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[^{18}F](*R*)-5-chloro-1-(1-cyclopropyl-2-methoxyethyl)-3-(4-(2-fluoroethoxy)-2,5-dimethyl phenylamino)pyrazin-2(1*H*)-one: Introduction of N^3-phenylpyrazinones as potential CRF-R1 PET imaging agents

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ABSTRACT

Based on a favorable balance between CRF-R1 affinity, lipophilicity and metabolic stability, compound **10** was evaluated for potential development as PET radioligand. Compound [^{18}F]**10** was prepared with high radiochemical purity and showed promising binding properties in rat brain imaging experiments.

Corticotropin-releasing factor (CRF), a 41 amino acid-containing neuropeptide, mediates the endocrine, autonomic, and behavioral responses to stress.[1] CRF regulates the hypothalamic-pituitary-adrenal (HPA) axis through binding to the CRF receptor, a G-protein coupled receptor for which two subtypes have been identified (CRF-R1 and CRF-R2). In response to physical or psychological stress, CRF is released from the hypothalamus and binds to CRF receptors in the anterior pituitary, resulting in the secretion of adrenocorticotropin hormone (ACTH). Increased ACTH stimulates the release of cortisol from the adrenal gland.[2] In order to stabilize the HPA axis, cortisol exerts negative feedback control on the secretion of CRF in the hypothalamus.[3] Severe stress, resulting in a hyper-secretion of CRF and extended activation of the HPA axis, may lead to disorders such as anxiety or depression.[4–6] CRF receptor antagonists, in particular antagonists of the CRF-R1 subtype, may have potential as novel antidepressants and anxiolytics.

In support of the evaluation of potential CRF-R1 antagonist clinical candidates, efforts have been undertaken to develop an appropriate radioligand for a Positron Emission Tomography (PET) study that would provide a useful clinical tool to monitor levels of receptor occupancy. The earliest reported effort to develop CRF PET radioligands was in 2001, but the ^{18}F-labeled compound described suffered from high lipophilicity and poor brain penetration.[7] Later, Eckelman[8] disclosed [^{76}Br]-MJL-1-109-2, **1a** (Fig. 1), a high affinity CRF-R1 PET radioligand (K_i ~2 nM) with a reasonable lipophilicity ($c\log P = 3$). In rat biodistribution studies, this compound showed limited retention of activity in brain with cerebellum and cortex uptake of 0.29 ± 0.01% injected dose per gram (ID/g) and 0.32 ± 0.03% ID/g, respectively, after 30 min. However, no further experiments have been reported. Recently, Lang reported a radiosynthesis of a similar analog, [^{76}Br]-BMK-I-152, **1b**, with lower lipophilicity and improved brain penetration, but has yet to publish any in vivo results.[9] Kumar[10] evaluated [^{11}C]SN003 (**2**), and Sullivan[11] studied [^{11}C]R121920 (**3**) and [^{11}C]DMP696 (**4**) in baboons (Fig. 1). Each of these studies concluded that the lack of detectable specific binding may be due to lower density CRF-R1 receptors in primate brain as compared to rat or human brain. In addition, while these latter PET ligands had high affinities for the CFR-R1 receptor (K_i 1.7–4 nM) and reasonable lipophilicities ($\log D$

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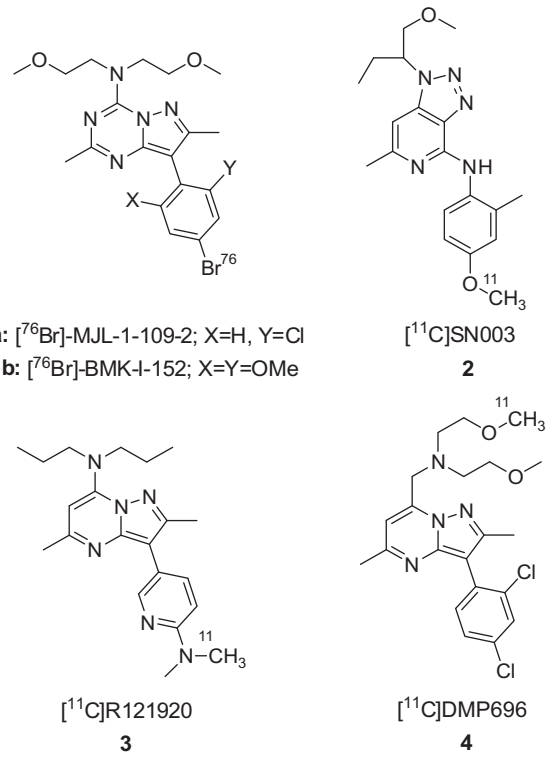


Figure 1. Previously reported potential CRF PET ligands.

