The Effects of Herkinorin, the First μ -Selective Ligand from a Salvinorin A-Derived Scaffold, in a Neuroendocrine Biomarker Assay in Nonhuman Primates

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ABSTRACT

Herkinorin is the first μ -opioid receptor-selective ligand from the salvinorin A diterpenoid scaffold. Herkinorin has relative $\mu > \kappa > \delta$ binding selectivity, and it can act as an agonist at both μ - and κ -receptors, in vitro. These studies were the first in vivo evaluation of the effects of herkinorin in nonhuman primates, using prolactin release, a neuroendocrine biomarker assay that is responsive to both μ - and κ -agonists, as well as to compounds with limited ability to cross the blood-brain barrier. In cumulative dosing studies (0.01–0.32 mg/kg i.v.), herkinorin produced only small effects in gonadally intact males (n=4), but a more robust effect in females (n=4). Time course studies with herkinorin (0.32 mg/kg) confirmed this greater

effectiveness in females and revealed a fast onset after i.v. administration (e.g., by 5–15 min). Antagonism experiments with different doses of nalmefene (0.01 and 0.1 mg/kg) caused dose-dependent and complete prevention of the effect of herkinorin in females. This is consistent with a principal μ -agonist effect of herkinorin, with likely partial contribution by κ -agonist effects. The peripherally selective antagonist quaternary naltrexone (1 mg/kg s.c.) caused approximately 70% reduction in the peak effect of herkinorin (0.32 mg/kg) in females, indicating that this effect of herkinorin is prominently mediated outside the blood-brain barrier.

Salvinorin A, a plant-derived hallucinogenic diterpene, is a highly selective κ-opioid agonist, and a novel template for semisynthetic opioid analogs (Roth et al., 2002; Prisinzano and Rothman, 2008). One of these novel analogs is herkinorin, the first salvinorin-derived compound with μ- over κ-selectivity reported in the literature (Harding et al., 2005). Herkinorin has approximately 8-fold selectivity for μ- over κ-receptors and approximately 98-fold selectivity for μ- over δ-receptors in competition binding assays (Harding et al., 2005). Herkinorin acts as a high-efficacy agonist at both μand κ-receptors in the guanosine 5'-O-(3-thio)triphosphate assay (Harding et al., 2005), with greater relative potency at μ-receptors. Herkinorin also displays some unique features in its interactions at the \(\mu\)-receptor, such as decreased agonist-induced internalization (Groer et al., 2007; Xu et al., 2007).

To date, there is no information on the in vivo effects of herkinorin in primates. The present study focuses on an initial evaluation of the in vivo opioid agonist effects of herkinorin in rhesus monkeys, using a translationally viable neuroendocrine biomarker assay, release of the anterior pituitary hormone prolactin. Different factors render this neuroendocrine biomarker a practical approach for initial evaluation of herkinorin: 1) prolactin levels are increased by both μ- and κ-agonists in mammals, including humans (δ -agonists seem not be to be active) (Hoehe et al., 1988; Ur et al., 1997; Kreek et al., 1999; Bowen et al., 2002; Butelman et al., 2007); and 2) effects of these opioids are at least partially mediated by opioid receptors outside the blood-brain barrier (e.g., in particular hypothalamic nuclei) (Merchenthaler, 1991; Butelman et al., 2004, 2008; Zheng et al., 2005), and they may therefore be used to determine the pharmacodynamic effects of compounds with limited ability to cross the blood-brain barrier.

This study presents the first direct evaluation of the pharmacodynamic effects of herkinorin in male and female nonhuman primates, and it also investigates potential μ -versus

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 κ -receptor effects of this novel compound, as well as activity outside the blood-brain barrier.

Materials and Methods

Subjects

Four captive-bred male and four female rhesus monkeys (Macaca mulatta; age range, approximately 8–12 years; weight range, 7.0–12.5 kg) were used. All subjects were gonadally intact. Unless otherwise stated, each study was carried out with an n=4 of either males or females. Females were studied in the follicular phase, estimated as days 1 to 12 from the onset of visible menses. Monkeys were singly housed in colony rooms maintained at 20 to 22°C, with controlled humidity and a 12:12-h light/dark cycle (lights on at 7:00 AM). Monkeys were fed approximately 12 jumbo primate chow biscuits, adjusted individually (PMI Feeds, Richmond, VA). Subjects also received appetitive treats, and multivitamins plus iron. An environmental enrichment plan was in place (e.g., music and videos). Water was freely available in home cages, via a waterspout.

