Synthesis, Binding Affinity, Radiolabeling, and MicroPET Evaluation of 4-(2-Substituted-4-substituted)-8-(dialkylamino)-6-methyl-1-substituted-3,4-dihydropyrido[2,3-*b*]pyrazin-2(1*H*)-ones as Ligands for Brain Corticotropin-Releasing Factor Type-1 (CRF₁) Receptors

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Abbreviations: CRF, corticotropin-releasing factor; CRF₁, CRF type-1 receptor;¹ PET, positron emission tomography; SPECT, single-photon emission computed tomography; BBB, blood-brain barrier; DMA, dimethylacetamide; SUV, standard uptake value;² TACs, time-activity curves; CPCU, chemical processing control unit; rcy, radiochemical yield; EOB, end-of-bombardment.

Keywords:

Corticotropin-releasing factor

CRF-1 receptor

Fluorine-18

PET imaging

Positron emission tomography

Abstract

Compounds **1** – **14** were synthesized in a search for high-affinity CRF₁ receptor ligands that could be radiolabeled with ¹¹C or ¹⁸F for use as positron emission tomography (PET) radiotracers. Derivatives of **2** were developed which contained amide *N*-fluoroalkyl substituents. Compounds [¹⁸F]**24** and [¹⁸F]**25** were found to have appropriate lipophilicities of log*P*_{7.4} = 2.2 but microPET imaging with [¹⁸F]**25** demonstrated limited brain uptake.

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The neuropeptide corticotropin-releasing factor (CRF)^{1,3} coordinates the neuroendocrine response to stress through the hypothalamic-pituitary-adrenal axis by acting on the CRF Type-1 (CRF₁) receptor.⁴ Dysregulation of brain CRF signaling has been proposed to be involved in stress, anxiety, depression, and addiction; and brain regional alterations in CRF₁ density have been detected in depressed patients and victims of suicide.⁵⁻⁸ This evidence has resulted in a more than 20-year search for small molecule, brain bioavailable CRF₁ antagonists that could be used as therapeutics for treating CRF₁-related disorders.⁹⁻¹¹ Therapeutic medication development can be uniquely aided by the availability of validated positron emission tomography (PET) and single-photon emission computed tomography (SPECT) radiotracers that can be used to both support target occupancy measurements of candidate therapeutics and to explore mechanisms of pathophysiology and treatment response by measurement of receptor density and expression levels.¹²⁻¹⁵ Numerous attempts to develop a viable brain CRF₁ receptor PET radiotracer have been reported over the years but no candidates to date have been successful due to problems such as high lipophilicity and inability to cross the blood-brain barrier (BBB), rapid metabolism, or insufficient specific receptor binding.¹⁶⁻¹⁹ As part of an effort to develop a viable brain CRF₁ receptor PET tracer we have been exploring various structural motifs²⁰ and report here our results with compounds **1** – **15** (Scheme 1), and derivatives thereof, which are based on a previous report that compounds **2**, **6**, and **9** displayed high binding affinity (*IC*₅₀ = 0.70, 0.49, and 0.92 nM, respectively) at the rat brain CRF₁ receptor.²¹ We anticipated that the reported high binding affinity of **2**, **6**, and **9** would enable radiotracer derivatives of this structural class to achieve specific binding at CRF₁ and that the presence of the carbonyl group would enhance water solubility, thereby reducing log*P* and increasing BBB passage.²²⁻²⁵ Furthermore, the synthetic route for this class of compounds²¹ (Scheme S1, Supplementary Material) allows the aromatic core and the *N,N*-dialkyl group to be held constant while the 2,4-substituted pendant aryl ring is varied to produce a library of high-affinity candidates that are amenable to radiolabeling with ¹¹C or ¹⁸F.

Scheme 1

The binding affinities of compounds **1** – **14** at the CRF_1 and CRF_2 receptors were determined by *in vitro* competition binding assays at 23 °C using HEK293T cells transfected with the human CRF_1 or CRF_2 receptor (Table 1). Compounds **1** – **14** all bound to the CRF_1 receptor with high affinity ($K_i = \sim 1.3 - 5.4$ nM). Thus, although these compounds have various combinations of substituents on the pendant aryl ring, they all have binding affinities within a narrow range that differs by ~ 4 nM. The difference in affinities of **2**, **6**, and **9** between our results and the previously reported results²¹ is presumably due to the difference between human and rat CRF_1 receptors as well as the fact that we are reporting K_i values and the previous work reported IC_{50} values. Sauvagine and R121919²⁶ were used as positive controls in our assays: R121919 was found to have an affinity of $K_i = 2.6 \pm 0.4$ nM for the CRF_1 receptor which is comparable to the previously reported values of $K_i = 3.5$ nM²⁶ and $K_i = 3.0 \pm 0.16$ nM;²⁷ and sauvagine was found to have an affinity of $K_i = 0.8 \pm 0.1$ nM which is in agreement with the previously reported value of $K_i = 0.8 - 1$ nM.²⁸ The competing radioligand for these studies, [¹²⁵I]-Tyr⁰-sauvagine, has a reported affinity of 0.2 – 0.4 nM for the CRF_1 receptor.²⁸ Compounds **1** – **3**, **6**, and **8** – **12** did not display binding affinity at the CRF_2 receptor (they were unable to significantly displace [¹²⁵I]antisauvagine-30),²⁹ thus demonstrating selectivity for the CRF_1 receptor and so further screening of compounds **4**, **5**, **7**, **13**, and **14** at the CRF_2 receptor was not performed.