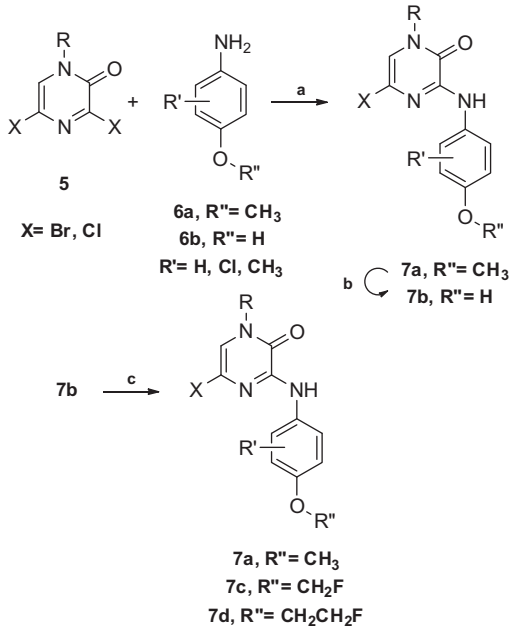
3.1–4), the studies found that all three ^{11}C radioligands underwent rapid metabolism in baboon, with compounds **3** and **4** showing only 50% of parent remaining after only 9 and 13 min respectively.[11]

To identify compounds as potential CRF PET radioligands, we hoped to achieve high CRF-R1 affinity (K_i < 5 nM) and provide analogs with reasonable protein binding (free fractions >1%). Suitable compounds would have high brain uptake and be stable with no radiolabeled metabolites in brain. Ideal compounds would also not be subject to efflux mechanisms. Each candidate molecule would make use of a facile synthesis of ^{11}C or ^{18}F -labeled derivatives. Lipophilicity, measured as an experimental octanol-water partition coefficient (log*D*),[12] was also an important consideration. It is well appreciated in the CRF antagonist field that the most potent compounds tend to be highly lipophilic (log*D* > 4).[2] For a suitable PET ligand, we set a minimum criteria of log*D* < 4.5, although a log*D* in the 1–2.5 range was preferred.[13]

Our previously reported series of N^3-phenylpyrazinone CRF-R1 antagonists[14] was chosen to explore the potential of identifying a suitable PET ligand. An extensive series of pyrazinone analogs (i.e., **7**, Scheme 1) had been prepared with several compounds analyzed to determine their lipophilicities. While CRF-R1 affinity in this series of compounds typically increased with higher lipophilicity, we focused our efforts on analogues with an N-1 side-chain bearing a methoxy group (in R, Scheme 1), since these derivatives had generally lower lipophilicity. The site chosen for introduction of a ^{11}C or ^{18}F label was the hydroxyl group on the 3-arylamino substituent. Here in we describe the properties and SAR of several analogs appropriate for labeling, and the radiosynthesis and initial evaluation of [^{18}F]**10** as a potential CRF-R1 PET imaging agent.

The preparation of these N^3-phenylpyrazinone analogs has been previously published.[14] Initially, 3-(4-methoxy)arylamino analogs (**6**, Scheme 1) were prepared from anilines obtained through commercial sources, then coupled to the pyrazinone cores (Scheme 1).

The preparation of phenol **7b** was achieved by coupling pyrazinone core **5** to a 4-methoxy aniline **6a**, followed by treatment of

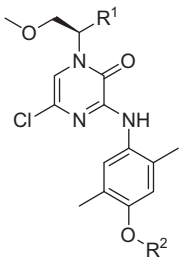


Scheme 1. Reagents and conditions: (a) NaHMDS, THF; (b) BBr_3, CH_2Cl_2, -78 °C; (c) CH_3I or FCH_2Br or FCH_2CH_2Br, KOH, DMSO, 65 °C.

