Regulation of Ingestive Behaviors in the Rat by GSK1521498, a Novel \( \mu \)-Opioid Receptor-Selective Inverse Agonist

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Received February 24, 2011; accepted June 27, 2011

**ABSTRACT**

\( \mu \)-Opioid receptor (MOR) agonism induces palatable food consumption principally through modulation of the rewarding properties of food. \( N-(3,5\)-difluoro-3-\( \{1H,1,2,4\)-triazol-3-yl\}-4-biphenyl)methyl\)-2,3-dihydro-1\( H \)-inden-2-amine (GSK1521498) is a novel opioid receptor inverse agonist that, on the basis of in vitro affinity assays, is greater than 10- or 50-fold selective for human or rat MOR, respectively, compared with \( \kappa \)-opioid receptors (KOR) and \( \delta \)-opioid receptors (DOR). Likewise, preferential MOR occupancy versus KOR and DOR was observed by autoradiography in brain slices from Long Evans rats dosed orally with the drug. GSK1521498 suppressed nocturnal food consumption of standard or palatable chow in lean and diet-induced obese (DIO) Long Evans rats. Both the dose-response relationship and time course of efficacy in lean rats fed palatable chow correlated with \( \mu \) receptor occupancy and the plasma concentration profile of the drug. Chronic oral administration of GSK1521498 induced body weight loss in DIO rats, which comprised fat mass reduction. The reduction in body weight was equivalent to the cumulative reduction in food consumption; thus, the effect of GSK1521498 on body weight is related to inhibition of food consumption. GSK1521498 suppressed the preference for sucrose-containing solutions in lean rats. In operant response models also using lean rats, GSK1521498 reduced the reinforcement efficacy of palatable food reward and enhanced satiety. In conclusion, GSK1521498 is a potent, MOR-selective inverse agonist that modulates the hedonic aspects of ingestion and, therefore, could represent a pharmacological treatment for obesity and binge-eating disorders.

**Introduction**

Obesity is a worldwide epidemic for which there are few pharmacologic treatment options. In the United States alone, two-thirds of the adult population is classified as overweight or obese. The etiology of the relatively recent surge in incidence is thought to be the availability of relatively inexpen-

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**ABBREVIATIONS:** MOR, \( \mu \)-opioid receptor; KOR, \( \kappa \)-opioid receptor; DOR, \( \delta \)-opioid receptor; DAMGO, \([\beta\text{-Ala}^2,\text{N-Me-Phe}^4,\text{Gly-ol}]\)-enkephalin; LY255582, \([3R,4R]-3,4\)-dimethyl-1-\([3S]-3\)-hydroxy-3-cyclohexyl-propyl\)-4-(3-hydroxyphenyl)piperidine; GSK1521498, \([N-(3,5\)-difluoro-3-\( \{1H,1,2,4\)-triazol-3-yl\}-4-biphenyl)methyl\)-2,3-dihydro-1\( H \)-inden-2-amine; CI-977, \([5R-(5a,7a,8b)]\)-N-methyl-N-[7-(1-pyrroldinyl)1-oxaspiro[4,5]dec-8-yl]-4-benzofuranacetamide; GSK1631668, \( N-(4-(1H-imidazol-4-yl)pyridin-5-yl)benzyl\)-2,3-dihydro-1\( H \)-inden-2-amine; GTP\( \gamma \)S, guanosine 5'-O-(3-thio)triphosphate; CHO, Chinese hamster ovary; DIO, diet-induced obese; FR\( x \), fixed-ratio schedule where each lever press is \( x \) rewards; ANOVA, analysis of variance.
reward (Mansour et al., 1995). Whereas all three receptors have been implicated in regulation of food intake, the MOR is thought to play the predominant role, because MOR knockout mice display reduced motivation to eat (Papaelo et al., 2007) and are resistant to diet-induced obesity (Tabarin et al., 2005). In addition, elevated MOR levels are observed in diet-induced obese Wistar rats (Smith et al., 2002) and in Osborne-Mendel rats, which are susceptible to diet-induced obesity (Barnes et al., 2006). Although MOR is present in areas of the brain that regulate consummatory behavior, such as the hypothalamus and nucleus tractus solitary, the expression is more prevalent in the limbic system (nucleus accumbens shell and ventral pallidum) (Smith and Berridge, 2007), implicating MOR in the regulation of hedonic aspects of food consumption. Morphine administered into the nucleus accumbens amplifies positive affective reactions to sucrose taste (Pecina and Berridge, 2000). Furthermore, intra-accumbens administration of the MOR-selective agonist ([d-Ala²,N-Methylglycine]-enkephalin (DAMGO) induces hyperphagia and enhances preference for high-fat and high-sugar content (Mucha and Iversen, 1986; Zhang and Kelley, 1997; Zhang et al., 1998; Will et al., 2003). MOR agonism in the nucleus accumbens communicates with other centers important to motivation and metabolic and autonomic regulation of food consumption as evidenced by the activation of hypothalamic, midbrain, and brainstem regions after DAMGO infusion into the ventrolateral striatum (Zhang and Kelley, 2000). In one study, MOR polymorphisms have been associated with obesity in humans (Xu et al., 2009).

It is now generally accepted that G protein-coupled receptor antagonists are either neutral antagonists, which only block agonist-induced effects, or inverse agonists, which reduce spontaneous constitutive activity as well as agonist effects (Kenakin, 1995). Clinical experience with neutral opioid receptor antagonists, such as naltrexone, demonstrates that although suppression of short-term food intake is observed (Drewnowski, 1997), a significant impact on body weight is not (Atkinson et al., 1985; Malcolm et al., 1985). It has been suggested that opioid receptor inverse agonists may be more effective than neutral antagonists in the treatment of obesity. Indeed, the opioid receptor inverse agonist, (3R,4R)-3,4-dimethyl-1-[(3S)-3-hydroxy-3-cyclohexyl-propyl]-4-(3-hydroxyphenyl)piperidine (LY255581), inhibited weight gain in obese Zucker rats over 30 days, whereas the effect of naltrexone was only observed for 7 days (Shaw, 1993). It should be noted that opioid receptor antagonists/inverse agonists appear to modulate the incentive value of palatable food and satiety, and, therefore, these agents may behave differently than anorectic agents such as phentermine.