Table 1

Results of *In Vitro* Competition Binding Assays at 23 °C Using Transfected Human CRF₁ and CRF₂ Receptors in HEK293T Cells.

compd	R ^o	R ^p	CRF ₁		CRF ₂	
			K _i (nM) ± SEM ^a	n =	% Displacement ^b	n =
1	Br	Br	1.3 ± 0.3	3	9.4 ± 2.3 %	2
2	Br	<i>i</i> -Pr	2.4 ± 0.2	3	0.7 ± 3.3 %	2
3	Et	Br	2.5 ± 0.7	6	2.1 ± 2.5 %	2
4	Br	Cl	2.5 ± 0.2	3	N/D	N/A
5	Cl	Br	2.7 ± 0.4	3	N/D	N/A
6	Cl	Cl	3.4 ± 0.4	3	0.9 ± 3.4 %	2
7	Me	I	3.4 ± 0.6	3	N/D	N/A
8	Me	Me	3.9 ± 1.2	3	6.8 ± 1.4 %	2
9	Me	OMe	4.0 ± 0.6	3	-2.3 ± 3.2 %	2
10	Br	<i>t</i> -Bu	4.2 ± 2.2	3	-3.8 ± 0.5 %	2
11	Me	Br	4.4 ± 0.9	3	5.9 ± 0.7 %	2
12	Br	Me	4.4 ± 1.1	3	5.5 ± 4.9%	2
13	Cl	Me	4.4 ± 0.4	3	N/D	N/A
14	Me	Cl	5.4 ± 0.9	3	N/D	N/A
R121919	N/A	N/A	2.6 ± 0.4	9	23.8 ± 0.3%	2
Sauvagine	N/A	N/A	0.8 ± 0.1	9	100.0 ± 0.0 %	2
Antisauvagine-30	N/A	N/A	N/D	N/A	99.0 ± 0.6 %	2

a) vs. [¹²⁵I]-Tyr⁰-sauvagine.²⁸ b) Mean % displacement of radiotracer ([¹²⁵I]antisauvagine-30)²⁹ specific binding at CRF₂ receptors by 1 μM of competing ligand. N/D = not determined. N/A = not applicable.

Compound **1** was found to have the highest binding affinity for the CRF₁ receptor followed by compounds **2** – **5**, but these compounds do not have a position available for radiolabeling with ¹¹C or ¹⁸F. Thus, we shifted our focus to derivatives that could potentially be radiolabeled on the amide nitrogen atom. Compound **16** (Scheme 2) was prepared by amide *N*-methylation of **1** with CH₃I. This resulted in only a small decrease in CRF₁ binding affinity (Table 2) relative to **1** (Table 1). Compounds **17** – **20** (Scheme 3) were synthesized (Scheme S2, Supplementary Material) to be used as intermediates in the synthesis of amide *N*-fluoroalkyl target compounds. The shorter *N,N*-dialkyl chains of **17** – **19** were expected to compensate for the increased lipophilicity that would result from adding alkyl groups to the amide nitrogen atom, while **20** was included to evaluate the effect on binding affinity of rearranging the methylene groups of the *N,N*-dialkyl substituent of **2** into a symmetrical substitution pattern. Furthermore, derivatives of **2** were used, rather than **1**, because 2-bromo-4-*iso*-propylaniline can be reacted neat without the need for solvent or NaH which simplified the synthetic procedure. Compounds **21** – **25** (Scheme 3) were synthesized (Scheme S2, Supplementary Material) by *N*-alkylation of the amide nitrogen atom.

