Small-Molecule Melanin-Concentrating Hormone-1 Receptor Antagonists Require Brain Penetration for Inhibition of Food Intake and Reduction in Body Weight

X. Eric Hu,1 John A. Wos, Martin E. Dowty,2 Paula M. Suchanek, Wei Ji, James B. Chambers, Stephen C. Benoit, Deborah J. Clegg, and Ofer Reizes3

Procter & Gamble Pharmaceuticals, Inc., Mason, Ohio (X.E.H, J.A.W., M.E.D., P.M.S., W.J., O.R.); Obesity Research Center, University of Cincinnati, Cincinnati, Ohio (J.B.C., S.C.B., D.J.C.); and Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio (O.R.)

Received August 17, 2007; accepted October 9, 2007

ABSTRACT

The melanin-concentrating hormone-1 receptor (MCH1R) is a G-protein-coupled receptor expressed in the brain and peripheral tissues that regulates energy storage and body weight. Here, we focused on discovery of the mechanism and site of action for a small-molecule MCH1R antagonist, which yields weight loss in a mouse model of human obesity. MCH1R is expressed throughout the brain but also found in peripheral tissues known to regulate fat storage and utilization, e.g., skeletal muscle and adipose tissue. Previous studies of MCH1R antagonist studies have not delineated the site that is critical for mediating the anorexigenic and weight-reducing actions. In this study, we evaluated the role of the brain and peripheral tissue receptors. We developed a novel nonbrain-permeable MCH antagonist analog with a carboxylic acid moiety to specifically test the site of action. Based on in vitro and in vivo assays, the analog is not able to cross the blood-brain barrier and does not lead to inhibition of food intake and reduced body weight. The data clearly demonstrate that MCH1R antagonists need access to the brain to reduce body weight and fat mass. The brain-permeable MCH1R antagonist leads to significant reduction in body weight and fat mass in diet-induced obese mice. The effect is dose-dependent and appears to be partially driven by a reduction in food intake. Finally, these studies show the utility of a medicinal chemistry approach to address an important biological and pharmacological question.

The obesity epidemic continues to increase in many developed and developing countries (Ogden et al., 2003; Haslam and James, 2005). The 2004 National Health and Nutrition Examination Survey indicated that more than 50% of the United States population is either obese or overweight (Hedley et al., 2004; Ogden et al., 2006). More importantly, the incidence of obese and overweight children is also on the rise. Obesity is associated with multiple metabolic disorders, including diabetes and cardiovascular disease (Ogden et al., 2003). There is a great interest in identifying behavioral and pharmacological approaches to reduce body weight in obese and overweight individuals.

Weight loss intervention strategies include targets for reducing hunger/appetite, enhancing satiety, increasing metabolism/energy utilization, blocking fat absorption, inhibiting adipose tissue differentiation, and inducing adipose tissue apoptosis. The current therapeutic approaches for weight loss include diet, exercise, and pharmacotherapy. Efficacy is very modest, with a high rate of recidivism at the end of the treatment period (Schnee et al., 2006). Orlistat (Xenical) and sibutramine (Meridia) are currently the only two approved pharmacotherapy agents on the market. Orlistat blocks absorption of fat from the intestine, whereas sibutramine reduces appetite by acting on brain pathways that regulate hunger (Bray and Ryan, 2007). These drugs show limited efficacy, and considerable attention is focused on the development of new reagents to decrease body weight.