7a with boron tribromide at low temperature (Scheme 1). Alternatively, the pyrazinone core **5** could be coupled directly to phenol **6b** to give **7b** directly, but the yields of this reaction were lower compared to the two step process. From phenol **7b**, **7a** (for a ^{11}C label), **7c** and **7d** were prepared by alkylation with methyl iodide, bromo fluoromethane, or 1-bromo-2-fluoroethane respectively, in the presence of potassium hydroxide in dimethyl sulfoxide with heating. This synthetic route (Scheme 1) provided a rapid, general method to use for the radiolabeling and evaluation of candidate compounds **8**–**17** as shown in Tables 1 and 2.

Key parameters for analogs **8**–**11** are shown in Table 1 and for analogs **12**–**17** in Table 2. The CRF-R1 binding affinities of these analogs were determined by measuring the inhibition of specific binding of [^{125}I]-*o*-CRF in a CRF-R1 receptor binding assay using rat frontal cortex homogenate.[15] The partition coefficients (log*D*) of these and previous analogs were determined by classical octanol-phosphate buffer (pH∼7.4) partitioning methodology.[16]

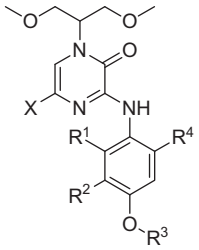
Table 1
CRF-R1 binding affinities, phase distribution coefficients and the metabolic stabilities of selected initial analogs



Compounds	R^1	R^2	IC_{50},nM	log*D*	hLM/rLM[a]
8	c-Pr	Me	0.26	4.4	0.17/0.23
9	c-Pr	CH_2F	4.1	3.1	–/–
10	c-Pr	CH_2CH_2F	2.4	3.7	0.07/0.12
11	Me	Me	0.88	3.4	0.06/0.19

[a] Rate of compound depletion (pmol/min/mg protein); see Ref. 19.

Table 2
CRF-R1 binding affinities, phase distribution coefficients and the metabolic stabilities of later synthesized, more polar analogs



Compounds	X	$R^1/R^2/R^3/R^4$	IC_{50},nM	logD	hLM/rLM[a]
12	Cl	Cl/H/Me/Cl	5.0	3.4	0.03/0.17
13	Cl	Cl/H/CH_2F/Cl	5.4	3.1	–/–
14	Cl	Cl/H/CH_2CH_2F/Cl	210	3.1	0.07/0.12
15	Br	Me/H/Me/Me	7.8	2.8	–/–
16	Br	Me/H/CH_2F/Me	4.3	2.9	0.22/0.26
17	Br	H/Me/Me/Me	0.68	3.8	0.07/0.14

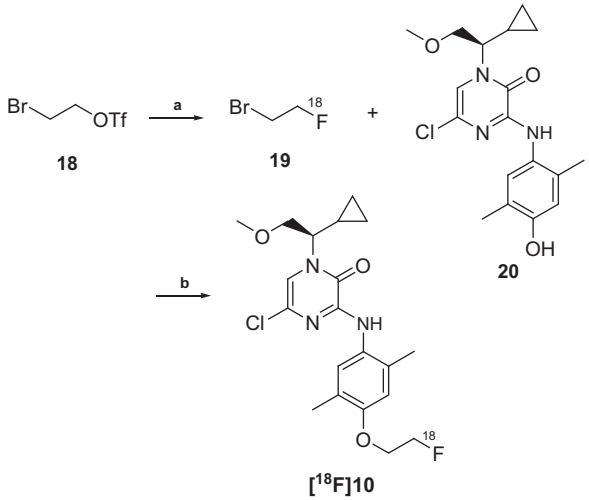
[a] Rate of compound depletion (pmol/min/mg protein); see Ref. 19.

Initial pyrazinone analogs **8**–**11** had good CRF-R1 binding affinities due to a less polar top amine side chain. Compound **8** was found to have a rat plasma protein free fraction of 2.1%.[17] Compound **10**[18] displayed good binding affinity to the CRF-R1 receptor, but was 10-fold less potent than its methoxy counterpart **8**. The presence of the fluoroethoxy group in **10**, however, lowered the log*D* by nearly 0.7 of a unit relative to **8**, and improved metabolic stability[19] in both human and rat liver microsomes when compared to **8**. Compound **11** was more potent at CRF-R1 and slightly more polar than **10**, but was less metabolically stable in rat. Thus, analog **10** offered a balance between binding potency, lipophilicity and metabolic stability when compared with **8** or **11.**