Subjects had complex history of pharmacological exposure (primarily opioid), but they had no history of any chronic or high-frequency exposure to any agent. Consecutive experiments in the same subject were separated by at least 72 h; the order of experiments was unsystematic among subjects. All studies were carried out over the course of several months while all subjects were in stable colony rooms. Studies were reviewed by the Rockefeller University Animal Care and Use Committee, in accordance with the Guide for the Care and Use of Animals (National Academies Press; Washington, DC, 1996).

Procedure for Neuroendocrine Experiments

Chair-trained monkeys were tested after repeated exposure to the experimental situation. Monkeys were chaired and transferred to the experimental room between 9:45 AM and 10:30 AM each test day, approximately. An indwelling catheter (24-gauge, Angiocath; BD Medical Systems-Infusion Therapy, Sandy, UT) was placed in a superficial leg vein and secured with elastic tape. An injection port (Terumo Medical Corporation, Elkton, MD) was attached to the catheter; port and catheter were flushed (0.3 ml of 50 U/ml heparinized saline) before use and after blood sampling or injection. Approximately 20 min after catheter placement, two baseline blood samples of approximately 1.5 ml were collected, 5 min apart from each other (defined as -10 and -5 min, relative to the onset of drug injection). Samples were kept at room temperature until the time of spinning (3000 rpm at 4°C) and serum separation. Serum samples were kept at -40°C until time of analysis (typically within 2 weeks of collection). Samples were analyzed in duplicate with a standard human prolactin immunoradiometric kit (DPC-Siemens Medical Solutions Diagnostics, Los Angeles, CA), following manufacturer's instructions. Studies report high protein homology in human versus rhesus monkey prolactin, as well as antibody cross-reactivity (Brown and Bethea, 1994; Pecins-Thompson et al., 1996). The reported sensitivity limit of this assay was 0.1 ng/ml; each individual kit was calibrated with known standards, in the range 2 to 200 ng/ml. The intraand interassay coefficients of variation with this kit in the laboratory were approximately 3 and 11%, respectively.

Cumulative Dosing Procedure. Monkeys were tested in a cumulative dosing or time course procedure. A cumulative dosing procedure was used to produce a rapid initial estimate of the potency and effectiveness of herkinorin within a single session. Cumulative dosing procedures have been used previously to efficiently measure potency and effectiveness of μ - and κ -agonists in this biomarker assay (Bowen et al., 2002; Butelman et al., 2002). Cumulative dosing studies commenced with baseline preinjection sample collection (two separate samples taken approximately 10 and 5 min before the onset of dosing), followed by four consecutive 30-min interinjection cycles, with agonist doses increasing in 0.5 log unit steps. Blood sampling

occurred 15 min after each injection. Herkinorin was studied herein up to the largest dose that could be administered due to solubility limitations (0.32 mg/kg). Herkinorin was then compared with salvinorin A (its structurally related parent compound, a selective κ -agonist) and loperamide (a peripherally selective μ -agonist), under similar conditions. A vehicle control study was also carried out, with four consecutive vehicle injections under identical timing conditions.

Time Course Procedure. After baseline sample collection, a single agonist dose (e.g., of herkinorin) was administered i.v., followed by sampling at 5, 15, 30, 60, 90, and 120 min after administration. In antagonism experiments, a single dose of antagonist (s.c. nalmefene or quaternary naltrexone) was administered 30 min before an agonist, followed by testing as described above. In these antagonism experiments, a single sample was also taken 20 min after administration of the antagonist alone (i.e., during the pretreatment period).

Design

Cumulative Dose-Effect Curve Studies. Cumulative dose-effect curve studies were carried out in males and females for herkinorin (0.01–0.32 mg/kg i.v.) compared with repeated vehicle injection, for control purposes. The effects of loperamide [0.01–0.32 mg/kg (males and females)] and salvinorin A [0.001–0.032 mg/kg (males) and 0.00032–0.01 mg/kg (females), based on prior studies; Butelman et al., 2007, 2008] were also studied under identical cumulative dosing procedures.

