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Characterization of a novel and selective CB1 antagonist as a radioligand for receptor occupancy studies

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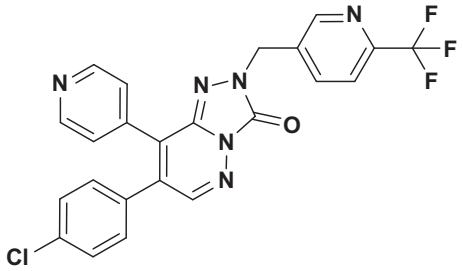
ABSTRACT

Obesity remains a significant public health issue leading to Type II diabetes and cardiovascular disease. CB1 antagonists have been shown to suppress appetite and reduce body weight in animal models as well as in humans. Evaluation of pre-clinical CB1 antagonists to establish relationships between in vitro affinity and in vivo efficacy parameters are enhanced by ex vivo receptor occupancy data. Synthesis and biological evaluation of a novel and highly selective radiolabeled CB1 antagonist is described. The radioligand was used to conduct ex vivo receptor occupancy studies.

The biological effects of the endocannabinoids are primarily mediated by the CB1 and CB2 receptors in the central nervous system and periphery and may be modulated by additional proteins as well.[1] CB1 is highly expressed in the brain, with the highest levels of expression in the hippocampus, cerebellum, substantia nigra, basal ganglia, caudate putamen, and pre-frontal cortex as determined by receptor autoradiography and immunohistochemical analysis.[2] The CB1 receptor has been implicated in the regulation of food intake and body weight.[3] Indeed, antagonists to the CB1 receptor such as rimonabant, taranabant, and ibipinabant (SLV-319) have proven to be clinically effective in the treatment of obesity.[4] BMS-725519 **1** was identified as compound exhibiting properties suitable for pre-clinical development for obesity (Fig. 1).

In support of studies to understand the relationships between receptor affinity, protein binding and drug exposure on in vivo efficacy, as well as to assist with human dose projections we wished to characterize BMS-725519's potential as a radioligand. The compound's excellent binding affinity coupled with well-balanced physicochemical properties, including calculated Log*P* and protein binding, made it a good candidate for radiolabeling and assessment in receptor autoradiography/occupancy studies. While the CB1 radioligand [^{3}H]SR141716A was commercially available, and used by our group to assess receptor binding affinity and ex vivo occupancy, we wished to characterize the properties of tritiated BMS-725519 as an additional in vitro tool and potential PET ligand option as compounds such as [^{18}F]MK-9470 and MePPEP were not commercially available to us. While it is always advisable to utilize radioligands and test drugs from distinct chemical classes to



BMS-725519 **1**

^{3}H-SR-141716 binding rCB1	12.4 nM
^{3}H-SR-141716 binding hCB1	4.6 nM
^{3}H-CP55940 binding hCB2	>10,000 nM
cLogP	2.37
% Protein binding (h, r)	95, 92

Figure 1. The structure, in vitro binding affinities, calculated Log*P* and in vitro protein binding values for BMS-725519.

