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Letters

Autoradiographic Visualization of Corticotropin Releasing Hormone Type 1 Receptors with a Nonpeptide Ligand: Synthesis of [^{76}Br]MJL-1-109-2

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Abstract: A high-affinity, nonpeptide radioligand for the CRHR1 was synthesized and showed distribution in rat brain consistent with CRHR1 using in vitro autoradiography. This is the first nonpeptide radiotracer combining high affinity and appropriate lipophilicity that penetrates the blood−brain barrier and hence has the potential to be used for PET imaging studies. In vivo visualization of changes in the CRH1 receptor or its occupancy would further the understanding of the pathophysiology of stress related diseases.

Introduction. Corticotropin releasing hormone (CRH) was first isolated in 1981 from ovine hypothalamus extracts and characterized as a 41 amino acid neuropeptide.[1] CRH acts as a major regulator of the hypothalamic−pituitary−adrenal (HPA) axis coordinating neuroendocrine, autonomic, immune, and behavioral responses to stress.[1−3] It is prevalent in the central nervous system (CNS) where it acts as a neurotransmitter.[4−6] Two classes of G-protein-coupled CRH receptors (CRHR1 and CRHR2) have been characterized and cloned from mouse, rat, and human.[7−12] Overstimulation of CRH type 1 receptors (CRHR1) in the brain has been associated with mental disorders such as anxiety,[13] depression,[14] and drug withdrawal syndromes.[13,15−17] A specific radiolabeled CRHR1 ligand for in vivo imaging methods such as positron emission tomography (PET) or single photon emission computed tomography (SPECT) could be an extremely useful tool for monitoring changes in CRH concentration or CRHR1 density noninvasively.[18] This approach would provide a valuable diagnostic and prognostic tool for several stress-related mental disorders. Here we report the design and synthesis of an agent that has the potential to enable examination of the regulation of CRHR1 receptors in the normal, abnormal, and drug-altered CNS by in vitro and possibly in vivo imaging techniques.

Rationale. Since ex vivo autoradiography studies are most predictive of the potential for in vivo PET imaging of a radioligand by providing its quantitative distribution in the brain, our initial work was directed towards the analysis of potential radioligands using ex vivo autoradiography.

Extensive structure−activity relationship (SAR) studies resulted in the discovery of very selective and high affinity CRHR1 antagonists such as the pyrrolopyrimidine derivatives CP-154,526 (**1**) and antalarmin (**2**) (Figure 1).[19]

Since the radioisotopes of halogen atoms provide suitable radiolabels, our first efforts to develop a candidate for imaging studies resulted in derivatives of

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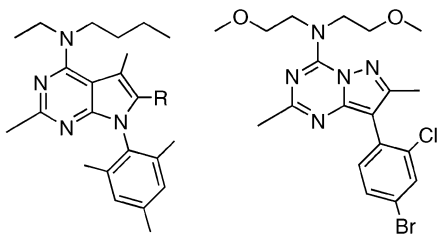

Figure 1. Structures of compounds **1**−**3**.

2 where fluorine atoms were introduced in the alkylamino side chains.[20] A fluoro derivative of **2** was found to show subnanomolar affinity for the CRHR1[20,21] and was tritium labeled.[20,21] However, its poor aqueous solubility, minimal blood−brain barrier (BBB) penetration, and low accumulation in key areas of the brain combined with high nonspecific binding proved that it was unsuitable as radioligand. Similar results were obtained with derivatives of **1**.[22] Further attempts to develop compounds with reduced lipophilicity resulted in compounds with concomitantly decreased receptor affinity.[23] In one example, iodine was introduced into the phenyl moiety of an analogue of **2** leading to a iodopyrimidine derivative with a K_i of only 14 nM.[23] This relatively poor affinity rendered the ligand unsuitable for in vivo imaging based on our preliminary autoradiography studies.