Time Course Studies. The time course effects of herkinorin were studied in male subjects, at the largest dose that could be administered, as limited by solubility (0.32 mg/kg i.v., compared with vehicle i.v.). This was followed by a similar determination of the effects of this dose in females.

Because males demonstrated only a small effect of herkinorin up to the largest dose that could be studied, antagonism experiments described below were only carried out in females. Nalmefene (0.01 or 0.1 mg/kg s.c.) was thus administered as a pretreatment to herkinorin (0.32 mg/kg i.v.). Nalmefene has relative $\mu\text{-}$ over $\kappa\text{-}selectivity$ as an antagonist in primates (France and Gerak, 1994). In prior studies, the lower nalmefene dose (0.01 mg/kg) was sufficient to block the effects of selective $\mu\text{-}agonists$ (France and Gerak, 1994), whereas the larger dose (0.1 mg/kg) was necessary to block the effects of $\kappa\text{-}agonists$ (Butelman et al., 2007). The effects of herkinorin (0.32 mg/kg) were also studied after pretreatment with the peripherally selective antagonist quaternary naltrexone (1 mg/kg s.c.) (Valentino et al., 1983). In a comparison study, the effects of fentanyl (0.01 mg/kg i.v.) were studied after analogous pretreatment with a $\mu\text{-}selective$ dose of nalmefene (0.01 mg/kg).

Neuroendocrine Data Analysis

Raw individual prolactin values for each experiment were converted to change in prolactin levels from individual preinjection baseline (i.e., $\Delta nanograms$ per milliliter) by subtracting mean preinjection baseline value for each subject. Data were then analyzed with two-way repeated measures analysis of variances (ANOVAs) (e.g., time \times drug condition) using SigmaStat 3.1 (Systat Software, Inc., San Jose, CA), followed by Newman-Keuls post hoc testing. Values are presented to two decimal places, and the level of significance (α) for all comparisons was set at 0.05.

Drugs

Herkinorin was synthesized in the laboratory of Dr. Thomas E. Prisinzano (Harding et al., 2005), and it was dissolved daily in 10% dimethyl sulfoxide/10% Tween 80/sterile water (v/v). Salvinorin A (extracted from commercially obtained leaves from Ethnogens.com in Dr. Prisinzano's laboratory) and loperamide HCl (Sigma-Aldrich, St. Louis, MO) were dissolved in 10% ethanol/10% Tween 80/80% sterile water (v/v). Quaternary naltrexone methobromide (also known as methylnaltrexone) was kindly supplied by Dr. Chun-Su



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Yuan (Department of Anesthesiology, University of Chicago, Chicago, IL), and it was dissolved daily in sterile water. Nalmefene HCl (Baker-Norton, Miami, FL) was dissolved in sterile water. Drug doses are reported in the forms stated above. All drugs were injected in volumes of 0.05 to 0.3 ml/kg.

Results

Cumulative Dosing: Baseline and Vehicle Control Experiment

Mean baseline prolactin levels in a cumulative vehicle experiment were 6.8 ng/ml (S.E.M. 2.3) in males and 18.8 ng/ml (S.E.M. 6.1) in females. Four consecutive vehicle injections (30-min interinjection interval; sampling 15 min after injection) resulted in small decreases in prolactin levels. For example, after the fourth injection, mean prolactin values were $-2.6~\Delta \text{ng/ml}$ (S.E.M. 1.4) in males and $-3.3~\Delta \text{ng/ml}$ (S.E.M. 9.9) in females. Mean $\Delta \text{nanograms}$ per milliliter values over four consecutive cycles with vehicle administration are presented in Fig. 1.

Cumulative Dosing Studies

Herkinorin. Up to the largest dose that could be studied (0.32 mg/kg), herkinorin only caused a small increase in prolactin levels in males [mean 14.4 Δ ng/ml (S.E.M. 7.7)]. However, in females, a greater maximal effect was detected at the largest dose [mean 175.8 Δ ng/ml (S.E.M. 47.4)] (Fig. 1). See below for statistical analysis of these cumulative dose-effect curve data.