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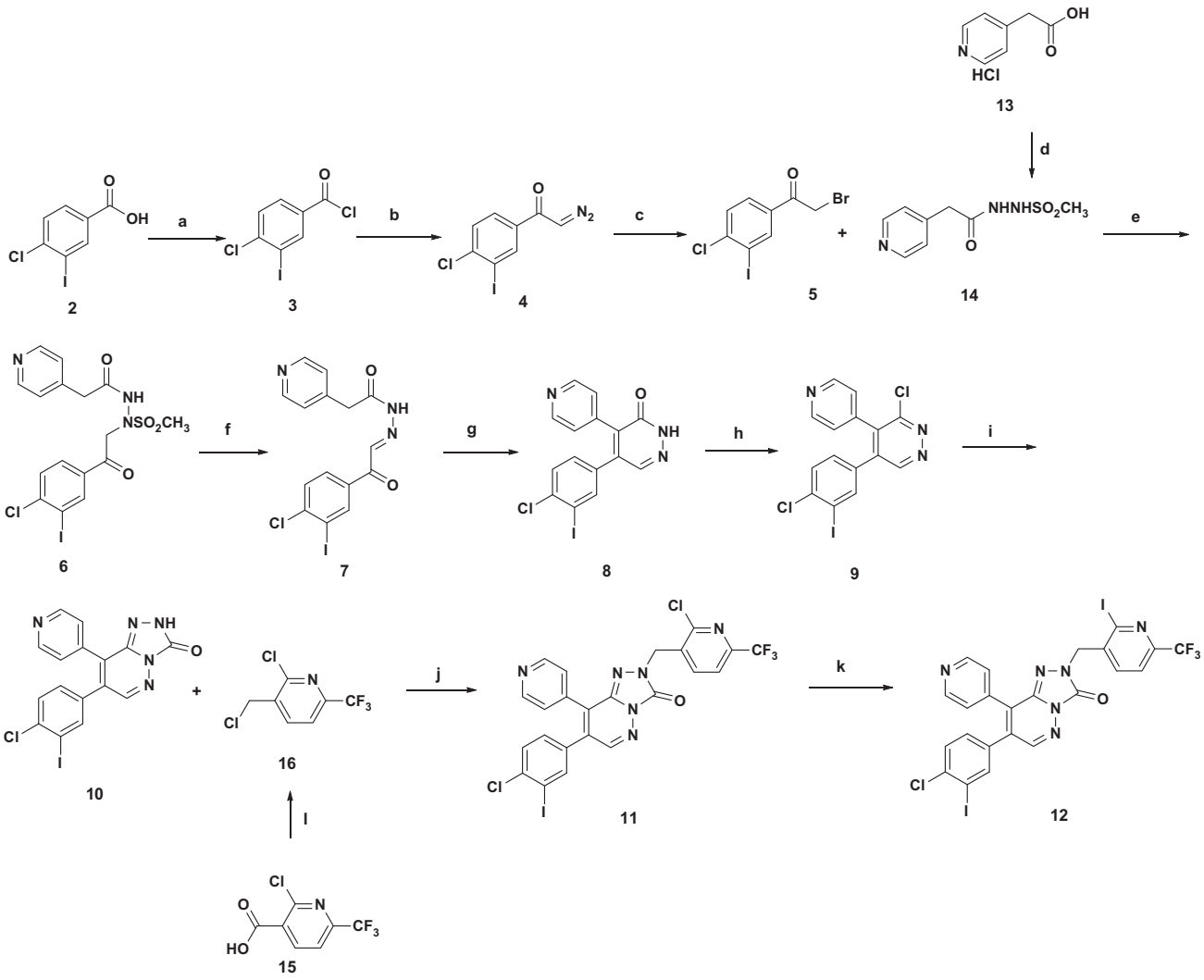


Scheme 1. Reagents and conditions: (a) $(COCl)_2$, $CH2Cl_2$, DMF(cat.); (b) diazomethyltrimethylsilane, CH_3CN; (c) HBr/HOAc; (d) $CH_3SO_2NHNH_2$, CDI, CH_3CN; (e) compd **13**, KO*t*Bu, DMF, −50 °C to rt; (f) NaOAc, rt; (g) DBU, 50 °C; (h) $POCl_3$; (i) (i) NH_2NH_2; (ii) CDI; (j) compd **15**, K_2CO_3, DMF, 80 °C; (k) NaI, TMS-Cl, CH_3CN, 180oC, 180 °C, microwave; (l) (i) BH_3-THF; (ii) $SOCl_2$, CH_2Cl_2.