Scheme 2

Replacing the *N*-butyl-*N*-ethyl substituent of **2** with *N,N*-dimethyl to give **17** resulted in a ~75-fold loss of binding affinity at CRF₁ (Table 2) compared to **2** (Table 1) due to the inability to fill the requisite lipophilic binding pocket on the receptor.^{21, 30, 31} When one of the *N*-methyl groups of **17** was replaced with *N*-ethyl to give **18** an ~11-fold gain in binding affinity was obtained relative to **17** and which corresponds to a ~6.8-fold loss in binding affinity relative to **2**. Compound **20**, which contains the same number of *N,N*-dialkyl methylene groups as **2** but which are arranged in a symmetrical fashion, had only a slight reduction in binding affinity relative to **2** which indicates that symmetrical *N,N*-dialkyl substitution can be tolerated by the CRF₁ receptor for this class of compounds as long as the lipophilic binding pocket on the receptor is filled. Amide *N*-alkylation of **17** to give **21** resulted in a ~3-fold increase in binding affinity relative to **17**, and when the *N,N*-dimethyl group of **21** was replaced with *N,N*-diethyl to give **22** this resulted in a ~8-fold increase in binding affinity relative to **21**. Replacing the *N*-fluoroethyl group of **21** with an *N*-(*E*)-fluoro-2-butenyl group³² to give **23** resulted in a ~4-fold increase in binding affinity while incorporation of an *N*-fluorobutyl group to give **24** resulted in a ~2.7-fold increase relative to **21**. Amide *N*-fluorobutylation of **18** to give **25** resulted in a negligible change in binding affinity relative to **18** but the presence of the *N*-ethyl group on **25** resulted in a slight increase in binding affinity relative to **24**. Thus, the significant loss of binding affinity that resulted from using an *N,N*-dimethyl group on **17** can be minimized through amide *N*-fluoroalkylation (which also provides a position for radiolabeling), and by replacing the *N,N*-dimethyl group with an *N*-ethyl-*N*-methyl or *N,N*-diethyl group.

Table 2

Results of *In Vitro* Competition Binding Assays at 23 °C Using Transfected Human CRF₁ Receptor in HEK293T Cells.

compd	R ^o	R ^p	R ¹	R ²	R ³	K _i (nM) ± SEM ^a	n =
16	Br	Br	Bu	Et	Me	2.4 ± 0.6	2
17	Br	<i>i</i> -Pr	Me	Me	H	180 ± 5	2
18	Br	<i>i</i> -Pr	Me	Et	H	16.4 ± 1.6	3
20	Br	<i>i</i> -Pr	Pr	Pr	H	3.1 ± 0.3	3
21	Br	<i>i</i> -Pr	Me	Me	CH ₂ CH ₂ F	59 ± 9	3
22	Br	<i>i</i> -Pr	Et	Et	CH ₂ CH ₂ F	7.3 ± 0.7	3
23	Br	<i>i</i> -Pr	Me	Me	(<i>E</i>)-CH ₂ CH=CHCH ₂ F	14.2 ± 2.9	3
24	Br	<i>i</i> -Pr	Me	Me	CH ₂ CH ₂ CH ₂ CH ₂ F	21.6 ± 2.8	3
25	Br	<i>i</i> -Pr	Me	Et	CH ₂ CH ₂ CH ₂ CH ₂ F	13.9 ± 1.9	3

a) vs. [¹²⁵I]-Tyr⁰-sauvagine.²⁸

- 17** $R^1 = \text{Me}, R^2 = \text{Me}, R^3 = \text{H}$
18 $R^1 = \text{Me}, R^2 = \text{Et}, R^3 = \text{H}$
19 $R^1 = \text{Et}, R^2 = \text{Et}, R^3 = \text{H}$
20 $R^1 = \text{Pr}, R^2 = \text{Pr}, R^3 = \text{H}$
21 $R^1 = \text{Me}, R^2 = \text{Me}, R^3 = \text{CH}_2\text{CH}_2\text{F}$
22 $R^1 = \text{Et}, R^2 = \text{Et}, R^3 = \text{CH}_2\text{CH}_2\text{F}$
23 $R^1 = \text{Me}, R^2 = \text{Me}, R^3 = (E)\text{-CH}_2\text{CH}=\text{CHCH}_2\text{F}$
24 $R^1 = \text{Me}, R^2 = \text{Me}, R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{F}$
25 $R^1 = \text{Me}, R^2 = \text{Et}, R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{F}$
26 $R^1 = \text{Me}, R^2 = \text{Me}, R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OTs}$
27 $R^1 = \text{Me}, R^2 = \text{Et}, R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OTs}$

Scheme 3

The radiolabeling precursors **26** and **27** (Scheme 3) were synthesized (Scheme S2, Supplementary Material) by *N*-alkylation of the amide nitrogen atoms of **17** and **18**, respectively, with 1,4-ditosyloxybutane.²⁰ Radiolabeling of [¹⁸F]**24** and [¹⁸F]**25** was performed in one-step with K¹⁸F/K_{2.2.2} (Scheme 4) from **26** and **27**, respectively. The log $P_{7.4}$ values of [¹⁸F]**24** and [¹⁸F]**25** (Table 3) were measured by the octanol/aqueous buffer shake-flask method^{33, 34} and were both found to be log $P_{7.4} = 2.2$ which is in the range for passive diffusion across the BBB.^{22, 23, 25}