During the development of **10** as a first candidate for radiolabeling, several newer analogs were synthesized in an effort to further lower log*D* values while retaining CRF-R1 potency. These compounds, which bear two methoxy groups on N-1, are shown in Table 2. By the incorporation of an additional oxygen on the top amine side chain, replacement of the core chlorine with bromine, plus the choice and movement of the substituents on the lower aniline ring moiety, log*D* values of <3 were obtained for compounds **15** and **16**, with single digit nanomolar CRF-R1 binding being retained. In addition, these compounds had free fractions ranging between 3-6%.[17] By replacement of the 4-methoxy group with fluoromethoxy as in compound **16**, affinity increases without a substantial increase of lipophilicity as compared to **15**. Compound **17** showed excellent affinity and metabolic stability, but the compromise was slightly higher lipophilicity than **12** and **13**. In all, compounds like **12**, **13**, and **15**–**17** were of interest due to their combined properties, particularly with their log*D* values being in a more desirable range for a potential PET ligand.

Based on its overall properties, the ^{18}F -labeled version of **10** was first chosen for further study in rats. The radiosynthesis of [^{18}F]**10**[20] is shown in Scheme 2. [^{18}F]**10** was routinely obtained in yields of 5–12 mCi, with specific activities of 0.25–0.50 Ci/μmol, and radiochemical purities >98%.

Compound [^{18}F]**10** was then characterized in a series of studies which looked at organ/tissue distribution, the possible formation of radiolabeled metabolites, and brain uptake imaging. Compound [^{18}F]**10** was first evaluated in a whole body biodistribution study (Fig. 2), with samples analyzed at 15 min and 60 min intervals. Compound levels were uniform across ten tissue types at both time points. Of particular interest was the relatively low distribution to the tibia, which demonstrated that a loss of fluorine locating to bone was not a significant issue with this compound, and thus suggested good stability of the radiolabel.



Scheme 2. Reagents and conditions: (a) freshly distilled **18**, $K^{18}F$, Kryptofix 222, 1,2-dichlorobenzene, 125 °C, under N_2, 10 min.; (b) solid KOH, DMSO, 60–70 °C, 12 min, water, C-18 Sep-Pak/acetonitrile, preparative HPLC.

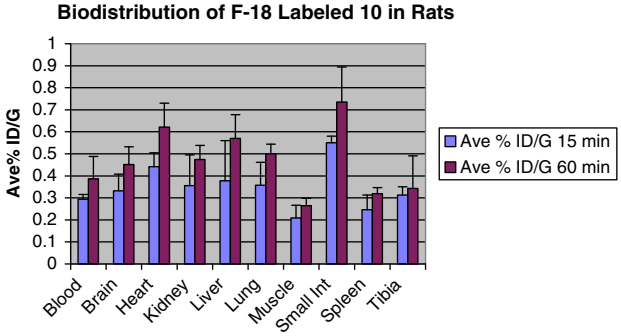


Figure 2. Multi-tissue biodistribution of [^{18}F]**10** in rats.

Metabolism studies were conducted 10 min post injection with [^{18}F]**10** (353 μCi per rat) to determine the nature of activity in brain and blood. Collected blood and brain samples were analyzed by HPLC (Fig. 3) and compared to the initial radioligand with a radiochemical purity (RCP) of 100 percent. After 10 min, 50 percent of the radioactivity found in the blood was due to a radiolabeled metabolite. Analysis of the brain sample at 10 min showed less than 10 percent of a similar R_t radioactive material, which was derived from blood present in the brain. The appearance of the substantial metabolite peak seen within the blood sample was not seen in the brain sample. This study demonstrated that only one labeled compound was the major component present in the brain after 10 min.

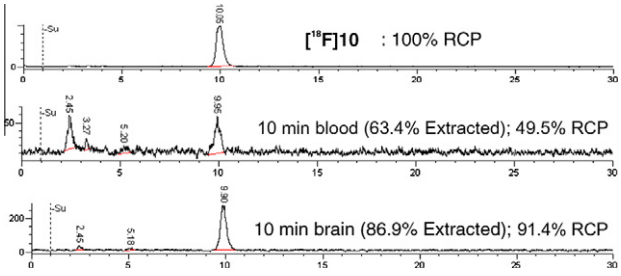


Figure 3. Nature of radioactivity in blood and brain for [^{18}F] **10** in rats.