ABBREVIATIONS: MCH, melanin-concentrating hormone; MCH1R, MCH-1 receptor; QMR, quantitative magnetic resonance; CTA, conditioned taste aversion; DIO, diet-induced obesity; DABA-821, 4’-trifluoromethyl-biphenyl-4-carboxylic acid [2-(4-diethylaminomethylphenyl)-ethyl]-methyl-amide (carboxy-DABA-822, 1-(4-{2-[(4’-trifluoromethylbiphenyl-4-carbonyl)-methyl-amino]-ethyl}-benzyl)-piperidine-4-carboxylic acid.
Over the past 5 years, the pharmaceutical and biotechnology industry has focused on neural targets that regulate appetite and satiety. Melanin-concentrating hormone (MCH) and its receptor, the MCH-1 receptor (MCH1R), have emerged as targets for weight reduction (Schwartz and Gel- ling, 2002). The MCH1R is a G-protein-coupled receptor, which is widely distributed in the brain; however, it is also expressed in peripheral organs, including adipose tissue and skeletal muscle (Schlumberger et al., 2002). Hypothalamic MCH and MCH1R expression are increased in states of food deprivation and fasting and rapidly decrease upon refeeding (Qu et al., 1996; Shimada et al., 1998; Ludwig et al., 2001). Central injections of MCH in rodents lead to an increase in food and water intake (Clegg et al., 2003), and chronic dosing leads to body weight gain (Shearman et al., 2003), suggesting a critical role of hypothalamic MCH signaling in the regulation of body weight. The converse is true as well; ablation of the MCH and MCH1R gene in both the periphery and brain in mice leads to reduction in body weight and resistance to weight gain on a high-fat diet (Shimada et al., 1998; Chen et al., 2002; Marsh et al., 2002). These data suggest a role for MCH antagonism as a potential pharmacotherapy in the treatment of obesity (Shimada et al., 1998; Ludwig et al., 2001; Chen et al., 2002; Marsh et al., 2002; Ito et al., 2003). Functional MCH1Rs are expressed in adipose tissue and MCH stimulates the secretion of leptin from cultured adipocytes. Given that MCH1R is expressed both in peripheral tissues as well as the brain, it is important for the development of pharmacotherapies to determine whether central or peripheral mechanisms are critical for the regulation of food intake, body weight, and energy expenditure.

Here, we report utilization of a small-molecule medicinal chemistry approach to delineate the site of action of an MCH1R antagonist in the reduction of food intake and body weight. We previously reported the identification of multiple chemical scaffolds that show high affinity toward the receptor (Kim et al., 2006; Warshakoon et al., 2006). This study focused on identifying which MCH1R site is critical for mediating the weight-reducing effects. We tested the hypothesis that MCH antagonists need to access the brain MCH1R population to regulate body weight. Our findings demonstrate, for the first time, that MCH1R antagonists are only efficacious when they have access to the brain.

Materials and Methods

Materials

Chemicals were obtained from Sigma-Aldrich (St Louis, MO), J.T. Baker (Phillipsburg, NJ), EM Science (Gibbstown, NJ), and Pierce (Rockford, IL). Plastic tubes were obtained from VWR International (West Chester, PA), Gilson (Middleton, WI), and National Scientific Supply Co, Inc. (San Rafael, CA). Mouse serum was obtained from Bioreclamation Inc. (Hicksville, NY). Rat Materials were obtained from Pel-Freez Biologicals (Rogers, AR). Rat National Scientific Supply Co, Inc. (San Rafael, CA). Mouse serum was obtained from Bioreclamation Inc. (Hicksville, NY). Hypothalamic MCH and MCH1R expression are increased in states of food deprivation and fasting and rapidly decrease upon refeeding (Qu et al., 1996; Shimada et al., 1998; Ludwig et al., 2001). Central injections of MCH in rodents lead to an increase in food and water intake (Clegg et al., 2003), and chronic dosing leads to body weight gain (Shearman et al., 2003), suggesting a critical role of hypothalamic MCH signaling in the regulation of body weight. The converse is true as well; ablation of the MCH and MCH1R gene in both the periphery and brain in mice leads to reduction in body weight and resistance to weight gain on a high-fat diet (Shimada et al., 1998; Chen et al., 2002; Marsh et al., 2002). These data suggest a role for MCH antagonism as a potential pharmacotherapy in the treatment of obesity (Shimada et al., 1998; Ludwig et al., 2001; Chen et al., 2002; Marsh et al., 2002; Ito et al., 2003). Functional MCH1Rs are expressed in adipose tissue and MCH stimulates the secretion of leptin from cultured adipocytes. Given that MCH1R is expressed both in peripheral tissues as well as the brain, it is important for the development of pharmacotherapies to determine whether central or peripheral mechanisms are critical for the regulation of food intake, body weight, and energy expenditure.

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Chemical Synthesis

Compounds were synthesized in a multiple step sequence starting from commercially available 4-aminoethylbenzoic acid (Scheme 1). The amine was protected with a Boc group. The corresponding benzoic acid (3) was treated with Mel/NaH followed by Dibal reduction to give a benzyl alcohol (4). This alcohol was converted to a biaryl amide (5) by removal of the Boc-protecting group and then coupling with a biaryl acid sequentially. The formation of a mesylate intermediate (6) followed by displacement with secondary amines furnished the synthesis of requisite amine products 1 and 2, which was obtained after base hydrolysis of methyl ester 7. The final products were purified by a prep- high-performance liquid chromatography method to offer ≥95% product purity for in vitro and in vivo studies. Details on chemical synthesis can be found in supplemental data.