Scheme 4

Table 3Log $P_{7.4}$ values.

compd	log $P_{7.4} \pm$ SD	n =
[^{18}F] 24	2.19 $\pm$ 0.01	4
[^{18}F] 25	2.22 $\pm$ 0.02	4

MicroPET imaging was performed in an anesthetized male cynomolgus monkey using a Siemens MicroPET Focus 220 as previously described.²⁰ The whole-brain time-activity curves (TACs) for [^{18}F]**25** (Figure 1) show that the radiotracer did not significantly enter the brain (SUV = <1) even though the log $P_{7.4}$ value of [^{18}F]**25** (Table 3) indicates that it should be able to diffuse across the BBB.^{22, 23, 25} The binding affinity of **25** (Table 2) may not be as strong as would be desired for a CRF₁ PET tracer, but this would not affect entry of the radiotracer into the brain, only retention of the radiotracer once in the brain. Thus, it would appear that the radiotracer was metabolized in the blood.

In summary, compounds **1** – **14** were found to have high binding affinities at the CRF₁ receptor with a range of K_i = ~1.3 – 5.4 nM, but the highest affinity compounds, **1** – **5**, do not have positions available for radiolabeling with ^{11}C or ^{18}F (although ^{76}Br derivatives are a possibility). Using shorter *N,N*-dialkyl chains allows for amide *N*-alkylation but these changes reduce the binding affinities at the CRF₁ receptor. One-step radiolabeling using amide *N*-butyltosylate precursors **26** and **27** was successful and yielded [^{18}F]**24** and [^{18}F]**25**, respectively. Compounds [^{18}F]**24** and [^{18}F]**25** had appropriate lipophilicities (log $P_{7.4}$ = 2.2) to support BBB permeability but microPET imaging with [^{18}F]**25** demonstrated minimal brain uptake. This program of synthesis, radiochemistry, in vitro characterization, and PET imaging, while failing to yield a valid brain CRF₁ receptor PET radioligand, further describes the theoretical and methodological complexities of developing such an in vivo molecular imaging probe.

Figure 1. MicroPET whole-brain TACs of [^{18}F]**25** in an anesthetized male cynomolgus monkey.

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1 $R^o = \text{Br}$, $R^p = \text{Br}$

2 $R^o = \text{Br}$, $R^p = i\text{-Pr}$

3 $R^o = \text{Et}$, $R^p = \text{Br}$

4 $R^o = \text{Br}$, $R^p = \text{Cl}$

5 $R^o = \text{Cl}$, $R^p = \text{Br}$

6 $R^o = \text{Cl}$, $R^p = \text{Cl}$

7 $R^o = \text{Me}$, $R^p = \text{I}$

8 $R^o = \text{Me}$, $R^p = \text{Me}$

9 $R^o = \text{Me}$, $R^p = \text{OMe}$

10 $R^o = \text{Br}$, $R^p = t\text{-Bu}$

11 $R^o = \text{Me}$, $R^p = \text{Br}$

12 $R^o = \text{Br}$, $R^p = \text{Me}$

13 $R^o = \text{Cl}$, $R^p = \text{Me}$

14 $R^o = \text{Me}$, $R^p = \text{Cl}$

15 $R^o = \text{Me}$, $R^p = \text{OH}$

Scheme 2

17 $R^1 = \text{Me}$, $R^2 = \text{Me}$, $R^3 = \text{H}$

18 $R^1 = \text{Me}$, $R^2 = \text{Et}$, $R^3 = \text{H}$

19 $R^1 = \text{Et}$, $R^2 = \text{Et}$, $R^3 = \text{H}$

20 $R^1 = \text{Pr}$, $R^2 = \text{Pr}$, $R^3 = \text{H}$

21 $R^1 = \text{Me}$, $R^2 = \text{Me}$, $R^3 = \text{CH}_2\text{CH}_2\text{F}$

22 $R^1 = \text{Et}$, $R^2 = \text{Et}$, $R^3 = \text{CH}_2\text{CH}_2\text{F}$

23 $R^1 = \text{Me}$, $R^2 = \text{Me}$, $R^3 = (E)\text{-CH}_2\text{CH}=\text{CHCH}_2\text{F}$

24 $R^1 = \text{Me}$, $R^2 = \text{Me}$, $R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{F}$

25 $R^1 = \text{Me}$, $R^2 = \text{Et}$, $R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{F}$

26 $R^1 = \text{Me}$, $R^2 = \text{Me}$, $R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OTs}$

27 $R^1 = \text{Me}$, $R^2 = \text{Et}$, $R^3 = \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OTs}$

Figure 1