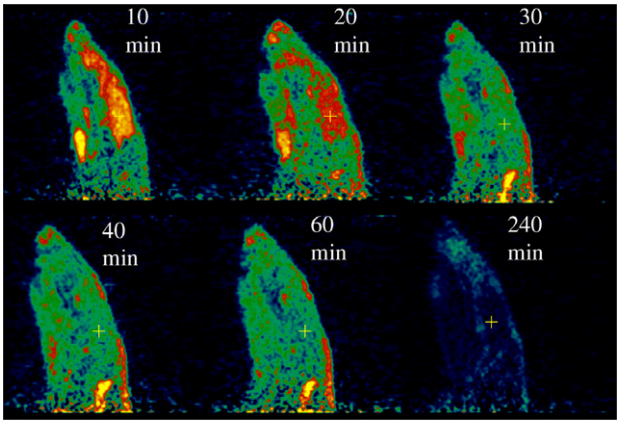


Figure 4. MicroPET sagittal images of [^{18}F] **10** in rat.

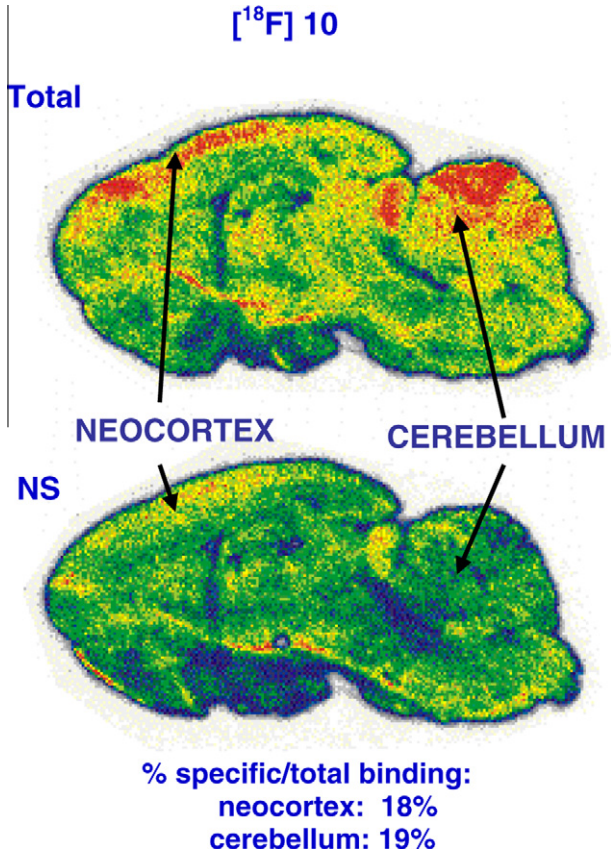


Figure 5. In vitro autoradiographic images of [^{18}F]**10** in rat brain slices.

Brain uptake was evaluated using in vivo MicroPET studies in rats. The sagittal images shown in Figure 4 are ear side views of rat head and neck. These studies showed rapid brain uptake within 10 min of dosing with subsequent rapid washout by 30 min. The MicroPET images also demonstrate very little defluorination of [^{18}F]**10** as evidenced by a lack of skull bone uptake of radioactivity.

Finally, an in vitro autoradiography imaging study[21] was carried out to determine the localization of the radioligand [^{18}F]**10** in rat brain slices. This study evaluated the uptake of the compound at the receptors and showed the extent of non-specific (NS) binding. Analysis of the sagittal images (Fig. 5) showed 18% specific binding for the neocortex region of the brain, and 19% specific binding for the cerebellum. Since [^{18}F]**10** showed low specific binding, we concluded that this particular compound was likely not an optimum tool, and that compounds with either higher CRF-R1 potencies and/or lower log*D* values may be needed.

In conclusion, we identified compound **10** with a balance between CRF-R1 affinity, low lipophilicity and metabolic stability for potential development as a PET radioligand. ^{18}F radiolabeled compound **10** was then prepared with high RCP, and was then studied in a series of rat experiments. Based on its promising properties, [^{18}F]**10** was evaluated in rat brain slice imaging experiments to determine if this compound could be advanced into further studies in primates. While [^{18}F]**10** did not prove to be the best tool to advance into primate studies, we were encouraged to continue to develop newer analogs from this series of compounds (see Table 2) with similar CRF-R1 affinity and even lower lipophilicity. These improved compounds, their radiosynthesis, and biological studies will be the subject of future publications.