In Vitro

MCH Binding and Function Assays. Compounds were assayed for binding and function using a human embryonic kidney 293 cell line that stably overexpresses the human MCH1R using established techniques. These cells were determined to express approximately 150 fmol receptor/mg protein with an affinity of 6.3 nM for the eukalyptus MCH used in the binding assays. Competitive binding assays were performed by incubating the cells with varying concentrations of compound in the presence of 25 nM europium-labeled MCH. Subsequently, cells were washed free of excess europium-labeled MCH, and residual bound MCH was quantified. Nonspecific binding was determined by incubating cells with 10 μM unlabeled MCH.

MCH activity was detected in the same cells expressing the MCH1R with a reporter construct that contains the serum response element regulating the expression of the firefly luciferase gene. The EC_{50} for MCH was determined to be 19 nM. Before the assay, cells were washed free of serum-containing media and incubated overnight in serum-free media. The next morning, cells were incubated with varying concentrations of the test compound (10 μM–100 μM) and 25 nM MCH for 4 h. Cells were then processed according to established protocols for luciferase activity as a measure of receptor activation.

Caco-2 Assay. Caco-2 cells (American Type Culture Collection, Manassas, VA), derived from human colorectal carcinoma, were seeded on porous transwell membranes at 20,000 cells/well and grown for 18 days before testing. Cells were washed three times with Dulbecco’s phosphate-buffered saline, pH 7.4, containing 0.5% bovine serum albumin and equilibrated for 30 min in a 37°C incubator (orbital shaker, 150 rpm). Test compound was added to individual wells, and radiolabeled mannitol (NEN Radiochemicals, Wellesley, MA) was included in each well as a paracellular permeability internal standard. Final solutions contained 10 μg/ml compound and 1 μCi/ml [3H]mannitol in Dulbecco’s phosphate-buffered saline with 1% dimethyl sulfoxide and 0.5% bovine serum albumin. Samples (200 μl) were collected at 120, 180, and 240 min during incubation from donor/receiver chambers, and aliquots were placed in 0.75-ml polypropylene tubes for liquid chromatography/tandem mass spectrometry analysis (100 μl) and scintillation counting (50 μl). Each sample was replaced with 200 μl of fresh vehicle. Two 10-μl samples were taken from the donor compartment at the beginning of the study and at the end of the study. One 10-μl sample was placed in a 0.75-ml polypropylene tube and diluted with 390 μl of bathing buffer to ensure good solubility of the concentrated sample after freezing and thawing and to allow a larger sample aliquot to be taken for analysis. The samples were frozen until analysis. Details on the liquid chromatography/tandem mass spectrometry analysis is provided online (supplemental data). Scintillation counting of the other 10 μl and the previous 50-μl samples was performed to determine [3H]mannitol permeability. Bidirectional [apical-basolateral (absorptive) and basolateral-apical (exosorptive)] permeability constants (P) were calculated at steady state according to Fick’s laws of diffusion: P = MD/ACo, where MD/dt is the mass of solute transported with time at steady state, A is the cross-sectional area of cells.
exposed to solute transport, and Co is the initial concentration of solute in the donor chamber.

**In Vivo**

**Diet-Induced Obese Mouse Studies.** Male mice (C57BL/6J; Jackson Laboratory, Bar Harbor, Maine) were obtained at 6 to 8 weeks of age and provided a high-fat diet (high fat, 45% kcal; Research Diets, Inc., New Brunswick, NJ) for 10 to 12 weeks before the study. Body weights and fat mass were monitored over this period. Upon their initial arrival to the facility, the animals were single housed in plastic cages on a reversed light cycle (11:00 AM, lights off; 11:00 PM, lights on). This research was conducted in accordance with Procter & Gamble’s policy on research involving animals with strict oversight for care and welfare. All studies were approved by Procter & Gamble’s Animal Care and Use Committee.