N-[(3,5-difluoro-3’-(1H-1,2,4-triazol-3-yl)-4-biphenyl) methyl]-2,3-dihydro-1H-inden-2-amine (GSK1521498; GlaxoSmithKline, Research Triangle Park, NC), is currently in clinical development for treatment of obesity and disorders characterized by compulsive eating. This report describes the preclinical characterization of opioid receptor pharmacology and selectivity profile and the pharmacokinetic properties of GSK1521498. The regulation of ingestive behaviors by GSK1521498 is explored in several paradigms as well as the translation of effects on consumption to reduction of body weight and fat mass.

**Materials and Methods**

**Drugs and Materials.** In vitro and in vivo studies were conducted with the dihydrochloride salt of GSK1521498, which was synthesized in the GlaxoSmithKline Department of Medicinal Chemistry (Research Triangle Park, NC) (Cowan, 2010). GSK1521498 was prepared in 0.5% hydroxypropyl methylcellulose-0.1% Tween 80, which served as the vehicle for oral administration for all studies. Vehicle and drug were administered orally in a volume of 2 mL/kg b.wt. Dose calculations were based on the dihydrochloride salt for all pharmacology studies.

DAMGO, [tyrosyl-3,5,6-H(N)], and deltorphin II (2-d-Ala²)-[tyrosyl-3,5,6-H] were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [³⁵S][5F]-[5α,7α,8β]-N-methyl-N-[7-(1-pyrrolidinyl)1-oxaspiro[4,5]yl]-4-benzofuranacetamide (CI-977) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK), and naloxone-HCl was from Enzo Life Sciences, Inc. (Farmindale, NY). Naltrexone HCl was from Sigma-Aldrich (St. Louis, MO).

Operant response chambers (MED Associates, St. Albans, VT) were custom built to contain the following: two retractable arm levers, two food reward pellet dispensers, one house light, one tone cue, one light cue for each lever, one head entry detector, and one pellet detector. All programs were written to provide both the tone and light cues for 1 s concurrent with the delivery of the food reward. The 45-mg palatable food rewards were custom made (Bio-Serv, Frenchtown, NJ) and contained the following ingredients: 341 g/kg dextrate, 36 g/kg m-dextrin, 200 g/kg casein, 3 g/kg cystine, 10 g/kg vitamin mix, 2 g/kg choline bit, 35 g/kg salt mix, 100 g/kg sucrose, 50 g/kg fiber, 215 g/kg hydrogenated vegetable oil, and 20 g/kg soybean oil.

**Opioid Antagonist GTP-γ-S Assay.** The assay was designed to measure the GDP-GTP exchange that occurs on the trimeric G protein using [³⁵S]GTP-γ-S, a poorly hydrolyzed, labeled analog of GTP with high affinity for the G protein. Addition of an agonist promotes increased G protein exchange at the G protein such that more [³⁵S]GTP-γ-S is bound to the G protein. For quantification of antagonistic activity, the addition of a 4 × EC₅₀ final concentration of agonist, DAMGO, dynorphin A, or Met-enkephalin for MOR, KOR, and DOR, respectively, was added. A separate plate with six curves of the standard agonist compound was run in the agonist mode in parallel with the antagonist assay. The EC₅₀ value and slope of agonist were used to calculate the negative log of the functional Kᵢ (pKᵢ).

Reactions were performed in white 384-well polystyrene low-volume plates in a final volume of 10 μL. Membranes expressing receptors and Wheat Germ Agglutinin LEADseeker bead solutions in assay buffer (20 mM HEPES, 10 mM MgCl₂, and 100 mM NaCl, pH 7.4, with KOH) were combined and shaken at room temperature for 30 min. GDP (10 μM final) was added to the mixture and then GTP-γ-S solution containing 4× EC₅₀ final agonist concentration. To quantify compound potencies, the mixture was added (10 μL) using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) to the compound plates containing 100 nL of test compound dissolved in 100% Me₂SO. Compounds under analysis were dissolved in Me₂SO to 3.0 mM and serially diluted 1:4 with Me₂SO through 11 dilutions. Then, 0.1 μL of each concentration was transferred to the corresponding well of an assay plate. This created a final compound concentration range from 0.000007 to 30 μM. The plates were sealed and centrifuged 500g for 2 min and incubated at 20°C for 5 h, and GTP-γ-S binding was then imaged using a ViewLux 1430 ultraHTS Microplate Imager (PerkinElmer Life and Analytical Sciences).

For assessment of affinity, opioid receptors were expressed at levels that did not result in measurable constitutive activity. Human MOR, KOR, and DOR were stably expressed in Chinese hamster ovary (CHO-K₁) cells (American Type Culture Collection, Manassas, MD) and rat MOR, KOR, and DOR were expressed in CHO-Gam E1A cells using the BacMam virus expression system described previously (Condraey et al., 1999). CHO-Gam E1A cells have improved BacMam transduction capabilities and were derived from CHO-K₁.
cells at GlaxoSmithKline. For assessment of inverse agonism, CHO-GamE1A cell membranes transfused using the BacMam system to overexpress human or rat MOR, KOR, and DOR were used. Altering the stoichiometry of receptor and G protein by enhancing receptor expression increases the number of active state receptors in the system, allowing for the assessment of constitutive activity (Chen et al., 1999).

Data analysis was performed with the Abase software package (version 5.4; IDBS, Surrey, UK). Raw counts from the ViewLux CCD imager were normalized and expressed as percentage inhibition using the formula: % response = 1 - ((U - C1)/(C2 - C1)) × 100, where U is the unknown value, C1 is the average of the low-signal (0%) control wells, and C2 is the average of the high-signal (100%) control wells. Curve-fitting was performed using the equation: 

\[ Y = A - \left(\frac{x - 1}{(x - 10^6)^2}\right) \]

where A is minimum response, B is maximum response, C is \(\log_{10} C_{50}\), D is slope factor, and x is \(\log_{10}\) compound concentration. The results were recorded as pIC\(_{50}\) values (−C in the above equation) and converted to fp\(_{K_i}\) by the equation:

\[ fp_{K_i} = -\log_{10}(IC_{50}^{(2 + (\frac{[E]/EC_{50}}{\log_{10} n})^{1/n}})) \]

where [E] is the concentration of agonist used for the antagonist assay (= 4 \times EC\(_{50}\) final assay concentration, where the EC\(_{50}\) was determined from the historical agonist curve data), EC\(_{50}\) is determined on assay day using a separate agonist plate containing >6 agonist curves, and n is the slope of the agonist curve.