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15. *CRF-R1 binding assay:* Frozen rat frontal cortex (source of CRF-R1 receptor) was thawed rapidly in assay buffer containing 50 mM HEPES (pH 7.0 at 23 °C) 10 mM $MgCl_2$, 2 mM EGTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.005% Triton X-100, 10U/mL bacitracin and 0.1% ovalbumin and homogenized. The suspension was centrifuged at 32,000*g* for 30 min. The resulting supernatant was discarded and the pellet re-suspended by homogenization in assay buffer and centrifuged again. The supernatant was discarded and the pellet re-suspended by homogenization in assay buffer and frozen at −70 °C. On the day of the experiment aliquots of the homogenate were thawed quickly and homogenate (25 μg/well rat frontal cortex) added to ligand (150 pM ^{125}I-*o*-CRF) and drugs in a total volume of 100 μL of assay buffer. The assay mixture was incubated for 2 h at 21 °C. Bound and free radioligand were then separated by rapid filtration, using glass fiber filters (Whatman GF/B, pretreated with 0.3% PEI) on a Brandel Cell Harvester. Filters were then washed multiple times with ice cold wash buffer (PBS w/o Ca^{2+} and Mg^{2+}, 0.01% Triton X-100 (pH 7.0 at 23 °C)). Nonspecific binding was defined using 1 μM DMP696. Filters were then counted in a Wallac Wizard gamma counter. Affinity is reported as an average from at least two test occasions, where each sample was evaluated at five concentrations. Materials: Rat frontal cortex was obtained from Analytical Biological Services, Inc. (Wilmington, DE). ^{125}I-*o*-CRF (2200 Ci/mmol) was obtained from PerkinElmer Life Sciences, Inc. (Boston, MA).
16. *Shake-flask logD determination assay for lipophilicity:* In a 250 mL separatory funnel were added 25 mM phosphate buffer (200 mL) and octanol (10 mL). The

two-phase system was mixed well and let stand overnight to allow complete saturation and separation of both layers. A sample (1.0 mg) was dissolved in octanol (1 mL) and transferred to a volumetric flask, containing 50 mL of the phosphate buffer, saturated with octanol, as described above. The resulting mixture was shaken intensely for 30–40 min and was allowed to stand until two layers separated completely. A sample of each layer was analyzed by an HPLC method twice. The sample area counts were used to calculate shake-flask log *D*.