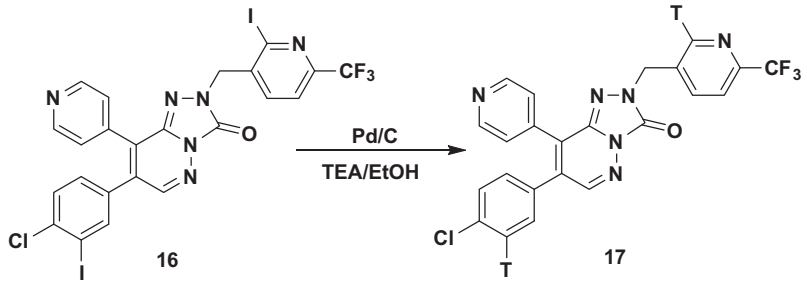
minimize non-selective interactions, resource constraints necessitated the use of unlabeled BMS-725519 as the test reagent for our characterization of [^{3}H]BMS-725519.

The synthesis of di-iodo analog is illustrated in scheme 1. Commercially available 4-chloro-3-iodobenzoic acid **2** was converted to compound **5** in 3 steps by reacting first with oxalyl chloride, followed by diazomethyltrimethylsilane and then HBr in acetic acid. Compound **5** then was reacted with compound **14** which was derived from 2-(pyridin-4-yl)acetic acid HCl salt to give compound **6**. Compound **6** was then treated first with NaOAc to afford compound **7** which was cyclized to form compound **8** under DBU. Compound **8** was then refluxed in $POCl_3$ to form compound **9**. The triazolopyridazinone **10** was constructed by reacting compound **9** with hydrazine first, followed by CDI. Compound **10** was then coupled with compound **16** which was synthesized from 2-chloro-6-(trifluoromethyl)nicotinic acid in 2 steps to give compound **11**. The second iodo was introduced by reacting compound **11** with NaI and TMS-Cl in acetonitrile at 180 °C in microwave reactor.

[^{3}H]BMS-725519 **17** (Scheme 2) was tested against membrane homogenates prepared from whole rat brain and CHO cells that recombinantly-expressed human CB1 receptor.[6] Saturation analysis revealed a K_d = 29.1 nM and a B_{max} of 2.16 pmol/mg protein for the CB1 receptor in rat brain (Fig. 2) and a K_d = 3.95 nM and a B_{max} of 41 pmol/mg protein for the recombinantly expressed human CB1 receptor.[5] Rimonabant and ibipinabant inhibited [^{3}H]BMS-725519 binding to human recombinant CB1 receptor in a concentration dependent manner with K_i values of 0.73 and 0.42 nM, respectively.[6] CP-55,940 and BMS-725519 were tested in competitive binding assays using [^{3}H]BMS-725519 on rat cerebellar tissue sections, yielding IC_{50} values of 4 and 37 nM, respectively.[7]

In vitro autoradiography studies revealed that [^{3}H]BMS-725519 produced selective labeling of distinct regions of the rat brain including the cerebellum, hippocampus, striatum and frontal cortex (Fig. 3).[7] The labeling pattern matches results from previously published studies with [^{3}H]CP-55940 and [^{3}H]SR141716 (rimonabant).[5] CB1 receptor autoradiographic labeling by [^{3}H]BMS-725519 was also characterized in wild-type and CB1 knockout mice. In wild-type animals the pattern of labeling was equivalent to that seen in the rat while in the KO animals specific labeling was absent (Fig. 4).[7]

Ex vivo binding studies were conducted on rat brain sections from animals treated with orally administered increasing doses of BMS-725519 (0.03, 0.3, 3.0 and 30.0 mg/kg p.o.).[8] As highlighted in Figure 5 it was possible to determine the level of displacement



Scheme 2. [^{3}H]BMS-725519 **17** was prepared by tritio-de-iodination of the di-iodinated analog **16** with carrier-free tritium gas in the presence of palladium/carbon, 10% at room temperature for 3 h yielding a specific activity of 29.0 Ci/mmol. The crude products were purified by HPLC to more than 99% radiochemical purity.