The mice were randomized into groups (n = 8) based on body weight as well as fat composition, which was determined using quantitative magnetic resonance (QMR; EchoMRI, Houston, TX). The QMR is a validated method for measuring body composition in rodents (Tinsley et al., 2004), and we performed our own validation study to confirm its utility in our studies (R. J. Sheldon, O. Reizes, and M. Cockman, unpublished data). Mice averaging 36 ± 5 g, 24% fat were used in the study. Mice were dosed with compound or vehicle (2% Tween 80/saline) via p.o. gavage or s.c. administration twice daily for a period of 4 or 10 days. Food intake and body weight were measured daily, and body composition was measured at the end of the study. On the last day of dosing, four mice from each dosing group were given a single dose, and blood samples from the four mice were then collected approximately 2 h after dosing to determine peak serum concentrations \(C_{\text{max}}\). The blood was put into serum separator tubes, placed on ice for 60 min, and centrifuged. The serum was poured off into a tube and frozen until prepared for analysis. Details of analytical analysis are provided in supplemental data. Statistical analysis (analysis of variance) of measured parameters was performed to determine significance of the compound’s effect.

**Intracerebroventricular Studies in Rats.** Adult male Long-Evans rats (Harlan, Indianapolis, IN) were individually housed in Plexiglas cages with sterile bedding and provided ad libitum access to pelleted chow (Purina, St. Louis, MO) and tap water. Rats were maintained on a 12/12-h light/dark cycle in a temperature-controlled, Association for Assessment and Accreditation of Laboratory Animal Care-accredited vivarium. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

All procedures were performed according to established protocols (Clegg et al., 2003). Surgeries were performed at least 7 days after rats arrived in the facility. Rats were anesthetized with 1 ml/kg i.p. injections of a mixture of 70 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 2 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA). Rats were placed in a stereotaxic instrument with the skull held horizontally, the sagittal sinus was displaced laterally, and a 21-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) was lowered directly on the midline, 2.2 mm posterior to bregma and 7.5 mm ventral to the dura, and fixed to the skull with anchor screws and dental acrylic. Obturators that extended 0.5 mm beyond the cannula tips were inserted. Once the rats regained their preoperative body weights following surgery, placement of the third ventricle cannulas was confirmed by injection of 10 ng of angiotensin II in 2 μl of normal saline in water-replete rats. Animals that did not drink at least 5 ml of water within 60 min were not used.

**Conditioned Taste Aversion Protocol.** The novel tastant was a highly palatable saccharin solution (0.15%). On day 1, each rat was weighed and assigned into one of four weight-matched groups, saline (0.9%), lithium chloride (10 mg/kg), vehicle (50 mM citrate, pH 3.5, 2% Tween 80), or MCH antagonist (10 mg/kg). Each rat was then water
deprived for 23 h beginning at 4:00 PM (2 h before lights off). On day 2, bottles of the saccharin solution were weighed and put on each animal's home cage at 3:00 PM (3 h before lights off). After 2 h, saccharin bottles were removed and weighed, and each animal was immediately given saline, LiCl, vehicle, or MCH antagonist, and given back a water bottle. On day 3, each water bottle was removed at 5:00 PM (1 h before lights off) and remained off for 23 h of deprivation. On day 4, a bottle of saccharin and a bottle of water were weighed and put on each cage at 4:00 PM (2 h before lights off). Fluid intakes were taken on both bottles at 6:00 PM, 8:00 PM, and 12:00 PM (day 5).

### Results

**MCH Antagonist Shows High Affinity toward the MCH1R.** MCH antagonist DABA-821 is a small molecule with MW of 505 (in an HCl salt form) and high affinity for the receptor (Table 1). DABA-821 bound to the MCH1R with a Ki of 39.3 nM and was a functional MCH antagonist with an IC50 of 14.0 nM (Table 1). This compound also showed greater than 25-fold selectivity for the MCH1R compared with a structurally related receptor, the serotonin receptor (Table 1). DABA-821 is a representative compound of a class of MCH1R antagonists that cause significant body weight and fat mass reduction in rodents.