17. *Protein binding studies:* Unbound fraction of test compounds in rats was determined in vitro by equilibrium dialysis using the Dianorm dialysis system. Rat plasma was spiked with the test compound and equilibrated against isotonic phosphate buffer for 3 h at 37 °C. Following the incubation period, plasma and buffer samples were analyzed for compound concentrations using LC/MS/MS. Unbound fraction was calculated based on the ratio between buffer concentration and the plasma concentration.
18. Analytical data for (*R*)-5-chloro-1-(1-cyclopropyl-2-methoxyethyl)-3-(4-(2-fluoroethoxy)-2,5-dimethylphenylamino)pyrazin-2(1*H*)-one: ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.35–0.32 (m, 1H), 0.50–0.48 (m, 1H), 5.88 (m, 1H), 0.76 (m, 1H), 0.86 (m, 1H), 2.24 (s, 3H), 2.28 (s, 3H), 3.33 (s, 3H), 3.68–3.64 (m, 1H), 3.75–3.71 (m, 1H), 4.16–4.13 (m, 1H), 4.22–4.20 (m, 2H), 4.68–4.66 (m, 1H), 4.80–4.78 (m, 1H), 6.65 (s, 1H), 6.68 (s, 1H), 7.24 (s, 1H), 8.01–7.92 (m, 1H). HRMS: 410.03 (MH^+); R_t 2.78 min.; analytical LC/MS: Phenomenex LUNA 4.6 × 50 mm; S5; inj. 5 μL; solvent A, CH_3OH(10%)/H_2O(90%) with TFA(0.1%); solvent B, CH_3OH(90%)/H_2O(10%)/with TFA(0.1%); starting%B = 0; final%B = 100; flow rate 4 mL/min; gradient time, 2 min; end time, 3 min; UV at 220 nm; purity 95%.
19. *Metabolic stability assay:* Estimates of rates of compound turnover (pmol/min/mg protein) were generated using human and rat liver microsomes (BD Gentest, Woburn, MA). The stability in liver microsomes was determined by a high-throughput in-house assay in which 3 μM of compound was incubated with 1.3 mM NADPH and 1 mg/ml of microsomal protein at 37 °C. Incubations were performed in sodium phosphate buffer (100 mM), at pH 7.4, and were terminated at 0 and 10 min following the start of the incubations. The final organic concentration of solvent was 0.015% DMSO and 0.985% acetonitrile. Compound concentrations were determined by LC/MS/MS.
20. *F-18 labeled **10**:* A 250 mCi sample of aqueous F-18 fluoride was obtained from P.E.T. NET Pharmaceutical Services, Milstein Hospital, New York, NY and applied to a previously activated QMA cartridge contained within our remote control radiosynthesis system. After passing N_2 through the cartridge, the activity was eluted from the cartridge and introduced into a 5 mL custom crimp seal vial by the addition of 0.1 mL of K_2CO_3 (6 mg/mL deionized water), 1 mL of the following stock solution (30 mg of K_2CO_3, 1 mL of deionized water, 165 mg of K_{222}, 14 mL of CH_3CN) followed by 1 mL of CH_3CN. The solution was then evaporated by placing the vial into a 90 °C oil bath, and applying a gentle stream of N_2 and a partial vacuum. When the volume of the liquid was reduced to less than 0.3 mL, a 0.5 mL aliquot of CH_3CN was added and the solution reduced by azeotropic distillation. This process was repeated 3 times in an effort to remove all traces of water. During the final procedure, the vial was removed from the oil bath before the solution had gone to dryness and the residue in the vial was placed under full vacuum at room temperature for 6 min. After 6 min, the vacuum was removed and 1,2-dichlorobenzene (300 μL) containing freshly distilled bromoethyl triflate (20 μL) was added to the vial. Formation and distillation of the F-18 bromofluoroethane was achieved by placing the reaction vial in a 130 °C oil bath and having a slow stream of N_2 gas, flow through the vial and into a receiving flask containing the corresponding phenol (2.3 mg), KOH (3.8 mg), DMSO (0.3 mL) and a small stirring bar. After 10 min, the N_2 gas flow was discontinued, the receiving flask sealed and the reaction mixture allowed to stir at 60–70 °C for 15 min. The vial was then cool to RT and diluted with 7 mL of water and passed through a C-18 SEP-PAK® cartridge. The cartridge rinsed with H_2O (10 mL) and the activity eluted from the cartridge with CH_3CN (2.5 mL). The sample was then diluted with H_2O (2.5 mL) and purified by semi-preparative HPLC (Zorbax Rx-C18, 5 micron column, 9.4 × 250 mm, 4.6 mL/min, 260 nm) with an isocratic mobile phase of 62% CH_3CN and 38% 0.1% TFA water. Under these conditions the radioactive product eluted from the column ~14 min. The radioactive product was collected and formulated by diluting the sample with H_2O (20 mL), concentrating the product on a C-18 SEP-PAK® cartridge, rinsing the cartridge with H_2O (5 mL) and eluting with EtOH (1 mL). The EtOH sample was then diluted with saline to yield a 10% EtOH/saline solution. In this procedure a sample of 9.2 mCi of HPLC purified product was available in 132 min.
21. [^{18}F]**10** in vitro autoradiographic studies: Fresh sagittal brain sections (20 μm thick) were obtained from adult male Sprague–Dawley rats. Sets of the brain sections were preincubated in buffer containing 50 mM HEPES, 10 mM $MgCl_2$, 0.2 M EGTA, pH 7.4) for 10 min. The sections were then incubated with 1.3 nM [^{18}F]**10** (1.3 mCi/mL) in the same buffer at 22–24 °C for 40 min. Nonspecific binding was defined by incubation of adjacent sections with [^{18}F]**10** in the presence of 1 μM DMP696, a selective CRF-R1 receptor antagonist. After incubation, slides were washed in ice-cold PBS (pH 7.4) for 2 min. The slides were then air dried and exposed to multi-purpose phosphor screens overnight and scanned by the Cyclone Storage Phosphor Imaging System (Packard Instruments Co.). The imaging of [^{18}F]**10** in the brain sections was analyzed using the OptiQuant acquisition and analysis program (Packard Instruments Co.).