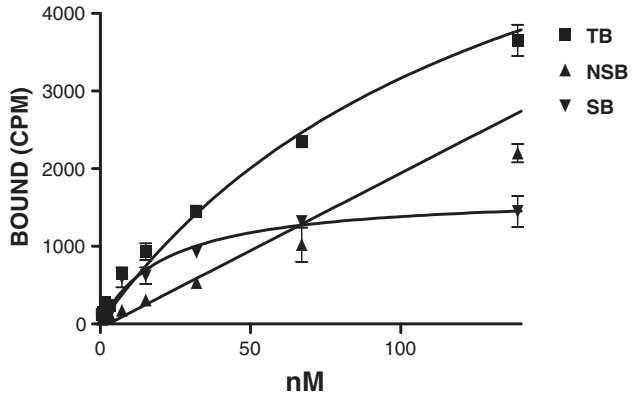


Figure 2. Representative saturation binding experiment (total $n = 2$) using [^{3}H]-BMS-725519 and 40 μg of rat brain membranes. TB = total binding; NSB = non specific binding defined by addition of 1000-fold excess of unlabeled BMS-725519; SB = specific binding.

of [^{3}H]BMS-725519 labeling by increasing doses of BMS-725519 in sections of rat brain, resulting in a determination of receptor occupancy.

When coupled with measures of food intake, plasma drug levels and brain drug levels, relationships between receptor occupancy and efficacy can be determined using this radioligand.[8,9] An in vivo study evaluating doses of 0.03, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 mg/kg was carried out to evaluate the relationships described above 6 h after dosing (Table 1). As calculated free plasma and brain drug levels increase there is a corresponding increase in both the suppression of food intake (as measured by the number of food pellets consumed) and receptor occupancy. The reduction in food intake appears to reach a plateau at the 10 and 30 mg/kg doses despite further increases in plasma and brain drug levels. Receptor occupancy also seems to be maximal at these two doses indicating that CB1 receptors may be fully occupied. While the absolute level of occupancy has not reached 100%, it is likely that the receptor occupancy at the highest doses corresponds to greater level of actual occupancy, possibly approaching 100%, given the fact that

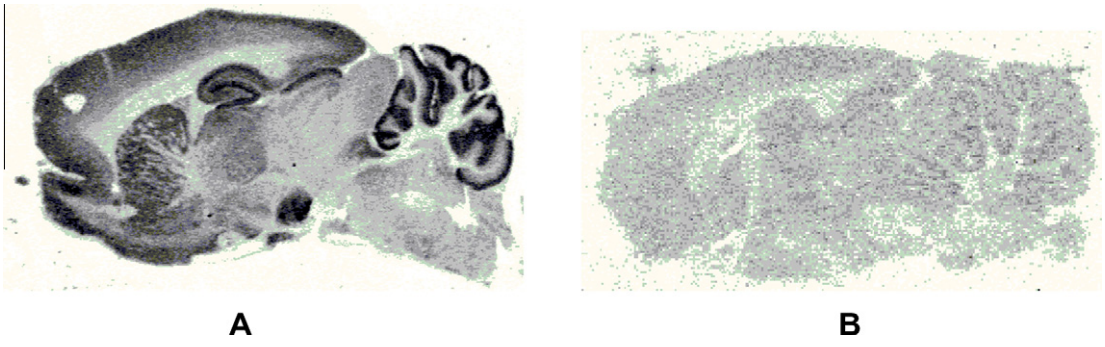


Figure 3. Representative autoradiogram of a sagittal section of rat brain incubated with 40 nM [^{3}H]-BMS-725519 (A) or section incubated with 40 nM [^{3}H]-BMS-725519 and 5 μM unlabelled BMS-725519 (B).

