid (Sigma #P8761), in Base Buffer (Hank's balanced salt solution, 25 mM HEPES, 0.1% BSA). Dye solution was allowed to 'load' for 1 h at room temperature in subdued light. Dye was subsequently removed and replaced with 75 mL of Base Buffer and 75 mL diluted test compound. Test compound dilution plates were prepared by serial diluting test and reference compounds from 100% DMSO stocks first 1:50 in Base Buffer and then serially (1:3.26) in Base Buffer containing 2% DMSO to generate twelve half log test concentrations. Compounds were preincubated with cells for 120 min prior to challenge with MCH. Unlike traditional FLIPR inhibition assays where the compounds are challenged with an EC_{50} agonist (MCH), in this hemiequilibria assay format compounds are challenged with an EC_{100} agonist (MCH). Compounds with similar binding affinities but with longer off-rates are better able to antagonize the EC_{100} challenge from MCH due to the transient kinetic nature of the FLIPR assay. Compounds of similar affinity but with slower off-rates will generate lower IC_{50} inhibition values when compared to similar compounds that have faster off-rates.
A manuscript describing a more detailed decription of the FLIPR hemiequilibrium assay and the association of slower apparent off-rates with reduced weight loss efficacy across multiple MCHR1 antagonist chemotypes is in preparation.