Our working hypothesis was that a compound with affinity for the CRHR1 receptor comparable to **2** ($K_i \sim$ 1 nM) coupled with a suitable lipophilicity (ClogP value $\sim$ 3) would provide a useful ligand for imaging studies. This hypothesis was based on the assumption that the relative lipophilicities of compounds were related to their calculated log *P* values (ClogP)[24] and that a sufficiently hydrophilic compound would cross the BBB more effectively than **2** (ClogP $\sim$ 7). With these design criteria in mind, we focused our work on a derivative of a previously described compound.[25] We retained the pyrazolo[1,5-*a*][1,3,5]triazine core of that molecule and its bis(2-methoxyethyl)amino group. Since earlier SAR studies stated that a 2,4-dichlorophenyl group significantly enhanced the binding affinity of the pyrazolo-[1,5-*a*][1,3,5]triazine to the CRH receptor,[25] we decided to include that substructure. However, we replaced the chlorine in the C4 position of the aromatic ring of the 2,4-dichlorophenyl moiety with bromine in order to obtain a compound MJL-1-109-2 (**3**) that would better serve our eventual purpose. The calculated lipophilicity of **3** was 3.05, and the K_i was found to be 1.9 nM,[23] similar to the data published by He et al. for the related dichloro compound.[25] Therefore we used unlabeled **3** as a precursor for the [^{76}Br]-labeled ligand for our preliminary in vitro autoradiographic study. A long-lived PET radionuclide ^{76}Br was selected for the radiolabeling since it allowed more time for nonspecific binding to clear, yielding higher target to nontarget ratios compared to other radionuclides (e.g., ^{18}F, which has a half-life of about 2 h vs ^{76}Br with 16 h).

Chemistry. Compound **3** was synthesized in six steps starting from (4-bromo-2-chlorophenyl)acetonitrile following a procedure published by He et al.[25] Treatment of bromo derivative **3** with *n*-BuLi at −78 °C followed by addition of tributyltin chloride gave the corresponding stannane in 58% yield. Conversion of the stannane by treatment with ^{76}Br (prepared as previously described by Kao et al.[26]) afforded the radioligand [^{76}Br]-**3**.

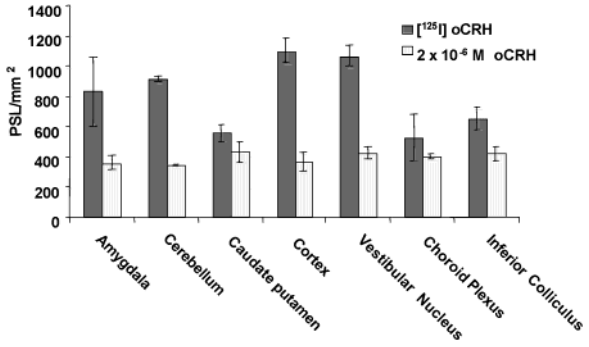

Figure 2. Rat brain regional distribution of CRHR1 determined by in vitro autoradiography with [^{125}I]oCRH.

In Vitro Autoradiography. The brains from Sprague−Dawley rats were rapidly frozen in dry ice, sagittally sectioned into 20 μm slices, and stored at −70 °C until use. The slide-mounted sections (from three different rats) were preincubated in buffer for 15 min and then incubated for 2 h with 0.6 nM [^{76}Br]-**3** (alone or with 10^{-6} M sauvagine, 10^{-6} M ovine corticotropin releasing hormone (oCRH), or 10^{-6} M unlabeled **3**, respectively) or 0.15 nM [^{125}I]Tyr^0-oCRF (alone or with 2×10^{-6} M oCRH). The slides were rinsed, air-dried, and placed on a phosphor-imaging plate. After scanning the plate, the CRHR1 receptor density was quantified by identifying ROI's expressed as photostimulated luminescence units per mm^2 (PSL/mm^2).

In Vivo Rat Biodistribution Studies. Rats were injected intravenously with approximately 3 μCi of [^{76}Br]-**3** and sacrificed after 30 min. Blood and brains were immediately excised from each animal. The brains were placed in 0.3 M sucrose on ice and dissected. All tissues were weighed, and the radioactive content of the blood and various tissues was assessed by gamma counting. Data were expressed as %ID/g (%injected dose/g of tissue).

Results and Discussion. In preliminary autoradiography studies, we established the distribution of CRHR1 with [^{125}I]oCRH, a known peptide CRHR1 radioligand. The regional brain localization of [^{125}I]-oCRH was determined by identifying and quantifying (PSL/mm^2) ROIs (Figure 2). The highest density of CRHR1 was observed in the cortex, the vestibular nucleus, and the cerebellum. Specific binding was found to range from 60% to 67% in these areas using 2×10^{-6} M unlabeled oCRH to determine nonspecific binding. The lowest specific binding was observed in the choroid plexus (24%) and the caudate putamen (22%), respectively. This regional rat brain localization of [^{125}I]oCRH was consistent with the distribution recorded by De Souza and Kuhar using ^{125}I-Tyr^{32}-oCRF, a CRHR1 specific ligand.[27]