**Animals.** All studies were conducted after review by the GlaxoSmithKline Institutional Animal Care and Use Committee and in accordance with the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Laboratory Animals, the Animal Welfare Act (U.S. Department of Agriculture), and the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Lean and diet-induced obese (DIO) Long Evans rats were purchased from Harlan (Indianapolis, IN). Male rats were used unless otherwise noted. Rats were housed at 72°F and 50% relative humidity with a 12-h light/dark cycle. For all studies except nocturnal food consumption, the rats were habituated in cages at GlaxoSmithKline facilities for at least 1 week before experimentation. Rats were given ad libitum access to water and either standard laboratory chow (Lab Diet 5001; PMI Nutrition International, Brentwood, MO) for lean rats or high-fat chow (TD95217; Harlan Laboratories, Madison, WI) for DIO rats. For nocturnal food consumption studies, rats were housed individually in chambers of a computerized feeding system from Accuscan Instruments, Inc. (Columbus, OH). The rats were given ad libitum access to water and either powdered Lab Diet 5001 or D12266B, a purified diet intended to mimic sweetened condensed milk (Research Diets, New Brunswick, NJ). DIO rats were provided with TD95217 at the Harlan facilities starting at 4 weeks of age and were maintained on that chow for 8 weeks. The rats were then transferred to GlaxoSmithKline where they were maintained on TD95217 until their average weight was approximately 500 g, and the body weight gain rate was slowing.

**Plasma Pharmacokinetic Analysis.** For intravenous administration, the dose formulation was administered to each animal via a femoral vein cannula (1 ml/kg of 2.6 mg/ml GSK1521498). After dose administration, the cannula was flushed with approximately 0.5 ml of heparinized saline. Blood samples (approximately 0.3 ml each) were collected from each animal at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h postdose. For oral administration, the dose formulation was administered via oral gavage (2 ml/kg of 0.5 mg/ml GSK1521498). Blood samples (approximately 0.3 ml each) were collected from each animal from a jugular vein cannula using a syringe at 0.25, 0.5, 1, 2, 4, 8, and 24 h postdose and transferred into labeled collection tubes containing tripotassium EDTA and chilled at 4°C. Plasma was harvested by centrifugation (4000g for 20 min at 4°C) within 10 min of blood collection and stored at −20°C until analysis.

For the brain distribution study, 30 mg/kg was administered via oral gavage (5 ml/kg of 6 mg/ml GSK1521498). Blood (approximately 0.3 ml each) and brain samples were collected from each animal at 1.5 and 8 h postdose. Plasma was harvested as described above, and brain samples were stored at −20°C until analysis.

To analyze for levels of GSK1521498, rat plasma samples were thawed at room temperature and vortex-mixed. Brain samples were homogenized in 5 volumes of water. Aliquots (10 μl of plasma or 20 μl of brain homogenate) of the study samples were extracted by protein precipitation with acetonitrile (200 μl containing an internal standard [100 ng/ml GSK1631668 (N-(4-(1H-imidazo[4,5-b]pyridin-5-yl)benzyl)-2,3-dihydro-1H-inden-2-amine)]) in 96-well plates. After the samples were vortexed for 40 s and centrifuged at 2304g at 4°C for 15 min, the acetonitrile supernatants (30 μl) were transferred to a clean 96-well plate containing 310 μl of water. The samples were then vortexed for 1 min, placed into a chilled autoinjector, and injected (10 μl) onto a 50 × 2 mm, 3-μm, Varian Polaris C18-Ether high-performance liquid chromatography column (Varian, Inc., Lake Forest, CA) using a gradient method. After an initial 0.5-min hold, the gradient increased linearly from 60% 5.2 mM ammonium acetate (pH 6) with 2% acetonitrile (A) and 40% methanol (B) to 95% B over 1 min and then was held at 95% B for 1.5 min. The analytical column was then reequilibrated at 40% B over the final minute. The flow rate was 0.6 ml/min. The flow was diverted to waste for the first 0.5 min, to the mass spectrometer for the next 3 min, and back to waste for the remaining run time. The total run time was 4 min. Analytes were detected using positive TurboLionSpray. GSK1521498 and the internal standard GSK1631668 were detected by multiple reaction monitoring of the transitions m/z 403.2 to m/z 289.9 and m/z 314.2 to m/z 208.0, respectively. GSK1521498 and GSK1631668 eluted at retention times of 2.0 and 1.7 min, respectively.

Triplicate plasma calibration curves for GSK1521498 ranged from 1 to 5000 ng/ml at 12 concentration levels, and 36 of 36 standards were within acceptable accuracy limits of ±20% of the nominal concentration. The correlation coefficient was 0.9973 using linear regression with 1/2 weighting. Plasma quality control samples were analyzed as 3 samples at each of 4 concentrations (10, 400, 1000, and 4000 ng/ml plasma) and 12 of 12 were within acceptable accuracy limits of ±20% of the nominal concentration.

The brain homogenate calibration curve for GSK1521498 ranged from 4 to 10,000 ng/g at 11 concentration levels, and 11 of 11 standards were within acceptable accuracy limits of ±20% of the nominal concentration. The correlation coefficient was 0.9953 using quadratic regression with 1/2 weighting.

The liquid chromatography-tandem mass spectroscopy system consisted of a CTC HTS PAL autoinjector (LEAP Technologies, Carrboro, NC), an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA), a switching valve (Valco Instrument Co. Inc., Houston, TX), and an API4000 triple quadrupole mass spectrometer (Applied Biosystems/MDX Scie, Concord, ON, Canada). Data were acquired, analyzed, and quantified using Analyst software version 1.4.1 (Applied Biosystems/MDS Sciex).

Noncompartmental pharmacokinetic parameters (terminal plasma half-life (t1/2), maximum plasma concentration (C\(_{\text{max}}\)), time of maximum plasma concentration (\(T_{\text{max}}\)), plasma clearance (CL), steady-state volume of distribution (\(V_{\text{ss}}\)), area under the plasma concentration-time curve extrapolated to infinite time (AUC\(_{\text{inf}}\)), and area under the plasma concentration-time curve for 24 h (AUC\(_{\text{24h}}\)) were calculated on the basis of the individual plasma concentration-time data using WinNonlin Professional 4.1 (Pharsight, Mountain View, CA). Oral bioavailability (F) was calculated using the following equation: F (%) = ([AUC\(_{\text{inf,p.o.}}\)/p.o. dose]/[AUC\(_{\text{inf,i.v.}}\)/i.v. dose]) × 100, where AUC\(_{\text{inf,p.o.}}\) was the individual AUC\(_{\text{inf}}\) after oral administration and AUC\(_{\text{inf,i.v.}}\) was the mean AUC\(_{\text{inf}}\) after intravenous administration.