Salvinorin A. In a comparative study, salvinorin A was studied in a similar cumulative dosing procedure in males versus females. Dose ranges were adjusted based on prior information of the greater effectiveness of a salvinorin A dose in males versus females (Butelman et al., 2007). Salvinorin A (0.001-0.032 mg/kg) caused robust dose-dependent effects in males [maximal mean 135.7 Δ ng/ml (S.E.M. 29.4), at 0.032 mg/kg]. Salvinorin A (0.00032-0.01 mg/kg) caused even more robust effect in females [maximal mean 314.9 Δ ng/ml (S.E.M. 90.6), at 0.01 mg/kg] (Fig. 1). See below for statistical analysis of these cumulative dose-effect curve data.

Loperamide. Loperamide caused dose-dependent prolactin increases in males [103 Δ ng/ml (S.E.M. 48.4), at 0.32 mg/kg]. Loperamide also caused robust, dose-dependent prolactin increases in females [maximal mean 202.1 Δ ng/ml (S.E.M. 23.7), at 0.32 mg/kg)] (Fig. 1). See below for statistical analysis of these cumulative dose-effect curve data.

Analysis of Dose-Effect Curve Data for All Compounds. For males and females, two-way repeated measures ANOVAs were carried out for drug (vehicle, herkinorin, salvi-

Fig. 1. Chemical structures of the parent compound salvinorin A (left) and its μ-selective analog herkinorin (right).

norin A, or loperamide) versus dose (as used in this four-cycle procedure), using Δ nanogram per milliliter values (i.e., after subtraction of individual preinjection baseline). The aim of this analysis was to describe the observed effects in the dose-effect curve studies, while taking into account individual baseline differences, and the slight decrease that is observed through repeated vehicle administration in this setting (see above).

In males, this drug \times dose ANOVA resulted in main effects of drug $[F(3,9)=9.94;\ p<0.003]$, dose $[F(3,9)=9.89;\ p<0.003]$, and their interaction $[F(9,27)=6.23;\ p<0.001]$. Herkinorin was not different from its cycle-matched vehicle control, in any of the doses tested. Salvinorin A was significantly different from vehicle at the two largest doses studied $(0.01\ \text{and}\ 0.032\ \text{mg/kg};\ q=5.60\ \text{and}\ 10.08,\ \text{respectively};\ \text{all}\ p<0.05)$. Loperamide was significantly different from vehicle only at largest dose studied $(0.32\ \text{mg/kg};\ q=7.70;\ p<0.05)$.

In females, the drug \times dose ANOVA also resulted in main effects of drug [F(3,9)=8.58; p<0.005], dose [F(3,9)=22.02; p<0.001], and their interaction [F(9,27)=7.74; p<0.001]. Herkinorin was significantly different from vehicle at the largest dose studied (0.32 mg/kg; q = 7.18; p<0.05). Salvinorin A was significantly different from vehicle at the two largest doses studied (0.0032 and 0.01 mg; q = 4.61 and 12.76, respectively; all p<0.05). Loperamide was different from vehicle only at the largest dose studied (0.32 mg/kg; q = 8.24; p<0.05).

Time Course Studies

Baseline and Vehicle Control Experiment. In the vehicle control time course experiment, males exhibited mean baseline preinjection prolactin values of 8.7 ng/ml (S.E.M. 1.1). Females had similar mean preinjection values 8.3 ng/ml (S.E.M. 1.9). Bolus administration of the vehicle used in herkinorin studies [1:1:8 dimethyl sulfoxide/Tween 80/sterile water (v/v)] resulted in slight gradual decreases in prolactin levels over the course of the experiment, reaching $-4~\Delta ng/ml$ (S.E.M. 1) for males and $-3.2~\Delta ng/ml$ (S.E.M. 0.6) for females, at the last (120 min) time point (Fig. 2).