**MCH Antagonist Reduces Body Weight and Fat Mass in Mouse Diet-Induced Obesity Model.** Chronic dosing of DABA-821 led to a dose-dependent reduction in body weight and fat mass. The antagonist was dosed over a 10-day period twice daily at 1, 3, 10, and 30 mg/kg (Fig. 1). DABA-821-treated mice showed pronounced weight loss even at a dose of 3 mg/kg compared with vehicle (Fig. 1A). There was also a reduction in weight from days 0 to 10 in the vehicle-treated mice, probably a result of minor stress due to the handling and dosing. During the 10 days of dosing, the 30 mg/kg dosed mice reduced their weight by 7.0 ± 0.5 g of weight or greater than 18% of their starting body weight. The weight loss was correlated with a dose-dependent decrease in cumulative food intake in the mice (Fig. 1B). At the end of the study, the 30 mg/kg group showed a 37.2 ± 3.9% reduction in cumulative food intake compared with the vehicle-treated mice. QMR (EchoMRI) was used to determine that the weight loss was due to fat mass decrease and was dose-dependent as well, with a maximum reduction in the 30 mg/kg group (Fig. 1C). The cumulative fat mass reduction was 0.9 ± 0.3 g in the vehicle-treated mice compared with a 5.9 ± 0.3 g reduction in the 30 mg/kg treated mice. In fact, even the 1 mg/kg treated mice showed a reduction of fat, 1.7 ± 0.3 g. There was no apparent reduction in lean mass in any group compared with the vehicle dosed mice (Fig. 1D). A full necropsy at the end of the dosing period revealed that the reduction in body weight was due solely to adipose tissue mass reduction and not due to reduction in other tissues such as skeletal muscle (data not shown).

**Conditioned Taste Aversion Analysis of DABA-821.** We next tested whether administration of the compound would condition an aversion to a novel taste, conditioned taste aversion (CTA). As depicted in Fig. 2, animals treated with saline and vehicle consumed significantly more saccharin than water during the 2-h test. Figure 2 depicts these data as a “preference ratio” (saccharin intake/saccharine intake).

### Table 1

<table>
<thead>
<tr>
<th>Molecular Mass</th>
<th>MCH1R IC50 (nM)</th>
<th>MCH1R Ki (nM)</th>
<th>Serotonin Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DABA-821</strong></td>
<td>505</td>
<td>14.0 ± 12.4</td>
<td>39.3 ± 15.0</td>
</tr>
<tr>
<td><strong>Carboxy-DABA-822</strong></td>
<td>511</td>
<td>35.6 ± 35.9</td>
<td>58.5 ± 22.6</td>
</tr>
</tbody>
</table>

![Fig. 1](https://jpet.aspetjournals.org/doi/fig/10.1124/jpet.117.209655)
water intake). Values above 50% indicate preference and values below 50% indicate aversion. This test revealed that animals treated with either the positive control (LiCl) or DABA-821 consumed significantly less saccharin than water during the observation period. However, the ratio for DABA-821 was significantly higher than for LiCl-treated rats, indicating a significantly weaker CTA. These data indicate that the compound indeed shows a modest CTA effect, although with repeated dosing the food intake effect decreases, yet weight loss continues, and reduced weight is maintained throughout the dosing period (Fig. 1B). The MCH1R antagonist food intake inhibition is greater at the beginning of the 10-day dosing than it is by the end of the dosing. The difference in daily food intake between vehicle and antagonist treated mice was greater at days 1 to 3 compared with days 6 to 9.

In Vitro Analysis of MCH Antagonist Carboxy-DABA-821, an Amphipathic Analog of DABA-821. Having established that this antagonist was an orally potent and efficacious compound in reducing food intake and body weight, we wanted to determine the compounds mode and site of action. It is a widely held assumption that the site of MCH and antagonist action on food intake and body weight must be the hypothalamus, although this is based on experiments where MCH is directly injected into the hypothalamus. Using medicinal chemistry, we tested this hypothesis. Based on its physical characteristics DABA-821 and related literature, analogs are predicted to have widespread tissue distribution.

Carboxy-DABA-821 was synthesized (Scheme 1) to evaluate whether brain accessibility is a necessary part of DABA-821’s efficacy. Carboxy-DABA-821 is a structural analog of DABA-821 but has incorporated an amphipathic moiety to restrict the compound’s access to the brain (Iwasaki et al., 1995). An additional minor modification was also made that included a biaryl substitution of para-CF\(_3\) in DABA-821 versus para-F biaryls in carboxy-DABA-821. This modification is not predicted to significantly impact in vivo efficacy based on other analog studies (data not shown). Carboxy-DABA-821 showed similar affinity (\(K_i = 58.5\) nM) and antagonism (IC\(_{50} = 35.6\) nM) as DABA-821 (Table 1). The compound was not evaluated in other selectivity assays.