**Nocturnal Food Consumption Protocol.** Rats were acclimated to the Accuscan apparatus for between 4 and 11 days and to oral gavage with vehicle for 3 days before the study. Experiments were limited to four groups of eight rats because the Accuscan apparatus contained 32 cages. The average food consumption over the 2 days before the study was used as baseline food consumption for each rat. Oral administration of vehicle or GSK1521498 was completed ap-

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proximately 1 to 2 h before the commencement of the dark cycle. Food hopper weight was measured every hour for 24 h by the computerized system. Manual weights of food in hoppers at the start of the study and end of study were taken. Rats were, on occasion, removed from the study either because of individual apparatus failure or because the difference between the manual and computer measures was greater than 3 g. Food spillage was monitored daily, and adjustments were made accordingly. Lean rats weighed approximately 320 g, whereas DIO rats weighed approximately 530 g at the time of the study.

Receptor Occupancy in Frozen Brain Slices. Rats were weighed, dosed orally with GSK1521498 (0.3–30 mg/kg) or vehicle at 8:00 to 9:00 AM, and tissues was collected 1.5, 8, or 24 h after administration of compound. To harvest brains, the rats were asphyxiated with CO2 and decapitated; skulls were opened, and brains were removed, immersed in precooled isopentane for 5 to 8 s, and stored at –80°C. MOR, KOR, and DOR occupancy was measured in frozen brain sections by ex vivo autoradiography (Foistel et al., 2006). Coronal sections (20 μm) were taken through the nucleus accumbens, dorsal endopiriform nucleus, or caudate/putamen for μ, κ, or δ receptor autoradiography, respectively, using a cryostat (Leica Microsystems, Inc., Bannockburn, IL). The sections were thaw-mounted onto Superfrost Plus slides and stored at –80°C until use.

For autoradiography, slides were thawed to room temperature under a gentle stream of cool air. A barrier was formed between sections designated for total and nonspecific binding by applying a line of DPX Mountant and allowing it to fully dry. The sections were covered with 0.2 to 0.3 ml of 50 mM Tris-HCl and 5 mM MgCl2 · 6H2O, pH 7.4, protease inhibitors, and 0.2% bovine serum albumin (assay buffer) containing either 5 nM [3H]DAMGO (MOR-selective agonist), 1.8 nM [3H]IC-977 (KOR-selective agonist), or 12 nM [3H]Deltorphin II (DOR-selective agonist) with or without 10 μM naloxone and incubated at room temperature for 10 (μ and δ) or 20 (κ) min in the dark. Excess cold naloxone was included to assess nonspecific binding. For assessment of ligand specificity, 100 nM DAMGO, norbinaltorphimine HCl, or [δ-Pen2,δ-Pen5]-enkephalin was used to assess nonspecific binding for MOR, KOR, or DOR, respectively. The slides were washed in ice-cold assay buffer (no ligands) for 30 s and then immersed in 50 mM Tris-HCl and 5 mM MgCl2 · 6H2O, pH 7.4 (wash buffer) for three 30-s washes. The sections were analyzed by removing all traces of DPX from the slides, allowing the slides to dry completely, covering the back of the slides with metallic electrical tape (as per the instrument manufacturer’s instructions), and removing all dust with clean, compressed air. The slides were imaged in a Beta Imager (Biospace Lab, Cambridge, MA) for 12 to 24 h. Beta Vision software was used to measure binding in the region of interest, captured as counts per minute and counts per minute per square millimeter. Specific binding was calculated by subtracting nonspecific binding from total binding of radioligand. The vehicle group represented the maximal possible occupancy for each radioligand, and the ability of GSK1521498 to displace radioligand binding in vivo was expressed as percentage DAMGO binding calculated as (specific binding for drug-treated rat/mean of specific binding for vehicle group) × 100.

Weight Loss Protocol. Three days before the study, rats were weighed, and body composition was assessed by quantitative magnetic resonance (EchoMRI-100 quantitative magnetic resonance system; Echomedical Systems, Houston, TX). Rats were randomized into groups based on percentage body fat such that average percentage body fat was not different between groups (eight rats per group). DIO rats weighed approximately 500 g, whereas lean control rats weighed approximately 440 g at the start of the study.

After randomization, rats were habituated to handling and oral gavage for 3 days and baseline food consumption was measured before the treatment regimen commenced. Rats were dosed once daily for 11 days, 1 h before the dark cycle commenced, with vehicle or GSK1521498. Body weight was measured 3 days a week on Monday, Wednesday, and Friday. Food hoppers were weighed daily to estimate daily consumption. Body composition was assessed again 18 h after the last dose, body weights were measured, and the rats were then rendered unconscious via CO2 inhalation. Blood was collected by cardiac puncture, and serum was prepared by allowing clot formation at room temperature for 20 min followed by centrifugation for 10 min at 1200g at 5°C.

Sucrose Preference. The experimental protocol was derived from methods reported in previous publications (Bolanos et al., 2003; Hommel et al., 2006). At the start of the study, each rat was given two bottles of ultrapure water and dosed with vehicle by oral gavage. Food spillage was recorded before provision to rats to assess overnight water intake. On day 2, the water bottles were weighed and then the positions of the bottles were switched before oral gavage with vehicle. Alternating the bottle positions controls for position bias. On the third day, the bottles were weighed and then emptied. The rats were then dosed with either vehicle or GSK1521498 by oral gavage and were provided with a bottle of ultrapure water and a bottle of 0.25% sucrose water. After the bottles were weighed, the ultrapure water and 0.25% sucrose-filled bottle were placed on the left and right sides of the cage, respectively. As before, the bottle weights were recorded the following day, GSK1521498 or vehicles were delivered by oral gavage, and the positions of the bottles were switched. The bottle weights were recorded again the next day and the bottles were emptied and refilled with either fresh ultrapure or 0.5% sucrose water, and the process described above was repeated. The procedure was then repeated a third and final time with 1.0% sucrose water. Bottle weight assessment and dosing occurred at approximately 2:00 PM each day. To calculate the sucrose preference score for each animal at each sucrose concentration, the volume consumed of the sucrose-water solution was divided by the total liquid intake (sucrose-water or water alone) over a 24-h period. Because each sucrose concentration was available for 2 days, the two 24-h periods were averaged together to get the final preference percentage.

