ABSTRACT

5-Hydroxytryptamine (5-HT)2A receptor inverse agonists are promising therapeutic agents for the treatment of sleep maintenance insomnias. Among these agents is nelotanserin, a potent, selective 5-HT2A inverse agonist. Both radioligand binding and functional inositol phosphate accumulation assays suggest that nelotanserin has low nanomolar potency on the 5-HT2A receptor with at least 30- and 5000-fold selectivity compared with 5-HT2C and 5-HT2B receptors, respectively. Nelotanserin dosed orally prevented (+)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI; 5-HT2A agonist)-induced hypolocomotion, increased sleep consolidation, and increased total nonrapid eye movement sleep time and deep sleep, the latter marked by increases in electroencephalogram (EEG) delta power. These effects on rat sleep were maintained after repeated subchronic dosing. In healthy human volunteers, nelotanserin was rapidly absorbed after oral administration and achieved maximum concentrations 1 h later. EEG effects occurred within 2 to 4 h after dosing, and were consistent with vigilance-lowering. A dose response of nelotanserin was assessed in a postnap insomnia model in healthy subjects. All doses (up to 40 mg) of nelotanserin significantly improved measures of sleep consolidation, including decreases in the number of stage shifts, number of awakenings after sleep onset, microarousal index, and number of sleep bouts, concomitant with increases in sleep bout duration. Nelotanserin did not affect total sleep time, or sleep onset latency. Furthermore, subjective pharmacodynamic effects observed the morning after dosing were minimal and had no functional consequences on psychomotor skills or memory. These studies point to an efficacy and safety profile for nelotanserin that might be ideally suited for the treatment of sleep maintenance insomnias.
1988; Landolt et al., 1999; Fish et al., 2005; Popa et al., 2005; Monti and Jantos, 2006). Indeed, several selective 5-HT_{2A} inverse agonists have entered clinical development for the treatment of insomnia; these include eplivanserin, volinanserin, pruvanserin, and nelotanserin (Teegarden et al., 2008).

Initial interest in 5-HT$_2$ receptors and their role in sleep arose from studies with selective 5-HT$_2$ modulators. For example, in both rodents and humans, slow-wave sleep (SWS) is increased by the selective 5-HT$_2$ receptor antagonist ritalinserin in a dose-dependent manner; in contrast, the selective 5-HT$_2$ receptor agonist meta-chlorophenylpiperazine produces the opposite effect (Idzikowski et al., 1986). Additional evidence supporting a role for 5-HT$_2$ receptors in sleep comes from the use of various antipsychotic agents known to promote SWS in both humans and rats (Bugovic and Wauquier, 1987; Sharpley et al., 1990; Monti and Monti, 2004). For example, the atypical antipsychotic drugs risperidone and olanzapine increase SWS in both schizophrenic and healthy subjects, respectively (Dursun et al., 1999; Sharpley et al., 2000; Yamashita et al., 2002). Nevertheless, because these agents lack selectivity, their sleep-promoting effects cannot be attributed to inhibition of 5-HT$_{2A}$ receptors alone.

Studies with transgenic animals, specifically 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor knockout mice, suggest that these receptors modulate sleep-wake states (Frank et al., 2002; Popa et al., 2005). Both knockout strains show a decrease in NREM sleep compared with wild-type controls. This biologically induced decrease is in contrast to the increase in NREM sleep induced by antagonists to these two receptors. For example, the selective 5-HT$_{2A}$ inverse agonist volinanserin increases NREM sleep in wild-type mice. This effect is absent in 5-HT$_{2A}$ receptor knockout mice, suggesting that volinanserin mediates its effects on sleep through this receptor site. These data, coupled with the baseline decreases in NREM sleep in the knockout mice, suggest that possible adaptive mechanisms are at play in the constitutive 5-HT$_{2A}$ receptor mutants. Indeed, both 5-HT$_{2C}$ and 5-HT$_{2B}$ ligands induce different responses on sleep parameters in the 5-HT$_{2A}$ knockout compared with control strains (Popa et al., 2005).