The ability of the compounds to traverse the blood-brain barrier was assessed using Caco-2 cells. The cells form a circumferential tight junctional cell monolayer similar to the blood-brain epithelium (Borchardt et al., 1996), thereby providing a good in vitro proxy assay for blood-brain barrier permeability (Abbott, 2004). Both antagonists were assessed experimentally in both the absorptive (i.e., apical to basolateral) and reverse direction (i.e., exsorption) to evaluate the mechanism of transport. DABA-821 showed high passive permeability in the assay (i.e., absorptive/exsorptive permeability coefficients of 9.5/8.0 \(\times 10^{-4}\) cm/min) with similar absorptive and exsorptive permeability, indicative of passive transport and suggestive of good brain penetration. Conversely, carboxy-DABA-822 showed higher exsorptive permeability relative to its absorptive permeability (i.e., 1.1/6.2 \(\times 10^{-4}\) cm/min), indicating that it was a likely substrate for the efflux transporter P-glycoprotein, which is a significant gatekeeper at the blood-brain barrier (Schinkel et al., 1996). These data suggested that carboxy-DABA-822 would have poor brain penetration. In fact, when the transport of carboxy-DABA-822 was studied in the presence of a P-glycoprotein inhibitor, the absorptive and exsorptive permeabilities normalized as expected (i.e., 1.9/2.1 \(\times 10^{-4}\) cm/min).

In Vivo Analysis of MCH Antagonists. We analyzed the in vivo activity of both MCH antagonists in diet-induced obese (DIO) mice to compare their efficacy. Although DABA-821 had been evaluated previously (Fig. 1), we reanalyzed the compound. DABA-821 was dosed orally twice daily at 20 mg/kg in DIO mice for a period of 4 days. As expected, DABA-821-treated mice showed a reduction in body weight and food intake (Fig. 3, A and B). Maximal reduction in body weight after 4 days was 2.6 ± 0.2 g. Cumulative food intake was reduced as well with a 41.2 ± 2.1% reduction compared with vehicle-treated mice. Body composition analysis using QMR showed a reduction in adipose mass (data not shown). Likewise, the efficacy of carboxy-DABA-822 was evaluated in DIO mice. The in vitro data indicated that the compound was a functional antagonist but was a substrate for P-glycoprotein in the Caco-2 assay. The compound was dosed orally twice daily at 20 mg/kg, and body weight and food intake were monitored (Fig. 3, C and D). In contrast to DABA-821, this molecule showed no in vivo activity. Neither food intake nor body weight was changed compared with the vehicle-dosed groups during the dosing period (Fig. 3, C and D).

Analysis of Blood and Brain Concentrations of the MCH1R Antagonists. The Caco-2 cell data indicated that DABA-821 compound should be brain-permeable, whereas the carboxy-DABA-822 should be brain impermeable. Indeed, this was confirmed by measuring the brain and serum concentration in oral and s.c. dosed DIO mice. Because the brain levels of dosed compound is ultimately driven by systemic serum levels dosing of compound can be achieved via either oral or s.c. route. After 4 days of dosing, mice were dosed a last time with compound and were euthanized 2 h later, followed by collection of blood and brain for analysis. DABA-821 was orally available with serum concentrations nearing 500 nM (243 ng/ml) after oral administration at 20 mg/kg (Table 2). Our studies evaluating the brain concentrations of DABA-821 were performed in s.c. dosed mice. We had independently determined that oral or s.c. dosing led to significant inhibition of food intake and reduction in body weight, although we observed that s.c. dosing led to higher...
serum levels of the compound (Table 2). It is noteworthy that as the compound concentration increased, so did the efficacy (data not shown). At 7 mg/kg s.c., the serum concentration of DABA-821 was approximately 1200 nM (612 ng/ml). Based on the favorable Caco-2 data, the brain concentrations of the compound dosed at 7 mg/kg s.c. were determined to be 2600 nM (1310 ng/ml). The 20 mg/kg s.c. dosed mice achieved even greater serum and brain concentrations (Table 2) and show dose proportionality. It is noteworthy that regardless of route of administration, the serum and brain concentrations correlated with efficacy of the DABA-821 compound.