Time Course Effects of Herkinorin. Up to the largest dose that could be administered (0.32 mg/kg i.v.), herkinorin caused a modest but significant time-dependent increase in serum prolactin levels in males (peak mean levels of 19.1 Δng/ml [S.E.M. 7.3]) (Fig. 2; note ordinate axis break). A rapid onset was observed in this effect, with peak effects observed at the earliest sample point (5 min after administration). A two-way repeated measures ANOVA [condition (vehicle or herkinorin) versus postinjection time] revealed a main effect of condition [F(1,3) = 12.2; p < 0.04], time [F(5,15) = 6.0; p < 0.003], and their interaction [F(5,15) =4.0; p < 0.02]. A Newman-Keuls test confirmed that herkinorin caused an increase in prolactin levels compared with vehicle at 5, 15, and 30 min after administration (q = 5.73, 5.41, and 5.0, respectively; all p < 0.05), but not at later time points (i.e., 60-120 min).

By contrast, the same herkinorin dose in females caused a greater maximal effect 5 min after administration [peak maximum 306.6 Δ ng/ml (S.E.M. 91)] (note ordinate axis break in Fig. 2). A two-way repeated measures ANOVA [condition (vehicle or herkinorin) versus post injection time] revealed a main effect of condition [F(1,3) = 15.34; p < 0.03],

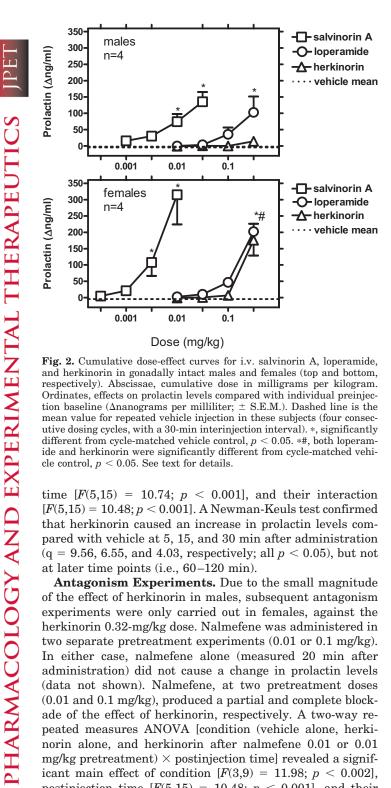


Fig. 2. Cumulative dose-effect curves for i.v. salvinorin A, loperamide, and herkinorin in gonadally intact males and females (top and bottom, respectively). Abscissae, cumulative dose in milligrams per kilogram. Ordinates, effects on prolactin levels compared with individual preiniection baseline (Δnanograms per milliliter; ± S.E.M.). Dashed line is the mean value for repeated vehicle injection in these subjects (four consecutive dosing cycles, with a 30-min interinjection interval). *, significantly different from cycle-matched vehicle control, p < 0.05. *#, both loperamide and herkinorin were significantly different from cycle-matched vehicle control, p < 0.05. See text for details.

time [F(5,15) = 10.74; p < 0.001], and their interaction [F(5,15) = 10.48; p < 0.001]. A Newman-Keuls test confirmed that herkinorin caused an increase in prolactin levels compared with vehicle at 5, 15, and 30 min after administration (q = 9.56, 6.55, and 4.03, respectively; all p < 0.05), but notat later time points (i.e., 60–120 min).

Antagonism Experiments. Due to the small magnitude of the effect of herkinorin in males, subsequent antagonism experiments were only carried out in females, against the herkinorin 0.32-mg/kg dose. Nalmefene was administered in two separate pretreatment experiments (0.01 or 0.1 mg/kg). In either case, nalmefene alone (measured 20 min after administration) did not cause a change in prolactin levels (data not shown). Nalmefene, at two pretreatment doses (0.01 and 0.1 mg/kg), produced a partial and complete blockade of the effect of herkinorin, respectively. A two-way repeated measures ANOVA [condition (vehicle alone, herkinorin alone, and herkinorin after nalmefene 0.01 or 0.01 mg/kg pretreatment) × postinjection time] revealed a significant main effect of condition [F(3,9) = 11.98; p < 0.002],postinjection time [F(5,15) = 10.48; p < 0.001], and their interaction [F(15,45) = 8.16; p < 0.001] (Fig. 3, left). Newman-Keuls tests revealed that there was a significant effect of nalmefene (0.01 mg/kg) pretreatment versus herkinorin alone at 5, 15, and 30 min after administration (q = 9.38, 5.95, and 3.94, respectively; all p < 0.05). The larger nalmefene pretreatment dose (0.1 mg/kg) also caused a significant difference versus herkinorin alone at 5, 15, and 30 min (q = 12.51, 8.61, and 5.37, respectively; all p < 0.05).

