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(1H)-ones as ligands for brain corticotropin-releasing factor type-1 (CRF1) receptors

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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(1H)-ones as Ligands for Brain Corticotropin-Releasing Factor Type-1 (CRF₁) Receptors

Jeffrey S. Stehouwer, Chase H. Bourke, Michael J. Owens, Ronald J. Voll, Clinton D. Kilts, and Mark M. Goodman

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 logP₇.₄ = 2.2 but microPET imaging with [¹⁸F]25 demonstrated limited brain uptake.

Keywords

Corticotropin-releasing factor; CRF-1 receptor; Fluorine-18; PET imaging; Positron emission tomography

The neuropeptide corticotropin-releasing factor (CRF) coordinates the neuroendocrine response to stress through the hypothalamic-pituitary-adrenal axis by acting on the CRF Type-1 (CRF₁) receptor. 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. 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. Therapeutic medication development can be uniquely aided by the availability of validated positron emission tomography (PET) and single-photon emission computed tomography

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(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.\textsuperscript{12–15} Numerous attempts to develop a viable brain CRF\textsubscript{1} 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.\textsuperscript{16–19} As part of an effort to develop a viable brain CRF\textsubscript{1} receptor PET tracer we have been exploring various structural motifs\textsuperscript{20} and report here our results with compounds \textbf{1–15} (Scheme 1), and derivatives thereof, which are based on a previous report that compounds \textbf{2}, \textbf{6}, and \textbf{9} displayed high binding affinity ($IC_{50} = 0.70\text{,} 0.49\text{,} \text{ and } 0.92$ nM, respectively) at the rat brain CRF\textsubscript{1} receptor.\textsuperscript{21} We anticipated that the reported high binding affinity of \textbf{2}, \textbf{6}, and \textbf{9} would enable radiotracer derivatives of this structural class to achieve specific binding at CRF\textsubscript{1} and that the presence of the carbonyl group would enhance water solubility, thereby reducing log$P$ and increasing BBB passage.\textsuperscript{22–25} Furthermore, the synthetic route for this class of compounds\textsuperscript{21} (Scheme S1, Supplementary Material) allows the aromatic core and the $N\text{-}N$-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 $^{11}$C or $^{18}$F.

The binding affinities of compounds \textbf{1–14} at the CRF\textsubscript{1} and CRF\textsubscript{2} receptors were determined by \textit{in vitro} competition binding assays at 23 °C using HEK293T cells transfected with the human CRF\textsubscript{1} or CRF\textsubscript{2} receptor (Table 1). Compounds \textbf{1–14} all bound to the CRF\textsubscript{1} receptor with high affinity ($K_i = 1.3–5.4$ 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 $\sim 4$ nM. The difference in affinities of \textbf{2}, \textbf{6}, and \textbf{9} between our results and the previously reported results\textsuperscript{21} is presumably due to the difference between human and rat CRF\textsubscript{1} receptors as well as the fact that we are reporting $K_i$ values and the previous work reported $IC_{50}$ values. Sauvagine and R121919\textsuperscript{26} 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\textsubscript{1} receptor which is comparable to the previously reported values of $K_i = 3.5$ nM\textsuperscript{26} and $K_i = 3.0 \pm 0.16$ nM;\textsuperscript{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.\textsuperscript{28} The competing radioligand for these studies, $[^{125}\text{I}]-\text{Tyr}^0\text{-sauvagine}$, has a reported affinity of 0.2–0.4 nM for the CRF\textsubscript{1} receptor.\textsuperscript{28} Compounds \textbf{1–3, 6}, and \textbf{8–12} did not display binding affinity at the CRF\textsubscript{2} receptor (they were unable to significantly displace $[^{125}\text{I}]\text{-antisauvagine}$-30),\textsuperscript{29} thus demonstrating selectivity for the CRF\textsubscript{1} receptor and so further screening of compounds \textbf{4, 5, 7, 13}, and \textbf{14} at the CRF\textsubscript{2} receptor was not performed. Compound \textbf{1} was found to have the highest binding affinity for the CRF\textsubscript{1} receptor followed by compounds \textbf{2–5}, but these compounds do not have a position available for radiolabeling with $^{11}$C or $^{18}$F. Thus, we shifted our focus to derivatives that could potentially be radiolabeled on the amide nitrogen atom. Compound \textbf{16} (Scheme 2) was prepared by amide $N$-methylation of \textbf{1} with CH$_3$I. This resulted in only a small decrease in CRF\textsubscript{1} binding affinity (Table 2) relative to \textbf{1} (Table 1). Compounds \textbf{17–20} (Scheme 3) were synthesized (Scheme S2, Supplementary Material) to be used as intermediates in the synthesis of amide

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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.

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-fluorobutylolation 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.