We next evaluated the serum and brain concentrations of the putative brain-impermeable, carboxy-DABA-822. This compound showed no efficacy yet achieved high serum concentrations, greater than 1500 nM (851 ng/ml), following multiple twice daily oral dosing. Despite the high serum concentrations, the compound showed very limited brain penetration, with concentrations only reaching ca. 20 nM (Table 2). The limited brain presence probably is a reflection of the blood/serum in the brain samples. The brain concentration of this compound was well below the concentration needed to inhibit the receptor (58.5 nM, Table 1). The result confirms the CaCO-2 data. Overall, these results suggested that good brain penetration was important for efficacy.

**In Vivo Activity of MCH Antagonists Directly Administered in the Brain.** Although the data above indicated that MCH antagonists needed to penetrate the brain to lead to a reduction in food intake and body weight, we confirmed the functional activity of these antagonists further by direct injection into the hypothalamus of rats. To gain accessibility to the hypothalamus, rats were implanted with third ventricle cannulas, and compounds were administered with the appropriate vehicle. Rats were injected with MCH, DABA-821, or both MCH and DABA-821, and food intake was monitored for 1 or 2 h (Fig. 4A). MCH stimulated food intake during the observation period, whereas the DABA-821 alone showed no effect compared with vehicle-treated rats. Pretreatment of rats with DABA-821 followed by MCH administration led to a significant inhibition of MCH-stimulated food intake (Fig. 4A). This result supports the hypothesis that this compound exerts its activity via inhibition of the MCH1R in the hypothalamus (Fig. 4A).

Carboxy-DABA-822 in vivo activity was evaluated via third ventricle i.c.v. administration as well. The in vitro binding and function data suggested that this compound would be active if it were accessible to the brain MCH1 receptors, so we directly assessed this question. As above, rats were i.c.v. injected with MCH, carboxy-DABA-822, or both MCH and carboxy-DABA-822, and then food intake was monitored for 1 or 2 h (Fig. 4B). MCH showed potent stimulation of food intake, whereas the antagonist showed little direct effect on food intake. When the compound was injected along with MCH, it was able to significantly inhibit MCH-induced food intake (Fig. 4B). In fact, i.c.v. injected carboxy-DABA-822 can lead to a reduction in body weight over a 24-h period (data not shown). Therefore, the compound is active in vivo when directly administered in the hypothalamus, the apparent site of action of the MCH-1R.

**Discussion**

Over the past few years, antagonism of the MCH1R has been a popular target for pharmacological treatment of obesity (Vasudevan et al., 2004; Kym et al., 2005; Morens et al., 2005; Palani et al., 2005a,b; Kim et al., 2006; Warshakoon et al., 2006). These reports describe various chemical scaffolds with both in vitro and in vivo activity, without definition of where the critical site of action is located. Very simply, these previous studies demonstrated that peripheral administration of small-molecule MCH antagonists leads to a reduction in food intake and body weight. Here, we present data that indicate a critical feature of the drugs appears to be that they cross the blood-brain barrier, demonstrating that brain MCH1R are critical for body weight regulation.
Our data show that antagonism of the MCH1R leads to a significant reduction in body weight and fat mass (Fig. 1). These effects appear to be only partially accounted for by reductions in food intake because the weight loss and maintenance of weight loss in the treated mice continues despite a reduced effect on food intake (Fig. 1). Using pharmacological reagents, we demonstrated for the first time that the site of action of small-molecule MCH antagonists is the brain (Fig. 3). In confirmation of these findings, we synthesized a brain-impermeable MCH antagonist and showed that this compound is not efficacious with respect to reductions in food intake and body weight even though it was peripherally available (Fig. 3). When this antagonist is injected directly into the ventricular system, it causes a reduction in food intake (Fig. 4B) and body weight (data not shown). Finally our data suggest that brain MCH1Rs, but not peripheral MCH1Rs, are the critical site of action with respect to body weight regulation.

The small-molecule MCH antagonist, DABA-821, reduces food intake and body weight. Similar to other compounds in the literature, this MCH antagonist reduces body weight and food intake in a dose-dependent manner (Borowsky et al., 2002; Kowalski et al., 2004; Mashiko et al., 2005). We found that it inhibits MCH-induced food intake when directly injected into the third ventricle with ready access to the hypothalamus (Fig. 4A). We determined that the compound, dosed orally, crossed the blood-brain barrier and that the concentration of the compound in the brain was greater than in the serum. The mechanism underlying the partitioning in the brain is unclear but may be indicative of some type of brain tissue binding of the molecule.