Operant Response Protocol. After 1 week of habituation, food intake was measured and recorded over a 3-day period, and the average food intake per rat per day was determined. A fixed-ratio schedule of reinforcement was used, in which each lever press resulted in one reward (FR1). The palatable food reward was a 45-mg pellet containing 23.3% fat and 10% sucrose by mass. The rats were then fed a 50% food restriction diet for 3 days to promote lever acquisition. This resulted in a temporary weight loss of approximately 10% body weight, which animals regained before drug testing began. The second day of the food restriction began day 1 of the operant response lever training. During the time of food restriction and training, animals could obtain additional food by learning to press the operant response lever. After day 4 of training, 25% of the rats, those with the lowest average number of rewards received from day 1 to day 4 of FR1 training, were eliminated from the experiment. Rats were trained during all three food-restricted days but then were allowed ad libitum access to standard chow in their home cage for the remainder of the experiments. Although animals were given ad libitum access to standard chow, they still were willing to press the lever to obtain the more palatable food reward. During training, animals were placed in the operant response chambers for approximately 30 min or until 120 food rewards were obtained, whichever came first. Total training time was approximately 3 weeks, and rats were trained on FR1 and were then switched to FR3 followed by FR5 and finally to the progressive-ratio training. Fixed-ratio training consisted of five sessions of FR1, two sessions of FR3, and a minimum of five sessions of FR5 to stabilize responding before testing began. Progressive-ratio training consisted of 5 sessions of FR1, two sessions of FR3, two sessions of FR5, and a minimum of seven sessions of PR to stabilize the responding before testing began. After the animals stabilized on the progressive-ratio protocol, compound testing began. Animals were given at least 7 days between experimental blocks. For the fixed-ratio study, on the test day rats were dosed approximately 1.5 h before being placed in the chambers for a
maximum of 30 min and could work to obtain up to 120 food rewards on an FR1 schedule. The auditory and visual cues were presented concurrently with each food reward. For the progressive-ratio protocol, on the test day, rats were dosed approximately 1.5 h before being placed in chambers and allowed to press on a progressive ratio. The schedule for the first through eighth reward was 2, 4, 7, 12, 17, 25, 38, and 50 presses. If the animals failed to obtain the reward within 10 min, they were considered to have reached their “breakpoint,” which caused the levers to retract and houselight to turn off. On average, only animals remained in the cages for approximately 30 min. All doses were counterbalanced over time, such that any specific trial contained all doses of drug, including controls. In addition, each group of animals was distinct, thus eliminating the risk of carryover effects.

**Statistical Analysis.** Statistical analyses were performed using JMP statistical software packages (release 8.0.2; SAS Institute, Cary, NC). In general, data are presented as means ± S.E.M. with n indicating the number of animals per group. Each of the reported studies was planned to compare specific doses of GSK1521498 with a control group via ANOVA or analysis of covariance with post hoc t tests, and the studies were powered accordingly. In this case, retrospective application of a procedure for testing multiple comparisons suggested by the data, e.g., Bonferroni, is not necessary and would decrease the power of the studies to detect the differences for which they were planned. Thus, all studies were evaluated via post hoc t tests after ANOVA or analysis of covariance with the results reported as $p < 0.05$, $p < 0.01$, and $p < 0.001$ to show the strength of evidence for each comparison. Differences between groups were considered significant when a value of $p < 0.05$ was achieved. One-way ANOVA followed by a post hoc t test was used to analyze nocturnal food consumption (Figs. 3 and 6) and body composition (Table 4). Two-way ANOVA with dose and time factors followed by post hoc t tests was used to analyze the receptor occupancy data, expressed as percentage of vehicle response (Fig. 5). A square root transformation was applied to the DAMGO binding data before analysis to meet the assumption of ANOVA. ANOVA with repeated measures followed by post hoc t tests was used for weight loss (Fig. 7a), sucrose preference (Fig. 8), and operant response (Fig. 9) experiments. For the analysis of balance of food intake versus body weight loss (Fig. 4), the mean loss of weight minus mean reduction in food consumption for each group was tested to determine whether the value was different from 0.

**Results**

**GSK1521498.** The chemical structure of the dihydrochloride salt of GSK1521498 is presented in Fig. 1. This salt form of GSK1521498 was used in all studies described herein. The pharmacokinetic characteristics of GSK1521498 after intravenous and oral administration to Long Evans rats are presented in Table 1. The excellent oral bioavailability and access to brain tissue as well as the low volume of distribution and clearance supported once-daily oral administration and optimal targeting of a central nervous system receptor.

**Receptor Pharmacology.** The potency of GSK1521498 at human and rat MOR, KOR, and DOR was tested in a GTPyS assay using membranes prepared from CHO-K1 cells expressing human opioid receptors or CHO-Gam E1A cells, which were BacMam-transduced with rat opioid receptors (Table 2). GSK1521498 is 14- and 20-fold selective for human MOR over KOR and DOR and is 52- and 66-fold selective for rat MOR over KOR and DOR. Naltrexone was less potent and selective for MOR than KOR and DOR. Naltrexone was approximately 4- and 10-fold selective at human MOR versus KOR and DOR and 20- and 25-fold selective at rat MOR compared with KOR and DOR.

Cell membranes from CHO-Gam E1A cells in which human and rat opioid receptors were expressed at a level that resulted in constitutive activity were used to assess inverse agonism of GSK1521498. As shown in Fig. 2a and b, GSK1521498 behaved as an inverse agonist at human and rat MOR, whereas naltrexone displayed activity characteristic of a neutral antagonist or partial agonist in this system. Likewise, GSK1521498 functioned as an inverse agonist at the human and rat KOR and DOR, whereas naltrexone was a neutral antagonist at DOR and a neutral antagonist/partial agonist at KOR (data not shown).