The discovery of more selective 5-HT$_{2A}$ inverse agonists has confirmed the role of this receptor in sleep modulation. For example, eplivanserin, a selective 5-HT$_{2A}$ inverse agonist, increases SWS and slow-wave EEG activity in humans and rats (Landolt et al., 1999; Francon et al., 2007). Likewise, volinanserin showed a dose-dependent increase in sleep consolidation characterized by a decrease in the number of NREM sleep bouts and concomitant increase in NREM sleep-bout length (Fish et al., 2005; Morairty et al., 2008).

We recently reported the discovery and synthesis of nelotanserin, a selective 5-HT$_{2A}$ inverse agonist (Teegarden et al., 2008). Nelotanserin induced dose-dependent sleep consolidation effects and increased markers of deep SWS in rats. Furthermore, when tested in healthy volunteers, nelotanserin was well tolerated and induced increases in sleep consolidation effects. These findings confirm and extend earlier reports of 5-HT$_{2A}$ inverse agonist effects on sleep maintenance, and point to the potential clinical utility of nelotanserin in the treatment of insomnias. This article details the preclinical and early clinical pharmacokinetic (PK) and pharmacodynamic (PD) results of nelotanserin.

### Materials and Methods

#### Preparation of Nelotanserin

Nelotanserin was discovered and synthesized at Arena Pharmaceuticals, Inc. (San Diego, CA). The chemical structure of nelotanserin (molecular weight, 437.24) is displayed in Fig. 1.

#### In Vitro Studies

**Drugs and Cell Culture Reagents.** The following reagents were purchased from commercial suppliers: HEK293 and COS7 cells (American Type Culture Collection, Rockville, MD); fetal calf serum, Dulbecco's modified Eagle's medium, Optimum I, and Lipofectamine (Invitrogen, Carlsbad, CA); myo[3H]inositol and [125I]DOI (PerkinElmer Life and Analytical Sciences, Waltham, MA). All 5-HT$_2$ antagonists and inverse agonists were purchased from either Sigma/RBI (Natick, MA) or from Tocris (Ellisville, MO).

**Cell Growth and Expression of Recombinant Human and Rat 5-HT$_2$ Receptors.** HEK293 cells stably expressing human 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ (unedited INI version) receptors were generated and used for all [125I]DOI competition binding assays as described previously. The same HEK293 expressing human 5-HT$_{2A}$ and 5-HT$_{2C}$, but not 5-HT$_{2B}$ receptors contained constitutive activity in the form of elevated basal IP accumulation, which allowed determination of potential inverse agonist activity of nelotanserin. Nelotanserin inverse agonist activity at 5-HT$_{2B}$ receptor was determined in HEK293 cells transiently transfected with the human 5-HT$_{2B}$ receptor.

Likewise, HEK293 cells stably expressing rat 5-HT$_{2A}$ receptor, or transiently expressing rat 5-HT$_{2A}$ or 5-HT$_{2C}$ were used for [125I]DOI competition studies as described previously (Thomsen et al., 2008). All functional IP accumulation studies for rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors were determined in HEK293 cells transiently expressing these receptors. Enhanced basal IP accumulation was not present in these cells, thus limiting our pharmacological evaluation of nelotanserin to antagonist mode (with an EC$_{50}$ concentration of 5-HT$_{2}$ present in the assay).

**Preparation of HEK293 Cell Plasma Membranes from Cells Expressing Recombinant Human and Rat 5-HT$_2$ Receptors.** Crude plasma membranes from HEK293 cells stably or transiently expressing recombinant human or rat 5-HT$_2$ receptors were prepared as described previously (Thomsen et al., 2008). Crude membrane pellets were prepared 24 h after transfection and stored at −80°C until they were used for radioligand-binding competition assays.

**[125I]DOI Binding to Recombinant Human and Rat 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ Receptors.** Radioligand-binding assays for human and rat 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ receptors were performed as described previously (Thomsen et al., 2008). Each nelotanserin radioligand competition study tested eight to ten different concentrations (determined in triplicate).