The MCH antagonist DABA-821 induces a modest CTA effect that may account for the reduction in food intake. However, caution must be taken with respect to the interpretation of CTA results. The fact that a substance supports CTA learning is not, in itself, proof of visceral illness nor does it support conclusions about potential drug regime compliance in humans (Benoit et al., 2003a,b). For example, all drugs of abuse, including alcohol, morphine, amphetamine, and cocaine, will cause robust CTAs, even though they are readily self-administered by rats (Benoit et al., 2003a,b). Furthermore, one of the most potent hypothalamic orexigenic neuropeptides, neuropeptide Y, also produces a robust CTA response (Woods et al., 1998; Madden et al., 1999). There are several potential interpretations of these data. However, the important point for our work is that the finding of a significant CTA does not in itself speak to the potential utility for this compound as an effective antiobesity agent.

To explore the site of action, we synthesized a structural analog of the active MCH antagonist, DABA-822. This molecule was synthesized by placing an amphipathic moiety on the compound, a modification predicted to reduce brain penetration. Indeed, this approach has been used previously to restrict compound access to the brain (Iwasaki et al., 1995). Our data indicate that adding the carboxylic acid moiety to the compound significantly reduces brain accessibility through P-glycoprotein efflux. It is noteworthy that the changes we made to the compound did not affect in vitro binding or function at the MCH1R. Finally, the compound was in vivo active when delivered directly into the hypothalamus. These data indicated that the MCH1R target for small-molecule MCH antagonists is in the brain.

The medicinal chemistry approach that we took supports a role for the brain MCH1R in the action of MCH antagonists. Although it has been speculated that these hypothalamic receptors are the site of action for antagonists in weight loss, there is no experimental evidence to support this hypothesis. The original studies of Borowsky et al. (2002) showed that peripheral administration of an MCH1R antagonist could inhibit the activity of MCH injected directly into the brain. The MCH antagonist was found to bind to sites in the brain and hypothalamus, apparently coincident with the sites of MCH receptor binding sites. Yet, there is no in vitro or in vivo evidence to support the compound penetrates the brain. Further evidence in support of the hypothesis relies on direct injection of MCH or MCH antagonists into the hypothalamus followed by evaluation of food intake and body weight (Bednarek et al., 2002; Hervieu, 2003; Kennedy et al., 2003; Shearman et al., 2003). Although informative, these data only indicate that the hypothalamus is one site of action for MCH and MCH antagonists, leaving open the role of the peripheral MCH1Rs. Our data help to more definitively delineate the site of action for the MCH1R antagonists.

These data support a role for MCH in the regulation of body...
weight. Our studies demonstrate the central nervous system receptors are important in the regulation of body weight. Furthermore, no in vivo role for the peripheral receptors was demonstrated. Peripheral MCH1 receptors have been shown to stimulate the secretion of leptin from isolated adipocytes and activate adipocyte signaling pathways (Bradley et al., 2000). Because the MCH1R in adipocytes stimulates the secretion of leptin, an MCH antagonist would be predicted to inhibit or suppress leptin secretion. As a consequence of suppressed leptin secretion, food intake would increase. Interestingly, DIO mice treated with carboxy-DABA-822 (restricted to the periphery) showed a small but not significant increase in food intake compared with vehicle-treated mice (Fig. 3). The significance of ery) showed a small but not significant increase in food intake mice treated with carboxy-DABA-822 (restricted to the periph-
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to delineate this function.

Our findings support the concept that the MCH1R is a good target for obesity therapy. Blockade of the receptor in mouse and rat obesity models lead to significant weight loss. Our studies clearly indicate that brain access is critical for efficacy of small-molecule MCH1R antagonists. The need to get these small-molecule antagonists into the brain may be one of the reasons that MCH antagonists have yet to reach clinical studies. Given the importance of finding efficacious pharmacological therapeutics for obesity, it is likely that MCH antagonists will one day be part of the clinical cadre of therapies.

Acknowledgments

We thank Karen Hodge, Cindy Obringer, and Mary Kay Dirr for excellent technical support.

References