**Correlation of Effect of GSK1521498 on Nocturnal Food Consumption with Receptor Occupancy and Plasma Exposure.** The interrelationship between GSK1521498 brain receptor occupancy and plasma concentration and the effect of GSK1521498 on food consumption are shown in Figs. 3 and 5 and Table 3. GSK1521498 dose-dependently suppressed nocturnal food consumption in lean male rats provided with

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>CL (ml·min⁻¹·kg⁻¹)</td>
<td>5.6</td>
</tr>
<tr>
<td>$V_{ee}$ (l/kg)</td>
<td>1.8</td>
</tr>
<tr>
<td>$t_{1/2}$ i.v. (h)</td>
<td>4.4</td>
</tr>
<tr>
<td>$C_{max}$ (ng/ml)</td>
<td>2000 ± 410</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>2–4</td>
</tr>
<tr>
<td>F at 10 mg/kg (%)</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>Brain/plasma ratio at 3 mg/kg</td>
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</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>GSK1521498</th>
<th>Naltrexone</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$f_{pK_1}$</td>
<td>SD</td>
</tr>
<tr>
<td>Human</td>
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<td></td>
</tr>
<tr>
<td>$\mu$</td>
<td>8.82</td>
<td>0.09</td>
</tr>
<tr>
<td>$\delta$</td>
<td>7.52</td>
<td>0.24</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>7.69</td>
<td>0.17</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\mu$</td>
<td>9.14</td>
<td>0.22</td>
</tr>
<tr>
<td>$\delta$</td>
<td>7.32</td>
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</tr>
<tr>
<td>$\kappa$</td>
<td>7.42</td>
<td>0.17</td>
</tr>
</tbody>
</table>
palatable chow (Fig. 3). In the vehicle-treated group, the rats began to eat when lights were extinguished at the 2-h time point, which was approximately 2 h after rats were dosed. Food consumption reached a plateau 12 h later when the light cycle began (14-h time point). At early time points, all three doses of GSK1521498 suppressed food consumption to the same extent; however, the effect waned in a dose-dependent manner until lights were turned on and the rats entered the sleep cycle. The efficacy of GSK1521498 was not affected by gender because results similar to those in Fig. 3 were also observed in intact or ovariectomized female rats fed palatable chow (data not shown). The suppression of food consumption by 30 mg/kg GSK1521498 in this model was of a magnitude similar to a maximally efficacious oral dose (30 mg/kg) of rimonabant, a cannabinoid receptor 1 inverse agonist previously marketed for treatment of obesity (data not shown).

The time course of the plasma drug concentrations after administration of oral doses of 1 and 10 mg/kg GSK1521498 is shown in Table 3. Maximal plasma concentrations for both doses were achieved by approximately 2 h postdose. The maximal plasma concentration of the 10 mg/kg dose was approximately 10-fold higher than that of the 1 mg/kg dose, indicating the escalation was linear in this dose range. At 8 h, the plasma drug levels were starting to decline, which correlates with the timing of the separation of the efficacy curves for the 0.3, 3, and 30 mg/kg doses in Fig. 3. By 24 h, plasma concentrations at both doses had fallen to 5% of the maximal levels; however, the plasma concentration in the 10 mg/kg dose group was still approximately 25% of the maximal exposure of the 1 mg/kg dose.

The displacement of radioligands specific for MOR, KOR, and DOR by GSK1521498 in brain regions enriched for each receptor was assessed to compare the time course and dose response of receptor occupancy with that of food consumption. The specificity of the radioligands [3H]DAMGO, [3H]CI-977, and [3H]deltorphin II for MOR, KOR, and DOR, respectively, was assessed in nucleus accumbens, dorsal endopiriform nucleus, and caudate/putamen, respectively (Fig. 4). Each radioligand was highly selective for its respective receptor subtype as indicated by the similarity of total binding to specific binding in the presence of high concentrations of receptor-selective ligands for either of the other two opioid receptors. As shown in Fig. 5A, GSK1521498 dose-dependently displaced the MOR occupancy of [3H]DAMGO.
The maximum \[^{3}H\]DAMGO displacement at each dose was present at the first time point (1.5 h after dosing). Maximal suppression of food consumption was noted during this time frame with the 0.3 mg/kg dose for which approximately 60% displacement was observed. Diminished signal was observed for all doses at 8 h, which is consistent with the plasma drug level time course and the divergence of the efficacy curves from one another in the food consumption study. At the 24 h time point, \[^{3}H\]DAMGO displacement was not different from that of vehicle for the 0.3, 1, and 3 mg/kg doses and had decreased in a dose-dependent manner in the 10 and 30 mg/kg groups. The signal at 24 h in the 10 mg/kg dose group is consistent with the remaining plasma drug levels at this time point. \[^{3}H\]DAMGO displacement did not fall below 60% for the 30 mg/kg dose group even at the 24-h time point, which is consistent with the sustained efficacy observed at the end of the dark cycle (14 h) in the food consumption study. Displacement of radioligand at KOR and DOR was not different from that of vehicle at the 0.3 and 3 mg/kg doses at any time point (Fig. 5, b and c), and these doses were fully efficacious in the early hours of the nocturnal food consumption study. Although the 1 mg/kg dose at the 8-h time point was statistically different (\(p = 0.03\)) from vehicle at KOR, this result was not consistent with either the 1.5-h time point or the other effect at this time point for the 0.3, 3, or 10 mg/kg doses, suggesting that the mild significance may be due to random variability and the small sample size (\(n = 3\)). Statistically significant displacement of radioligand at KOR and DOR was observed in the 10 and 30 mg/kg dose groups at the 1.5-h time point; however, the magnitude of displacement observed at MOR was not observed.

**Reduction of Food Consumption and Translation to Weight Loss in DIO Rats.** GSK1521498 (0.3, 3, and 30 mg/kg) inhibited palatable food consumption in a dose-dependent manner in DIO rats fed palatable chow (Fig. 6a). The same group of DIO rats was then given ad libitum access to standard chow (Fig. 6b). Under these conditions, the ED\(_{50}\) for inhibition of food intake is 6.2 mg/kg compared with an ED\(_{50}\) of only 1.6 mg/kg in rats with access to palatable chow (Fig. 6a).

Oral administration of 3, 10, or 30 mg/kg GSK1521498 to DIO rats once daily for 12 days resulted in dose-responsive induction of weight loss (Fig. 7a). As shown in Table 4, there was a dose-dependent reduction of fat mass, and the effects of the 10 and 30 mg/kg doses were different from those of vehicle (\(p = 0.05\) for 3 mg/kg group). At the highest dose (30 mg/kg), body weight loss reached a plateau at a fat mass level greater than that of lean rats fed standard chow. Lean mass was not different from vehicle in any dose group; thus, the body weight reduction was predicated on reduction of fat mass. The linear correlation of mean body weight loss and mean fat loss across the treatment groups was highly positive (0.98) with \(p < 0.05\). In contrast, the correlation between body weight loss and lean loss (0.32) was not significantly different from 0 (\(p = 0.7\)). These correlation coefficients align with the analyses, which showed significant differences in body weight loss and fat mass loss across treatment without a significant reduction in lean mass.