**Radioligand-Binding Assays for Additional Human 5-HT Receptors and Neurontitransmitter Transporters.** Nelotanserin competition for radioligand binding to human 5-HT$_{1A}$, 5-HT$_{3}$, 5-HT$_{4C}$, 5-HT$_{5A}$, 5-HT$_{5C}$, and 5-HT$_{7}$ receptors, as well as dopamine and norepinephrine transporters, were performed at Cerep, Inc. (Poitiers, France).

**5-HT$_{2}$ IP Accumulation Assays.** Human and rat 5-HT$_{2}$ receptor IP accumulation assays were performed as described previously (Thomsen et al., 2008). Evaluations of potential antagonist activity were
DOI administration. Inhibition of this response with selected 5-HT2A
receptors to a simple hyperbolic function for determination of
Kd and \(B_{\text{max}}\) values (data not shown). \(K_d\) values were calculated by
use of the Cheng-Prusoff equation (Cheng and Prusoff, 1973). For IP
accumulation, \(EC_{50}\) values were also determined by fitting data to
sigmoidal function (variable slope) by use of the same nonlinear
least-squares curve-fitting program.

In Vivo Studies

Animal Subjects. Adult male Sprague-Dawley rats (Harlan, San
Diego, CA) were used for all in vivo studies. These rats were main-
tained in an environment controlled for temperature and light (lights
on: 2:00 PM to 2:00 AM), and provided food and water ad libitum.

Rat Pharmacokinetic Evaluation. Nelotanserin was formu-
lated in 80% Tween 80 and 20% phosphate-buffered saline and orally
administered to rats at a dosing volume of 5 ml/kg. Heparinized plasma
samples and brain extracts were collected and analyzed for
nelotanserin by use of a selective Liquid chromatography tandem
mass spectrometry method. In brief, nelotanserin was separated
from matrix proteins in rat plasma and brain homogenates with the
addition of acetonitrile at 2-fold the tissue volume, followed by cen-
trifugation. The supernatants collected from the processed plasma
and brain samples were injected onto an high-performance liquid
chromatography system equipped with a Scieix API 3000 mass spec-
 trometer (AME Bioscience, Toreo, Norway). Quantitation was per-
formed with regression analyses of external calibration standards.

Noncompartmental PK analysis was performed with a commercial
software package (WinNonlin Professional version 4.1.1.b; Pharsight,
Mountain View, CA). The PK parameters evaluated include maxi-
 mum plasma concentration (\(C_{\text{max}}\)), time to maximum plasma con-
centration (\(t_{\text{max}}\)), terminal phase plasma half-life (\(t_{1/2}\)), area under the
plasma exposure curve from the time of dosing to the last sample
collected (\(AUC_{\text{last}}\)), area under the plasma exposure curve from the
time of dosing extrapolated to infinity (\(AUC_{\text{inf}}\)), and mean resid-
tency time from the time of dosing to the last sample collected
(\(MRT_{\text{last}}\)).

Inhibition of DOI-Induced Decreases in Rearing. Before
evaluation in rat sleep, nelotanserin was tested in a DOI-screening
assay. DOI is a 5-HT2 partial agonist that induces a biphasic loco-
motor response (hypolocomotion followed by hyperlocomotion) in rats
(Wing et al., 1990). The hypolocomotive response is readily measured
motor response (hypolocomotion followed by hyperlocomotion) in rats
plasma exposure curve from the time of dosing to the last sample
collected (\(AUC_{\text{last}}\)), area under the plasma exposure curve from the
time of dosing extrapolated to infinity (\(AUC_{\text{inf}}\)), and mean resid-
tency time from the time of dosing to the last sample collected
(\(MRT_{\text{last}}\)).

Inhibition of DOI-Induced Decreases in Rearing. Before
evaluation in rat sleep, nelotanserin was tested in a DOI-screening
assay. DOI is a 5-HT2 partial agonist that induces a biphasic loco-
motor response (hypolocmation followed by hyperlocomotion) in rats
(Wing et al., 1990). The hypolocomotive response is readily measured
by counting the number of times an animal rears within 10 min after
DOI administration. Inhibition of this response with selected 5-HT2A
inverse agonists suggests activity at central 5-HT2A receptors.