In a comparison experiment, the μ-selective agonist fent-

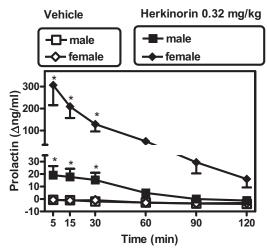


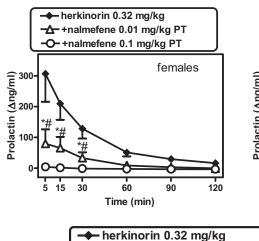
Fig. 3. Time course of herkinorin (0.32 mg/kg i.v.) in males and females, compared with i.v. vehicle, 5 to 120 min after injection. Abscissa, time from bolus i.v. injection (minutes). Ordinate, effects on prolactin levels compared with individual preinjection baseline (Δnanograms per milliliter; ± S.E.M.). Please note break in ordinate axis, to illustrate difference between effects in males versus females. *, significantly different from respective vehicle time point. See text for details.

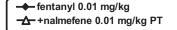
anyl (0.01 mg/kg) produced prolactin-releasing effects of a similar magnitude to those of herkinorin, but it was fully blocked by the smaller dose of nalmefene (0.01 mg/kg) (Fig. 3, right). A two-way repeated measures ANOVA [condition (vehicle alone, fentanyl alone, fentanyl after nalmefene 0.01 mg/kg pretreatment) × postinjection timel revealed a significant main effect of postinjection time [F(5,15) = 3.89]p < 0.02] and condition \times postinjection time interaction [F(10,30) = 3.06; p < 0.009]. Newman-Keuls tests revealed that nalmefene (0.01 mg/kg) pretreatment resulted in significant differences versus fentanyl alone at 5 and 15 min (q = 3.87 and 5.68; all p < 0.05).

The effects of herkinorin (0.32 mg/kg) were also studied after pretreatment with the peripherally selective antagonist quaternary naltrexone (1 mg/kg). Quaternary naltrexone alone did not have an effect on prolactin levels (20 min after administration; data not shown). Quaternary naltrexone caused a partial reduction in herkinorin's effect (Fig. 4). A two-way repeated measures ANOVA [condition (herkinorin alone versus herkinorin after quaternary naltrexone pretreatment) × postinjection time] revealed a significant main effect of postinjection time [F(5,15)] = 8.13; p < 0.001] and condition \times postinjection time interaction [F(5,15) = 5.87; p < 0.003]. Newman-Keuls comparisons confirmed that quaternary naltrexone pretreatment caused a blockade in the effect of herkinorin at 5 and 15 min (q = 6.89 and 4.0, respectively; all p < 0.05) (Fig. 5). Larger quaternary naltrexone doses were not probed herein due to supply limitations.

Discussion

Herkinorin is the first compound derived from the salvinorin A diterpenoid scaffold to have μ- over κ-selectivity (Harding et al., 2005). The present neuroendocrine biomarker assay (prolactin release) is responsive to both μ- and κ-agonists in humans and nonhuman primates and humans; therefore, it is a translationally viable assay to study the in





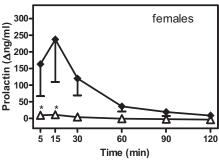


Fig. 4. Antagonism of the effects of herkinorin (0.32 mg/kg) in females by nalmefene (0.01 or 0.1 mg/kg) or of fentanyl (0.01 mg/kg) by nalmefene (0.01 mg/kg; right). Nalmefene alone, measured 20 min after administration, had no effect on prolactin levels (data not shown). See Fig. 2 for other details. *#, either dose of nalmefene was significantly different from herkinorin alone, p < 0.05. See text for details

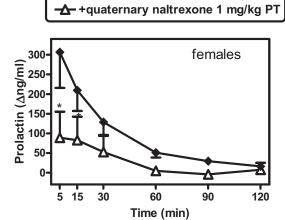


Fig. 5. Antagonism of the effects of herkinorin (0.32 mg/kg) in females by quaternary naltrexone (1 mg/kg s.c.). Quaternary naltrexone alone, measured 20 min after administration, had not effect on prolactin levels (data not shown). *, significantly different from herkinorin alone, p < 0.05. See Fig. 2 and text for details.

vivo pharmacology of this novel compound (Hoehe et al., 1988; Bart et al., 2003).