The amount of body weight loss was compared with the cumulative suppression of food consumption during the study (Fig. 7b). The loss in body weight for each rat was converted to kilocalories using a conversion factor of 9 kcal/g b.wt. because the body weight loss comprised fat mass. The weight loss in kilocalories was not different from the reduction in food intake expressed in kilocalories, suggesting that body weight loss is related to reduced caloric intake.

**GSK1521498 Regulates Sucrose Preference, Food Reinforcement Efficacy, and Satiety.** An escalating concentration, two-bottle choice sucrose preference paradigm was used to demonstrate the effects of GSK1521498 on carbohydrate preference (Fig. 8). Preference peaked at approximately 80 to 90% for the highest sucrose concentration (1%) in the vehicle group. Rimonabant (30 mg/kg) was used as a positive control because it has been previously demonstrated to be effective in reducing sucrose preference. Sucrose preference at both the 0.5 and 1% concentration was significantly lower compared with vehicle in a statistically significant manner (Fig. 8a).
suppressed by 1 and 10 mg/kg GSK1521498, and the effect was similar to that of 30 mg/kg rimonabant.

Food reinforcement efficacy was assessed in a progressive-ratio model in which the number of lever presses required for delivery of a palatable food reward increased with each successive reward. GSK1521498 significantly suppressed the number of lever presses on the first day of dosing in a progressive-ratio paradigm at doses of 1, 3, and 10 mg/kg (Fig. 9a). Although the trend was still present on days 2 and 3 of dosing, the effect of all three doses was diminished. The difference from the vehicle group was not statistically significant with the exception of the 10 mg/kg dose on test day 3, although \( p = 0.05 \) on test day 2 for this dose. The initial time course of lever-pressing was not affected by GSK1521498, which indicates that the rats are not compromised by drug treatment such that they are unable to perform the task (data not shown). The effect of GSK1521498 on satiety was assessed using a fixed-ratio schedule of reinforcement. In the FR1 interval paradigm, doses of 1, 3, and 10 mg/kg GSK1521498 inhibited the total number of lever presses by 50 to 75% over a period of 3 consecutive days relative to that for the vehicle group (Fig. 9b). The vehicle group averaged between 86 and 95 lever presses during the 3 test days, the 1 mg/kg dose group achieved 40 to 43 lever presses/day, and the 3 and 10 mg/kg groups achieved 24 to 37 lever presses/day.

Secondary Pharmacology. Adverse neurological effects were not observed in an Irwin screen in which male Sprague-Dawley rats were dosed orally with 5 and 50 mg/kg GSK1521498 once daily for 3 days. GSK1521498 treatment did not cause significant changes in arterial blood pressure, core body temperature, or activity after 3 daily oral doses of 3 or 30 mg/kg per day of telemetered Sprague-Dawley rats.
The endogenous opioid system was first implicated in the control of ingestion by the discovery that morphine could promote food intake (Martin et al., 1963). Numerous subsequent studies have demonstrated that opioids primarily regulate ingestive behavior related to palatability of foods (i.e., incentive value) rather than subsistence intake and that the effects are mediated through central reward systems (Levine and Billington, 1997; Kelley and Berridge, 2002). MOR plays a critical role in modulating hedonic and motivational components of palatable food intake (Zhang and Kelley, 1997; Zhang et al., 1998; Pecina and Berridge, 2000; Will et al., 2003).

LY255582 was the first opioid inverse agonist to be developed for the treatment of obesity. This compound has been carefully studied for its ability to regulate palatable food consumption and body weight in rats (Mitch et al., 1993; Shaw, 1993; Statnick et al., 2003). LY255582 has subnanomolar potency for rat MOR and is 5- and 13-fold selective over KOR and DOR, respectively; thus, it is considered to be relatively nonselective. In DIO Long Evans rats, the maximal suppression of 24-h food intake was approximately 50 to 60% compared with that for vehicle. Striatal opioid receptor occupancy by LY255582 correlated with the reduction in food consumption; however, neither the radioligand used to assess occupancy (diprenorphine) nor LY255582 is selective, so the receptor(s) mediating the efficacy could not be identified. A subsequent study, which examined localization of LY255582 in mouse brain by autoradiography, suggested that the effects on food intake and body weight were mediated by the
combined effect at $\mu$, $\kappa$, and $\delta$ receptors (Gackenheimer et al., 2005).

The goal of our program was to identify a MOR-selective molecule to avoid undesired adverse events that might be elicited by inverse agonism at KOR and DOR. For instance, increased levels of anxiety and depressive phenotype are observed in DOR knockout mice, and KOR knockout mice experience increased nociception for specific pain responses (Gaveriaux-Ruff and Kieffer, 2002). GSK1521498 appeared to have potency for rat MOR similar to that for LY255582; however, it was more selective for rat MOR versus KOR and DOR (greater than 50-fold). It should be noted that the potency and selectivity of the two compounds were assessed by different methodologies. LY255582 was assessed in a binding assay using rat brain homogenates, whereas GSK1521498 was tested in a functional system using membranes from cells expressing recombinant rat receptors. Both GSK1521498 and LY255582 suppressed 24-h food consumption in the DIO Long Evans rat model; however, the maximal reduction achieved with LY255582 was 50%, whereas that with GSK1521498 was greater than 85%. A possible explanation for the disparity is that MOR occupancy in the maximally efficacious LY255582 dose groups (10 and 20 mg/kg) was less than that observed with GSK1521498 (10 and 30 mg/kg).

The data in Figs. 3 and 5 and Table 3 show consistency between the patterns of MOR occupancy, plasma drug concentrations, and the time course and dose-response relationship of palatable food consumption. An oral dose as low as 0.3 mg/kg was sufficient to maximally suppress food intake over the first few hours of the nocturnal cycle coincident with maximal plasma drug levels and approximately 60% MOR occupancy. Neither KOR nor DOR occupancy by GSK1521498 was observed at this dose, which suggests that maximal efficacy in the food consumption model can be mediated solely by MOR occupancy. Strong suppression of food intake was present throughout the nocturnal cycle for the 30 mg/kg dose group, in which MOR occupancy did not fall below 60% for the 24-h period. The 0.3 mg/kg dose groups exhibited efficacy in the food intake model similar to that of the 30 mg/kg group at early time points when occupancy was approximately 60%. We conclude that at least 60% MOR occupancy is sufficient for robust efficacy in this model. The correlation of MOR occupancy with efficacy indicates that techniques such as positron emission tomography imaging can be used to estimate efficacious dose and plasma drug levels in humans. In addition, plasma drug levels should serve as an easily measurable surrogate for MOR occupancy in the clinic.