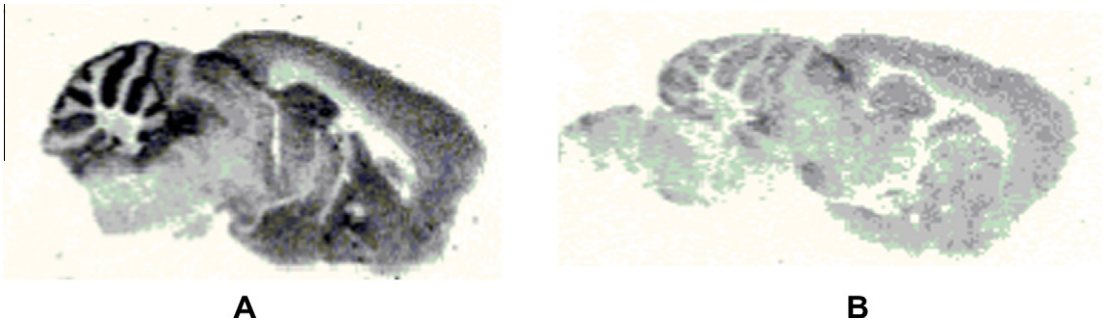


Figure 4. Ex vivo autoradiography of mouse brain sagittal sections of either wild-type (A) or CB1 knockout (B) animals.

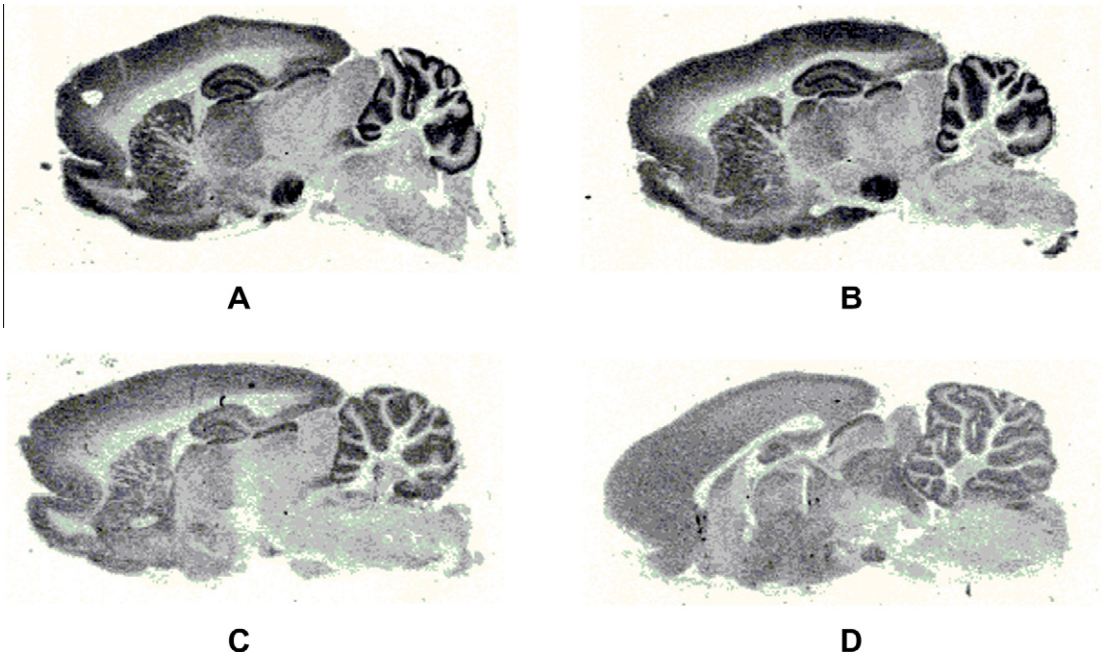


Figure 5. Ex vivo autoradiography of rat brain sagittal sections following drug treatment. Rats were administered vehicle (A), 0.03 mpk (B), or 3 mpk (C) or 30 mpk (D) of BMS-725519 and then processed for receptor autoradiography with [^{3}H]BMS-725519.