In cumulative dosing studies in males (0.01–0.32 mg/kg i.v.), herkinorin only caused a small, and nonstatistically significant effect, up to the largest dose that could be studied under the present solubility conditions. For comparison, the parent compound salvinorin A displayed greater potency and efficacy than herkinorin in males. The peripherally selective $\mu\text{-agonist}$ loperamide also displayed greater effectiveness than herkinorin in males.

The effect of herkinorin in females was more robust. The parent compound salvinorin A was approximately 30-fold more potent than herkinorin in females. The peripherally selective μ -agonist loperamide was approximately equipotent and equieffective to herkinorin in this determination in females.

The relative ineffectiveness of herkinorin in males could be potentially interpreted as a sign of partial agonist effects in this assay, compared with loperamide and salvinorin A (both of these latter ligands have high efficacy and selectivity at their respective receptor targets, the $\mu\text{-}$ and $\kappa\text{-}receptors$, respectively) (DeHaven-Hudkins et al., 1999; Roth et al., 2002). However, herkinorin also has relatively high efficacy at both $\mu\text{-}$ and $\kappa\text{-}receptors$ in vitro (with greater relative potency at μ over κ) (Harding et al., 2005). Furthermore, recent unpub-

lished pilot studies with herkinorin (0.32 mg/kg) as a pretreatment to either salvinorin A (0.032 mg/kg) or loperamide (0.32 mg/kg) were not consistent with partial agonist effects by herkinorin (i.e., no herkinorin-induced blockade of salvinorin A or loperamide was observed). Thus, based on these findings, it is more likely that the present lack of herkinorin effectiveness in males is due to practical limits to reach a sufficiently high dose in vivo, as limited by solubility.

To further investigate the agonist effects of herkinorin, time course studies at the largest herkinorin dose were carried out (0.32 mg/kg, measured 5-120 min after i.v. bolus). These findings were generally consistent with the cumulative dose-effect curve study. In the time course studies, a small but significant effect was observed in males, whereas a robust effect was observed in females. Herkinorin displayed a fast onset after i.v. administration (peak values were observed at 5–15 min). Intriguingly, although the effectiveness of herkinorin was greater in females than in males, the duration of action of herkinorin was similar in subjects of either sex. Specifically, herkinorin was significantly different from vehicle only up to 30 min after administration in either females or males. These are the first studies to compare the effects of herkinorin in male and female subjects of any species, to our knowledge. Similarly to the parent compound salvinorin A, herkinorin displayed robustly increased effectiveness in females versus males. These studies are consistent with research in humans, showing that opioid agonists can cause more robust effects on this neuroendocrine endpoint in females than in males (Kreek et al., 1999).

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The demonstration that a compound can cause prolactin release is not sufficient to implicate opioid receptors, because several types of compounds in addition to opioids also have this effect (including dopaminergic antagonists or serotonergic agonists; see, e.g., Aloi et al., 1984; Nordström and Farde, 1998). Consequently, antagonism studies were designed to determine whether opioid (μ - and or κ -receptor) mechanisms were involved in the effects of herkinorin. Antagonism studies such as apparent p A_2 or p K_B analyses were not undertaken herein, because the solubility of herkinorin under these conditions precluded investigation of surmountability. In addition, antagonism studies were only undertaken in females, due to the small magnitude of the observed effect of herkinorin in males.