Nelotanserin or vehicle was administered first, followed (25 min
later) by DOI. Ten minutes after DOI administration each rat was
placed in a standard clear plastic cage surrounded by a stainless steel
frame, which, when broken, register both fine and gross movement.
Ten minutes of rearing activity was recorded for each animal dosed
with vehicle or nelotanserin and DOI.

Rat Sleep. The effects of nelotanserin on rat sleep were evaluated
in two studies. The first study measured the acute effects of nelotan-
serin (vehicle, 1, 3, and 10 mg/kg), and the second measured the
subchronic effects of nelotanserin (vehicle, 10 mg/kg).

Acute dose-response study. All rats used in the acute-sleep studies
were prepared with plastic implants designed to provide continuous
EEG and electromyogram (EMG) recordings (Plastics One, Inc.,
Roanoke, VA). To this end, rats were anesthetized with a ketamine
(80 mg/kg)/xylazine (12 mg/kg) cocktail, fitted into a stereotaxic
apparatus, and a dorsal midline incision along the top of the head
was used to expose the skull. Four stainless steel spring top screws
(\#800) were implanted to penetrate the skull and contact, but not
disturb, the dura. These served as epidural electrodes to which the
EEG leads were attached. Two of these screws were implanted on the
left [bregma (\(\beta\)) –1.0 mm anterio-posterior (AP), \(\beta\) –3.0 mm medio-
lateral (ML); and lambda (\(\lambda\)) +1.0 mm AP, \(\lambda\) –4.0 mm] and used to
record alpha and delta EEG. The remaining two screws were im-
planted on the right (\(\beta\) +3.0 mm AP, \(\beta\) +1.0 mm ML, and \(\lambda\) +4.0 mm
AP, \(\lambda\) +1.0 mm) and used to record theta waves. Finally, two elec-
rodes for EMG recordings were implanted into dorsal neck muscles
and secured with sutures. The plastic connector from which the six
receptor electrodes were connected was then secured to the skull with
cement (Garant; 3M, St. Paul, MN), and the scalp was sutured around
the implant to allow protrusion of the lead connectors. All
animals were treated with buprenex (0.3 mg/kg i.m.) for 2 days
postoperative recovery. Rats were allowed at least 2 weeks recovery
before experimentation. At test, the rats were housed in plastic
 testing boxes (34 cm x 24 cm x 50 cm; Dragonfly Inc., Ridgeley, WV
with free access to food and water. After box placement, the skull
implants were connected to cable attached to a swivel system that
allowed the rats to move freely within the cage, and transmitted data
to a data collection system (Embria A10; Embria Systems, Broomfield,
CO). EEG and EMG recordings were then digitized and analyzed by
use of Somnologica Science Software (Embria Systems).

The acute effects of nelotanserin on rat sleep were tested by use of
a repeated-measures design wherein each rat was dosed orally with
either vehicle polyethylene glycol 400 or one of three concentrations
of nelotanserin (1, 3, or 10 mg/kg) administered 3 days apart in
pseudo-random order. All doses were administered 2 h before lights
on. Sleep recordings were initiated immediately after dosing and
continued for 24 h.

Subchronic repeated-dose study. The effects of repeated doses of
nelotanserin on rat sleep were tested in animals implanted with
telemetric transmitters (model F50-EEE; Data Sciences Interna-
tional, St. Paul, MN). Similar to the tethered system described in the
previous section, these transmitters contain six electrodes designed
to transmit EEG and EMG signals. These electrodes were implanted
by use of the same coordinates and procedures described above, with
the exception that the telemetry transmitter was implanted subcu-
taneously in the flank region, and there was no need for a skull cap
to accommodate hard wire connections. The rats were returned to their
home cages (housed individually) for recovery and subsequent sleep
study recordings. Telemetric data were captured using Data Quest
3.0 software (Data Sciences International) and analyzed by use of
Somnologica Science Software (Embria Systems).