Table 1
Effects of BMS-725519 on receptor occupancy and efficacy, and the determination of plasma and brain exposures

P.O. Dose (mg/kg)	0.03	0.1	0.3	1.0	3.0	10	30
Receptor occupancy (%)	12.7	14.3	20.6	29.8	55.6	64.5	74.1
Free plasma exposure (nM)	1.5	5.4	15.3	49.4	112	531	1047
Free brain exposure (nM)	0.93	2.6	6.4	18.6	19.9	99.7	308
Reduction in food intake (%)	0	0	22.3	23.7	41.5	67.4	72.5

dissociation of receptor-bound drug is known to occur during the in vitro processing of tissue sections.

In summary we have developed a selective radioligand for assessing CB1 receptor occupancy that is similar to other available CB1 radioligands. As part of our evaluation we determined that [^{3}H]BMS-725519 could facilitate the development of an understanding of relationships of receptor affinity, drug exposure and receptor occupancy in the brain to develop human dose projections in support of pre-clinical development studies.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.016.

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6. Brain tissue or cells scraped from plates were homogenized manually by hand in 10 volumes of the cold buffer with 10 mM HEPES and 1 mM EGTA, pH 7.4, containing complete protease inhibitor cocktail, 1 mM dithiothreitol, and 10% sucrose. The homogenate was centrifuged at 1000 g for 10 min at 40 °C. Supernatant was collected and centrifuged at 100,000 g for 20 min. The pellet was re-suspended in buffer containing 20 mM HEPES, 1 mM EGTA, pH 7.4, 1 mM DTT, and 1 mM $MgCl_2$. Saturation analyses were carried out to determine the dissociation constant, K_d of [^{3}H]BMS-725519. Specifically, increasing concentrations of the radioligand was added to the assay mixture and the specific bound was measured using cold BMS-725519 as the non-specific. The binding reaction was incubated for 90 min at room temperature and then terminated by transferring the reaction mixtures onto GF/B filter plates using a Packard Cell Harvester. The filter plates were then washed and the contents of the plates were counted on a Packard TopCount Scintillation Counter.
7. Male Sprague-Dawley rats or wild-type or CB1 receptor knockout mice (obtained from the laboratory of Andreas Zimmer) were sacrificed by decapitation. Brains were collected, incubated in pre-chilled (−20 °C) 2-methyl butane for 2–3 min and stored at −80 °C until use. The samples were sectioned to 20 μm at −20 °C using a cryostat (Jung Frigo-Cut, 2800E) and stored at −80 °C. For binding studies, the slide-mounted sections were brought to 22–24 °C, dried, and pre-incubated in the binding buffer containing 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 0.25% BSA, 1 mM leupeptin for 2 min. The sections were then incubated in the same solution containing 2.1–385 nM [^{3}H]-BMS-725519 for 45 min at 22–24 °C. The adjacent sections were incubated under the same conditions in the presence of 10 μM unlabelled BMS-725519 to define nonspecific binding. Concentration-related displacements of BMS-725519 and CP-55940 were assessed by incubating the compounds at a

concentration of 0.01–10,000 nM in the total binding buffer with 40 nM [^{3}H]BMS-725519. After incubation, the sections were rinsed in phosphate buffer saline three times, 2 min each, and rinsed quickly in de-ionized H_2O. The sections were then placed in cassettes against a tritium-sensitive screen for 2–3 days. The captured storage phosphor images were analyzed using OptiQuant Acquisition and Analysis software (Perkin Elmer Life Science).