Nalmefene has a relative μ - over κ -selectivity as an antagonist in nonhuman primates (France and Gerak, 1994; Butelman et al., 2002), and it does not in itself cause prolactin release in nonhuman primates (as found in the present study

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and Butelman et al., 1999; Mello et al., 2000). It is interesting to note that nalmefene exhibits partial κ-agonist effects in cloned human κ-receptors in the guanosine 5'-O-(3-thio)triphosphate assay, and it does cause detectable prolactin release in humans (Bart et al., 2005). In the present study, 0.01 mg/kg nalmefene produced a partial reduction (by approximately 75%, from a mean peak of 306.6 to 79.8 Δng/ml) in the effects of herkinorin in females. The larger dose of nalmefene (0.1 mg/kg) was able to produce essentially complete blockade of this effect (by approximately 98%, to a mean peak of 4.4 Δ ng/ml). Given prior data on doses of nalmefene active against μ- and κ-opioid ligands in rhesus monkeys (France and Gerak, 1994; Butelman et al., 2002, 2007), this suggests that the effects of herkinorin are mediated principally by μ-receptors and that κ-receptors may also be partially involved. These data are consistent with the in vitro profile of herkinorin, which shows relative (approximately 8-fold) binding selectivity for μ - over κ -receptors (Harding et al., 2005). As a direct confirmation in this setting, the smaller dose of nalmefene (0.01 mg/kg) was indeed sufficient to cause complete blockade of the effects of the selective µ-agonist fentanyl (also see Butelman et al., 2008). This confirms that an agonist thought to act solely through \(\mu\)-receptors in this assay is fully blocked by the smaller nalmefene dose under these experimental conditions. Antagonism experiments with more selective κ-opioid antagonists (e.g., norbinaltorphimine) were not undertaken due to the potential for ultralong duration of such antagonists on neuroendocrine endpoints (at least several weeks) (Pascoe et al., 2008).

As recently shown and confirmed herein (e.g., with loperamide), µ-receptors located outside the blood-brain barrier (probably in the hypothalamus) can mediate prolactin-releasing effects in primates (Merchenthaler, 1991; Zheng et al., 2005; Butelman et al., 2008). For comparison, we recently reported that fentanyl may produce this effect by acting on μ-receptor populations inside as well as outside the bloodbrain barrier (Butelman et al., 2008). In this study, the peripherally selective opioid antagonist quaternary naltrexone (methylnaltrexone; 1 mg/kg) (Valentino et al., 1983; Yuan et al., 2002) caused a partial blockade of the peak effect of herkinorin (by approximately 70%, to a mean peak of 88.3 Δng/ml). This confirms that herkinorin may cause the present effect by acting at least partially on opioid receptors outside the blood-brain barrier (Zheng et al., 2005). Based on prior studies (Butelman et al., 2004) and recent pilot data, we have also found that quaternary naltrexone is not able to fully block the effects of κ -agonists in this endpoint. Thus, it may not be excluded that the part of the effect of herkinorin that was insensitive to quaternary naltrexone may have been mediated by κ -receptors, rather than by central μ -receptors (Butelman et al., 2008). Such a conclusion would in fact be consistent with the present nalmefene antagonism data (see

To our knowledge, this is the first direct dose-effect curve comparison of prolactin-releasing effects of μ - and κ -opioids in gonadally intact male and female primates. The present study confirms that κ -opioids (e.g., salvinorin A) cause prolactin-releasing effects of both greater potency and greater apparent efficacy in females than in males (Kreek et al., 1999). Sex differences for a peripherally selective μ -opioid (loperamide) were more modest, and observable mostly as a difference in maximal effect. Overall, the present dose-effect

curve determinations support the use of this translationally viable neuroendocrine biomarker for the study of novel μ -and κ -opioid analogs in primates. These findings support use of the biomarker in human clinical populations, as a means to determine responsiveness of either $\mu\text{-}$ or $\kappa\text{-}receptor$ pools, and their association to disease status.

This is also the first evaluation of herkinorin, the first μ -selective ligand from the salvinorin scaffold (Harding et al., 2005), in nonhuman primates, and the first evaluation of its neuroendocrine effects in any species. This study shows that herkinorin exhibits moderate potency in vivo and that its effectiveness in this neuroendocrine biomarker is greater in females than in males. Antagonism studies confirm that herkinorin can produce μ -agonist effects in vivo, although it may have moderate μ - over κ -selectivity in this respect. Last, studies with quaternary naltrexone suggest that herkinorin may produce these effects by acting at least partially on opioid receptors located outside the blood-brain barrier. Additional studies to improve the pharmacokinetic properties and μ -selectivity of the herkinorin template are ongoing.

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