This study was designed to provide insight into the effects of
repeated dosing on the sustained efficacy of nelotanserin. To this
end, the effects of nelotanserin on rat sleep were compared between
two groups that received once-a-day oral doses of either vehicle or
nelotanserin (10 mg/kg) for 6 consecutive days. All doses were ad-
ministered 2 h before lights on. Sleep recordings were initiated
immediately after the first dose and continued for 7 consecutive
days. Recordings from days 1, 3, 5, and 7 (recovery day) were scored
and analyzed.

Polysomnographic Data Analysis. All polysomnogram (PSG)
recordings were blinded before scoring to avoid experimenter bias.
EEG and EMG data were scored visually in 10-s epochs for waking,
REM sleep, and NREM sleep. Scored data were analyzed and ex-
pressed as time spent in each state per half-hour. Likewise, bout
length and number of bouts for each state were clustered into half-
hour bins. A "bout" consisted of a minimum of two consecutive epochs
of a given state. EEG delta power (1–4 Hz) within NREM sleep was
also analyzed in half-hour bins. The EEG spectra during NREM
sleep were obtained with a fast Fourier transform of EEG waveforms
on all epochs. Data were analyzed by use of analysis of variance.
When statistical significance was found from our analyses of vari-
 ance, t tests were performed comparing all nelotanserin treatment groups with vehicle.

**Evaluation of Nelotanserin in Healthy Volunteers**

The single-dose nelotanserin PK and PD data were derived from two phase 1 studies, nelotanserin-001 and nelotanserin-002. These studies were conducted in healthy volunteers with no pre-existing sleep disorders. The protocol and informed consent documents for each study were approved by an independent institutional review board. The research was conducted in accordance with the declaration of Helsinki and Good Clinical Practice guidelines.

**Nelotanserin-001 Study Design.** Forty-five healthy male adults aged 18 to 45 years were randomly assigned to a single-dose, double-blind, placebo-controlled, dose-escalation study. The doses of nelotanserin tested were 10, 20, 40, 80, and 160 mg. Within each dose cohort, nine subjects were randomly assigned in a 2:1 ratio to receive either nelotanserin or placebo. Standard PK and safety measurements were performed throughout the study. In addition, detailed PD measurements were performed and included: day-time EEG, Leeds psychomotor tests [Multiple Choice Reaction Time (MCRT) and Critical Flicker Fusion Test (CFFT)], memory test (Rey Auditory Verbal Learning Test, or RAVLT), vigilance and mood scales [Addiction Research Center Inventory (ARCI-49) scale, Bond and Lader Visual Analog Scale (VAS), Digit Symbol Substitution Test (DSST)], and subjective sleep assessment scale (Leeds Sleep Evaluation Questionnaire, or LSEQ).

**Nelotanserin-002 Study Design.** The second of the two phase 1 studies was a randomized, single-dose, double-blind, placebo-controlled study with a four-way crossover to assess the safety and pharmacological effects of nelotanserin. In this study, subjects aged 45 to 65 years were randomly assigned to receive a single dose of on of three doses of nelotanserin (10, 20, or 40 mg) or placebo in a random order and were then crossed over after a minimum 5-day washout period. To assess the effects of nelotanserin on sleep in healthy subjects, a postnap insomnia model as described by Mathias et al. (2001) was used. During screening, subjects had night consecutive nights of PSG monitoring. The first night was to rule out any comorbid sleep disorders such as obstructive sleep apnea and periodic limb movement disorder. The other screening nights were to establish baseline and also ensure that subjects were asleep for at least 30 min during the 2-h nap period. During each of the four treatment periods, a single night of PSG recording was performed. Nelotanserin or placebo was administered at 10:30 PM, 30 min before the initiation of PSG recordings, which were carried through to 7:00 AM the following morning. In addition to PSG measurement, the LSEQ, Leeds psychomotor test, word-pair retrieval test, and Bond and Lader vigilance and mood scales were performed.