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/K22,2 (Scheme 4) from 26 and 27, respectively. The logP7,4 values of [18F]24 and [18F]25 (Table 3) were measured by the octanol/aqueous buffer shake-flask method33, 34 and were both found to be logP7,4 = 2.2 which is in the range for passive diffusion across the BBB.22, 23, 25

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 [18F]25 (Figure 1) show that the radiotracer did not significantly enter the brain (SUV = <1) even though the logP7,4 value of [18F]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 CRF$_1$ receptor with a range of $K_i = \sim 1.3–5.4$ 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 CRF$_1$ receptor. One-step radiolabeling using amide $N$-butyltosylate precursors 26 and 27 was successful and yielded $[^{18}\text{F}]24$ and $[^{18}\text{F}]25$, respectively. Compounds $[^{18}\text{F}]24$ and $[^{18}\text{F}]25$ had appropriate lipophilicities ($\log P_{7.4} = 2.2$) to support BBB permeability but microPET imaging with $[^{18}\text{F}]25$ demonstrated minimal brain uptake. This program of synthesis, radiochemistry, in vitro characterization, and PET imaging, while failing to yield a valid brain CRF$_1$ receptor PET radioligand, further describes the theoretical and methodological complexities of developing such an in vivo molecular imaging probe.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**Abbreviations**

- CRF: corticotropin-releasing factor
- CRF$_1$: 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

**References**

Figure 1.
MicroPET whole-brain TACs of $[^{18}\text{F}]25$ in an anesthetized male cynomolgus monkey.
Scheme 1.
Scheme 2.
Scheme 3.

17 $R^1 = $ Me, $R^2 = $ Me, $R^3 = $ H
18 $R^1 = $ Me, $R^2 = $ Et, $R^3 = $ H
19 $R^1 = $ Et, $R^2 = $ Et, $R^3 = $ H
20 $R^1 = $ Pr, $R^2 = $ Pr, $R^3 = $ H
21 $R^1 = $ Me, $R^2 = $ Me, $R^3 = $ CH$_2$CH$_2$F
22 $R^1 = $ Et, $R^2 = $ Et, $R^3 = $ CH$_2$CH$_2$F
23 $R^1 = $ Me, $R^2 = $ Me, $R^3 = $ (E)-CH$_2$CH=CHCH$_2$F
24 $R^1 = $ Me, $R^2 = $ Me, $R^3 = $ CH$_2$CH$_2$CH$_2$CH$_2$F
25 $R^1 = $ Me, $R^2 = $ Et, $R^3 = $ CH$_2$CH$_2$CH$_2$CH$_2$F
26 $R^1 = $ Me, $R^2 = $ Me, $R^3 = $ CH$_2$CH$_2$CH$_2$CH$_2$OTs
27 $R^1 = $ Me, $R^2 = $ Et, $R^3 = $ CH$_2$CH$_2$CH$_2$OTs
Scheme 4.
### Table 1

Results of *In Vitro* Competition Binding Assays at 23 °C Using Transfected Human CRF<sub>1</sub> and CRF<sub>2</sub> Receptors in HEK293T Cells.

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

<sup>a</sup> vs. [<sup>125</sup>I]-Tyr<sup>0</sup>-Sauvagine.<sup>28</sup>

<sup>b</sup> Mean % displacement of radiotracer ([<sup>125</sup>I]antisauvagine-30)<sup>29</sup> specific binding at CRF<sub>2</sub> receptors by 1 μM of competing ligand.

N/D = not determined. N/A = not applicable.
**Table 2**

Results of *In Vitro* Competition Binding Assays at 23 °C Using Transfected Human CRF<sub>1</sub> Receptor in HEK293T Cells.

<table>
<thead>
<tr>
<th>compd</th>
<th>R&lt;sup&gt;a&lt;/sup&gt;</th>
<th>R&lt;sup&gt;b&lt;/sup&gt;</th>
<th>R&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>R&lt;sup&gt;3&lt;/sup&gt;</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (nM) ± SEM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>n =</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Br</td>
<td>Br</td>
<td>Bu</td>
<td>Et</td>
<td>Me</td>
<td>2.4 ± 0.6</td>
<td>2</td>
</tr>
<tr>
<td>17</td>
<td>Br</td>
<td>i-Pr</td>
<td>Me</td>
<td>Me</td>
<td>H</td>
<td>180 ± 5</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>Br</td>
<td>i-Pr</td>
<td>Me</td>
<td>Et</td>
<td>H</td>
<td>16.4 ± 1.6</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>Br</td>
<td>i-Pr</td>
<td>Pr</td>
<td>Pr</td>
<td>H</td>
<td>3.1 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>21</td>
<td>Br</td>
<td>i-Pr</td>
<td>Me</td>
<td>Me</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;F</td>
<td>59 ± 9</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>Br</td>
<td>i-Pr</td>
<td>Et</td>
<td>Et</td>
<td>CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;F</td>
<td>7.3 ± 0.7</td>
<td>3</td>
</tr>
<tr>
<td>23</td>
<td>Br</td>
<td>i-Pr</td>
<td>Me</td>
<td>Me</td>
<td>(E)-CH&lt;sub&gt;2&lt;/sub&gt;CH=CHCH&lt;sub&gt;2&lt;/sub&gt;F</td>
<td>14.2 ± 2.9</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>Br</td>
<td>i-Pr</td>
<td>Me</td>
<td>Me</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;CHF</td>
<td>21.6 ± 2.8</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>Br</td>
<td>i-Pr</td>
<td>Me</td>
<td>Et</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;F</td>
<td>13.9 ± 1.9</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> vs. [<sup>125</sup>I]-Tyr<sup>0</sup>-sauvagine<sup>28</sup>
Table 3

Log $P_{7,4}$ values.

<table>
<thead>
<tr>
<th>compd</th>
<th>$\log P_{7,4} \pm SD$</th>
<th>$n$</th>
</tr>
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<tbody>
<tr>
<td>$[^{18}F]24$</td>
<td>2.19 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>$[^{18}F]25$</td>
<td>2.22 ± 0.02</td>
<td>4</td>
</tr>
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