The suppression of nocturnal intake of palatable food observed in DIO rats translated into induction of weight loss. Weight loss reached a plateau before the fat mass level was reduced to that of lean rats fed standard chow. This pattern has been observed in clinical studies with antiobesity agents such as rimonabant. The plateau effect was also observed with a 30 mg/kg dose of rimonabant in this model, and the maximal percentage weight loss from baseline was similar to that observed with 30 mg/kg GSK1521498 (data not shown). Because the cumulative reduction in food consumption was equivalent to the loss of weight, we conclude that GSK1521498 induced fat mass loss through the suppression of food consumption. It is not possible to compare the magnitude of weight loss efficacy for GSK1521498 with that of LY255582 because the models used were inherently different (Shaw, 1993; Statnick et al., 2003). Obese Zucker rats continue to gain weight and treatment of the DIO Long Evans rats started early in the weight gain curve; thus, LY255582 inhibited the progressive weight gain compared with that in the vehicle group. The DIO Long Evans rats used in the present study were permitted to become heavier before the start of the experiment such that the rate of gain had slowed and induction of weight loss could be measured. Although GSK1521498 strongly suppressed palatable food consumption in DIO rats, the same rats that were then provided with standard chow and subjected to caloric restriction during the 24 h before the nocturnal food consumption experiment were less sensitive to GSK1521498. In fact, the lowest dose of 0.3 mg/kg was no longer active in this model. This observation may have implications for how this mechanism should be best assessed in clinical studies with regard to the composition of diet and restriction of calories.

Opioid receptor antagonists/inverse agonists are thought to modulate satiety and motivation or desire to eat palatable food. A progressive-ratio model was used to assess the reinforcement efficacy for food reward, whereas a fixed-ratio schedule was used to assess effects on satiety when food reward was obtained with a minimal amount of work (Maccioni et al., 2008; Nair et al., 2008). GSK1521498 inhibited reinforcement efficacy in the progressive-ratio model and suppressed performance in an FR1 schedule compared with vehicle. The efficacy in the FR1 paradigm suggests that GSK1521498 enhanced the development of satiety. The amount of lever-pressing in the GSK1521498-treated rat FR1 paradigm was far greater than that observed for the progressive-ratio model; therefore, the efficacy of GSK1521498 in the latter model cannot be explained simply by an effect on satiety but rather by suppression of the motivation to work for reward or the sensitivity to reward. Durability of efficacy over 3 days of treatment, especially at the lower doses was greater in the FR1 schedule than in the progressive-ratio paradigm. Of interest, we have observed that rimonabant (30 mg/kg) reduced lever pressing in the progressive-ratio schedule to the same extent as GSK1521498 on day 1 of drug testing, but a significant difference from vehicle on days 2 and 3 of dosing was not observed (data not shown). There may be a compensatory mechanism that limits the persistence of the effect on reinforcement, whereas the effect on satiety is sustained. In the operant response setting, initiation of lever pressing was not disrupted; thus, the rats were not neurologically impaired such that they were unable to work for rewards. Neurologic function was also not found to be impaired in an Irwin screen in rats treated with GSK1521498 (data not shown). Finally, GSK1521498 altered the choice to imbibe palatable food as demonstrated by the diminished preference for sucrose-containing solutions.

At present, there are few pharmacotherapeutic options for obesity therapy. GSK1521498 may be clinically useful for the treatment of obesity and other disorders characterized by compulsive eating including binge-eating disorder, because it robustly inhibits ingestion of palatable food under normal conditions of nocturnal consumption, induces satiety, and inhibits the preference for and desire to seek palatable food. This molecule may, therefore, be effective in modulating choice of nutrients and desire for fat and sugar in the clinic. Because GSK1521498 also suppressed consumption of standard chow, we expect that binge eating of any type of food could be suppressed.
Received 26 October 2017; accepted 30 July 2018.

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GSK5121498 is currently in clinical development for obesity therapy and has indeed been shown to significantly reduce hedonic taste preference and caloric intake, particularly for high-fat/high-sugar foods in subjects with body mass index between 25 and 35 kg/m² (Nathan et al., 2011). In a human functional magnetic resonance imaging study, GSK5121498 attenuated palatable food reward-related amygdala activation, whereas naltrexone was inactive (Rabiner et al., 2011); thus, this novel μ opioid receptor-selective inverse agonist may have a clinical profile different from that of naltrexone when used for treatment of obesity and binge-eating disorders.

Acknowledgments

We thank Terry Kenakin for his advice on assessment of constitutive receptor activity; Mike Jeune, Kevin Hedeen, and Sabrina Rogers for their assistance with drug metabolism and pharmacokinetics analysis and formulations; Richard Cox for advice on receptor occupancy experiments; Mandy Bergquist and Robert Ward for advice and assistance with statistical analysis; Carol Sable for assistance with manuscript preparation; and Edward Bullmore and Pradeep Nathan for critical review of the manuscript.

Authorship Contributions

Participated in research design: Ignar, Goetz, Epperly, Shearer, Sorensen, Speake, and Hommel. Conducted experiments: Noble, Carbello, Stroup, Fisher, Brainard, Larkin, Sorensen, and Hommel. Contributed new reagents or analytic tools: Fisher and Larkin. Performed data analysis: Ignar, Goetz, Noble, Carbello, Stroup, Fisher, Boucheron, Brainard, Epperly, Shearer, Speake, and Hommel. Wrote or contributed to the writing of the manuscript: Ignar, Goetz, Boucheron, Epperly, Shearer, and Hommel.

References


Chen G, Jayawikreme C, Way J, Queen K, Watson C, Ignar D, Chen WJ, Ignar, Goetz, Epperly, Shearer, and Hommel. Contributed new reagents or analytic tools: Fisher and Larkin. Performed data analysis: Ignar, Goetz, Noble, Carbello, Stroup, Fisher, Boucheron, Brainard, Epperly, Shearer, Speake, and Hommel. Wrote or contributed to the writing of the manuscript: Ignar, Goetz, Boucheron, Epperly, Shearer, and Hommel.

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