**Results**

**In Vitro Pharmacology**

**Radioligand Binding and Functional Activity of Nelotanserin at Human and Rat 5-HT Receptors.** Nelotanserin displays high affinity for recombinant human 5-HT~2A~ receptors ($K_{i} = 0.35$ nM), moderate affinity for human 5-HT~2C~ receptors ($K_{i} = 100$ nM), and low affinity for human 5-HT~2B~ receptors (2000 nM) stably expressed in HEK293 cells. A summary of mean $K_{i}$ values obtained from multiple determinations is provided in Table 1. The results suggest that nelotanserin has a 262-fold higher affinity for human 5-HT~2A~ than 5-HT~2C~ receptors and a 6610-fold higher affinity for human 5-HT~2A~ than 5-HT~2B~ receptors. The $K_{i}$ of nelotanserin for rat 5-HT~2A~ is approximately 6-fold higher than the $K_{i}$ for human 5-HT~2A~ receptors, whereas the $K_{i}$ values of nelotanserin for rat 5-HT~2B~ and 5-HT~2C~ are similar to the $K_{i}$ values for human 5-HT~2B~ and 5-HT~2C~ receptors (Table 1). In addition to evaluating nelotanserin affinity by use of the 5-HT~2~ agonist [125I]DOI as radioligand, nelotanserin competition studies using antagonist/inverse agonist radioligands for human 5-HT~2A~ ([3H]ketanserin) and 5-HT~2C~ ([3H]mesulergine) receptors gave similar $K_{i}$ values (data not shown).

**Table 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[125I]DOI RBA $K_i$</th>
<th>IP Assay $EC_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 5-HT<del>2A</del></td>
<td>0.4 ± 0.07 (29)</td>
<td>0.9 ± 0.1 (13)</td>
</tr>
<tr>
<td>Human 5-HT<del>2B</del></td>
<td>2644 ± 147 (26)</td>
<td>6856 ± 2575 (5)</td>
</tr>
<tr>
<td>Human 5-HT<del>2C</del></td>
<td>106 ± 5 (27)</td>
<td>30 ± 8 (4)</td>
</tr>
<tr>
<td>Rat 5-HT<del>2A</del></td>
<td>2.5 ± 1.0 (3)</td>
<td>4.7 ± 0.4 (3)</td>
</tr>
<tr>
<td>Rat 5-HT<del>2B</del></td>
<td>4851 ± 278 (2)</td>
<td>ND</td>
</tr>
<tr>
<td>Rat 5-HT<del>2C</del></td>
<td>221 ± 23 (3)</td>
<td>170 ± 39 (3)</td>
</tr>
</tbody>
</table>

RBA, radioligand-binding assay.

*Antagonist assay with 5 nM 5-HT (EC~50~) added before compound addition.

**Agonist activation of human and rat 5-HT~2~ receptors increase intracellular IP and calcium (Roth et al., 1998). Figure 2 shows representative nelotanserin inverse agonist dose-response curves for human 5-HT~2~ receptors. Results from IP accumulation assays suggest that nelotanserin is a potent 5-HT~2~ full inverse agonist ($IC_{50} = 1.7$ nM), a moderately potent 5-HT~2C~ partial inverse agonist ($IC_{50} = 79$ nM) (maximal response was 62% of the response obtained for the reference inverse agonist clozapine), and a weak 5-HT~2B~ inverse agonist ($IC_{50} = 791$ nM). Nelotanserin inverse agonist values obtained from multiple determinations provide mean $IC_{50}$ values of 0.9 ± 0.1 nM, 6856 ± 2575 nM, and 30 ± 8 nM for recombinant human 5-HT~2A~, 5-HT~2B~, and 5-HT~2C~ receptors, respectively.

The functional potency of nelotanserin for rat 5-HT~2A~ and 5-HT~2C~ receptors was evaluated in an IP accumulation assay in antagonist mode (in the presence of an $EC_{50}$ concentration of clozapine).
of 5-HT present in the assay). Nelotanserin blocked 5-HT-mediated IP accumulation with a potency of 4.7 ± 0.4 nM and 170 ± 39 nM for rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, respectively.