8. For the determination of receptor occupancy and plasma/brain exposures, BMS-725519 was orally administered to male Sprague-Dawley rats at 0.03, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 mg/kg one hour prior to the dark cycle (six animals per dose group). The vehicle used to make the dosing solution consisted of 10% DMAC/10% cremophor/10% ethanol and 70% water and the dosing volume was at 2 ml/kg. At 6 h post-dose, blood samples and brain tissues were collected from three animals per dose group for determination of exposures, and three rat brains were collected for the determination of CB1 receptor occupancy. Blood samples were centrifuged at 4 °C to obtain serum. Brain tissue for exposure determination was rinsed with ice-cold water and blotted dry to remove excess water. After recording the weights, the brains were homogenized using three volumes of water. The homogenized brains and the serum samples were stored at −20 °C before analysis by LC–MS. Free drug concentrations were calculated based on total drug levels × 8% unbound drug determined from in vitro protein binding studies (data in Fig. 1). Intact brains for the determination of receptor occupancy were rapidly frozen and sectioned as described in Ref.[7]. The radioligand binding procedures for receptor occupancy studies were similar to the in vitro with a few changes to minimize the loss of receptor occupancy of exogenously administered BMS-725519: The pre-incubation of the tissue sections in buffer was shortened to 30 s, [^{3}H]BMS-725519 incubation time was 6 min at 40 nM, non-specific binding was determined by adding 5 μM unlabelled BMS-725519 to the binding buffer, and post incubation washes were three times at 30 s each. The binding buffer and washing buffer were pre-chilled at 4 °C. Digital images were generated with a Cyclone storage phosphor-imaging system as described earlier. Radioligand binding density in a defined brain region (cerebellum) was measured as digital light units per millimeter squared (DLU/mm^2). All measurements were first subtracted by the background DLU/mm^2. For ex vivo binding studies, the percent of specific binding was calculated as the following: percent specific binding = (specific binding in drug treated)/(specific binding in vehicle treated) × 100%. The data were reported as the mean ± S.E.M.
9. For feeding studies male Sprague Dawley rats (~225 g starting weight) were obtained from Charles River Laboratories and housed on a 12:12 light/dark cycle in the BMS vivarium. Animals were acclimated to the facility for 10 days prior to use with standard rodent chow (Harlan Teklad) and water ad libitum. There were five groups: Vehicle (10%DMAC, 10%EtOH, 10% Cremophor,70% water; 2 ml/kg; p.o.) and 7 doses of Test Agent (BMS-725519; 0.03, 0.1, 0.3, 1, 3, 10 and 30 mg/kg; p.o.). There were six rats in each group, and all animals were tested simultaneously. Animals were trained to bar-press for food in an operant chamber (Coulbourn Instruments, Allentown, Pa) equipped with a lever, a food hopper, a water bottle with photocells and an infra-red activity monitor. The training sequence was initiated by depriving the animal of food for 16 h. At the end of that period, which coincided with the beginning of the dark cycle, the rat was placed into an operant chamber with 5–45 mg pellets (Research Diets purified food pellet) present in the feeding trough. Subjects could obtain additional pellets by pressing a lever situated adjacent to the trough. During the first 20 h of training one lever press was sufficient to deliver one pellet. During the second 20 h, three lever presses were required to obtain a pellet. At all times the animal had access to one water bottle situated on the opposite side of the cage. Photobeams positioned around the opening to the water bottle were broken by a nose-poke and served to provide a measure of licks taken by the animal. All testing sessions began at the start of the dark-cycle, the time at which nocturnal feeding begins. Animals were subject to two 20 h sessions with an interval of 4 h between sessions. The first session served as a baseline measure of feeding and animals were assigned to treatment groups in such a way that there were no differences in the average number of pellets eaten during the 20 h baseline period. Test compounds were delivered orally immediately prior to the start of the second session. The second session commenced with a 60 min lock-out period during which lever pressing did not result in pellet delivery. At the end of this period, rats could obtain food reward on an FR3 schedule for the remainder of the twenty hour session. Statistical evaluation of pellets consumed at each time interval after the lock-out period (1, 2, 4, 8, 12 and 20 h) was conducted using one-way ANOVA. Group differences were further assessed using Fishers PLSD as a post-hoc test.