The regional distribution of [^{76}Br]-**3** was visualized in rat brain by in vitro autoradiography. Figures 3 and 4 represent sagittal autoradiograms (1.8 mm and 3.6 mm, respectively, from the midline) from which the appropriate ROIs were determined and then quantified for the different regions (Figure 5). The highest levels

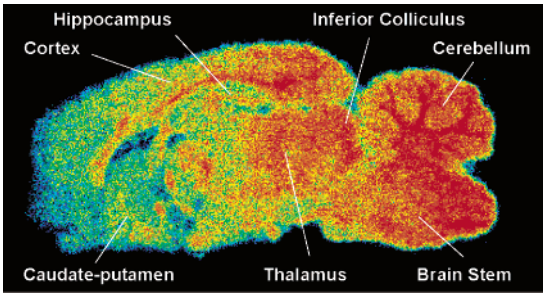

Figure 3. Regional rat brain localization of [^{76}Br]-**3** using in vitro autoradiography, 1.8 mm from the midline.

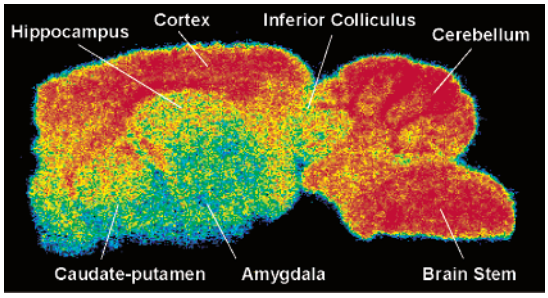

Figure 4. Regional rat brain localization of [^{76}Br]-**3** using in vitro autoradiography, 3.6 mm from midline.

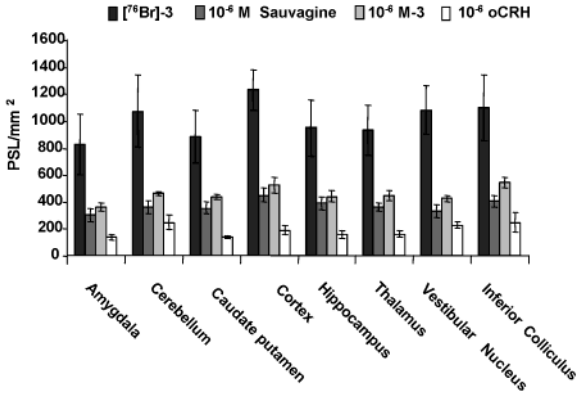

Figure 5. Determination of the specific binding of [^{76}Br]-**3** (carrier free) in the rat brain, from in vitro autoradiography studies. Error bars represent standard deviations of $n = 3$.

of radioactivity were observed in the cortex, the vestibular nucleus, the cerebellum, and the inferior colliculus indicating areas of high receptor density.

Addition of 10^{-6} M unlabeled **3** to [^{76}Br]-**3** revealed about 60% reduction of specific binding in the cerebellum, the vestibular nucleus, the cortex, and the inferior colliculus (Figure 5). Using 10^{-6} M oCRH with [^{76}Br]-**3** to determine nonspecific binding, specific binding ranged from 77% to 85% in all brain regions. When blocked with 10^{-6} M sauvagine (a peptide ligand that binds to both CRHR1 and CRHR2), the specific binding ranged from 59% to 70% in all brain regions.

The overall pattern of distribution of the [^{76}Br]-**3** in the brain compares favorably with the regional distribution of [^{125}I]oCRH and with published results[27] using ^{125}I-Tyr^{32}-oCRF providing further proof of CRHR1 distribution.

In vivo biodistribution studies in rats revealed that [^{76}Br]-**3** was able to penetrate the blood−brain barrier. After 30 min uptakes of 0.29±0.01% ID/g and 0.32 ±0.03% ID/g (mean ± SD; $n = 4$) were observed in the cerebellum and cortex, respectively.

Conclusion. A positron emitting radiobrominated ligand, [^{76}Br]-**3**, has been synthesized. This is the first nonpeptide radiotracer with high affinity ($K_i \sim 2$ nM) for CRHR1 receptors combined with appropriate lipophilicity (ClogP ~ 3), which crosses the BBB exhibiting potential for use in PET imaging studies. Its distribution in key areas of the brain proved to be consistent with human CRHR1 distribution in rat brain using in vitro autoradiography. Visualization of changes in CRHR1 density or occupancy could lead to improved understanding of stress-related mental conditions such as anxiety, depression, and drug abuse.

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Supporting Information Available: Experimental procedures and analytical data for **3** and [^{76}Br]-**3** as well as a description of the autoradiography experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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