**Competition of Nelotanserin for Radioligand Binding to Several Other Human 5-HT Receptors and Neurotransmitter Transporters.** To further evaluate the selectivity of nelotanserin for human 5-HT$_{2A}$ relative to other additional human serotonin receptors and neurotransmitter transporters, nelotanserin was evaluated in radioligand competition studies performed at Cerep, Inc (Poitiers, France; summarized in Supplemental Tables 1 and 2). Nelotanserin, at a final concentration of 1 µM, does not appreciably compete for binding to any of the additional serotonin receptors or neurotransmitter transporters evaluated. The selectivity of nelotanserin for 5-HT$_{2A}$ receptor relative to 61 human G protein-coupled receptors (GPCRs) was also determined, and nelotanserin did not appreciably inhibit radioligand binding to any of these GPCRs.

**In Vivo Studies**

**Rat PK.** Nelotanserin concentrations were determined in plasma derived from whole blood and brain tissue collected from male rats administered a single oral dose at 10 mg/kg. Nelotanserin absorption from the gastrointestinal tract into the systemic circulation resulted in a $C_{\text{max}}$ of 0.928 µg/ml (2.12 µM concentration) at 4 h after dosing, with a terminal elimination $t_\frac{1}{2}$ of 21.6 h. The time course for nelotanserin concentrations in the brain paralleled the plasma concentrations and reached almost 40% of plasma levels (rat PK data are summarized in Table 2 and Fig. 3).

**Reversal of DOI-Induced Hypolocomotion.** Nelotanserin dose-dependently prevented DOI-induced decrease in rearing (Fig. 4). DOI administered 10 min before locomotor testing induced a significant decrease in rearing compared with vehicle-treated controls. This decrease was reversed by pretreatment with nelotanserin 35 min before locomotor testing. Statistically significant reversal was observed at 3 and 10 mg/kg.

**Acute Effects of Nelotanserin on Rat Sleep.** The dose response established in the DOI reversal study was used to evaluate acute effects of nelotanserin on rat sleep parameters. Although PSG recordings were collected 24 h after compound administration, no differences between any of the groups were observed beyond the first 5 h; therefore, the analyses of these recordings were confined to the first 5 h immediately after dosing. This time period encompasses 2 h within the dark cycle followed by 3 h in the light cycle. Analysis of the data showed no clear effects of cycle on any of the parameters measured; therefore, the data for the first 5 h after dosing are combined.

Compared with vehicle treatment, nelotanserin treatment had no effect on measures of sleep onset latency, which was measured as the time to initiation of the first continuous minute of sleep (6 × 10 s epochs; data not shown). Nelotanserin induced a dose-dependent increase in total NREM sleep time with a concomitant decrease in both REM sleep and total wakefulness.

Nelotanserin dose-dependently increased sleep consolidation as defined by concomitant decreases in bout number and increases in bout duration. The most pronounced effects were observed for the NREM sleep state, during which nelotanserin induced statistically significant decreases in bout number in all three dosage groups, and statistically significant increases in bout duration for the two higher dosage groups (Fig. 5). In addition to sleep consolidation, nelotanserin induced a dose-dependent statistically significant increase in EEG delta power (Fig. 5).

**Subchronic Effects of Nelotanserin.** The beneficial effects of nelotanserin on rat sleep parameters, observed after acute dosing, which included increases in sleep consolidation and promotion of deep sleep, were explored after repeated subchronic dosing. Nelotanserin maintained efficacy through subchronic dosing, which included increases in NREM sleep consolidation and deep
sleep (EEG delta power). Nelotanserin induced a statistically significant increase in NREM sleep consolidation, with significant decreases in the number of sleep bouts concomitant with significant increases in bout length scored on days 1, 3, and 5 of treatment; this effect disappeared 24 h after cessation of treatment on day 7 (Fig. 6). Furthermore, nelotanserin induced significant increases in deep sleep as indicated by the increase in delta power observed on days 1, 3, and 5. Again this effect was no longer apparent after cessation of treatment on day 7, and it is noteworthy that there was no rebound in any of the parameters measured (Fig. 7).

**Evaluation of Nelotanserin in Healthy Volunteers**

**PK Results.** The PK data from the nelotanserin-001 study are summarized in Table 3. Nelotanserin was rapidly absorbed after oral administration and $C_{\text{max}}$ was reached 1 to 1.3 h after drug administration in all dose groups. The $t_{1/2}$ of nelotanserin in plasma was between 3.9 h and 10.7 h for the 10- to 40-mg doses, and 23 h for the 80- and 160-mg doses. However, the exposure to nelotanserin was not dose proportional over the tested dose range (10–160 mg) with $\text{AUC}_{(0-1)}$ and $\text{AUC}_{(0-\infty)}$ increasing proportionally with doses from 10 to 80 mg, and $C_{\text{max}}$ increasing proportionally with doses from 10 to 40 mg. The $\text{AUC}_{(0-\infty)}$ was to 729 ng·h/ml after the highest dose of 160 mg, and seemed to be the same as the value obtained with the 80-mg dose (701 ng·h/ml). This suggests that there is dose-limited absorption of nelotanserin in humans at the 80-mg dose.

The apparent clearance from plasma ($\text{CL/F}$) ranged from 147 liters/h to 264 liters/h and the apparent volume of distribution ($V_z/F$) from 1229 to 8799 liters, with a moderate...
Intersubject variability in all dose groups. These data and the moderate to high intersubject variability (percentage coefficients of variation up to 43% for $C_{\text{max}}$ and 107% for $C_{\text{max}}$) suggest that the PK variability between subjects is high.

**PD Results.** The duration of SWS was significantly increased by treatment with nelotanserin (Fig. 8) based on the EEG results. Compared with placebo, the study showed that 10-, 20-, and 40-mg doses of nelotanserin all increased SWS, with the most marked effect observed with the 40-mg dose. However, no clear dose-effect relationship was evident. Further analyses of stages 3 and 4 demonstrated that the most prominent effects of nelotanserin on SWS were during the second quartile of the night (data not shown).

An indirect consequence of the SWS enhancement during the first part of the night was a significant increase in REM latency. Other REM sleep parameters were unchanged with treatment. Nelotanserin did not influence total sleep time, indicating that the increase in SWS occurred at the expense of other sleep stages.

Together with an increase in SWS, statistically significant treatment effects were observed for number of stage shifts ($p < 0.0001$), number of awakenings after sleep onset ($p < 0.0001$), microarousal index ($p < 0.01$), and duration of bouts of sleep ($p < 0.0001$; Table 4). Analysis of each dose of nelotanserin compared with placebo showed that all three doses significantly decreased the number of stage shifts, number of awakenings after sleep onset, microarousal index, and number of bouts of sleep, whereas duration of bouts of sleep was significantly increased. No clear dose-effect relationship was evident from these parameters.

Results from the sleep EEG recordings showed that most of the EEG changes of nelotanserin occurred within the first 2

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Parameters (%CV)$^a$</th>
<th>Nelotanserin Dose Group ($n = 6$/Group)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mg</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>17.5 (62)</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.0</td>
</tr>
<tr>
<td>AUC$_{0-120}$ (ng·h/ml)</td>
<td>48.9 (50)</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (ng·h/ml)</td>
<td>51.4 (53)</td>
</tr>
<tr>
<td>AUC$_{0-\text{inf}}$ (ng·h/ml)</td>
<td>42.9 (15)$^d$</td>
</tr>
<tr>
<td>$t_{\text{1/2}}$ (h)</td>
<td>3.9 (41)$^d$</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>4.6 (17)$^d$</td>
</tr>
<tr>
<td>CL/F (liters/h)</td>
<td>237.2 (14)$^d$</td>
</tr>
<tr>
<td>Cl/F (liters/h)</td>
<td>1332.6 (44)$^d$</td>
</tr>
</tbody>
</table>

$^a$ % CV = coefficient of variation (100 × S.D./mean).
$^b$ The values stated in parentheses are percentages.
$^c$ Median.
$^d$ $n = 5$.

**TABLE 4**

Effect of nelotanserin on sleep continuity parameters

<table>
<thead>
<tr>
<th>PSG Parameter (Mean ± S.E.M.)</th>
<th>Baseline</th>
<th>Treatment ($n = 23$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>10 mg</td>
</tr>
<tr>
<td>Total sleep time (min)</td>
<td>378.2 ± 7.7</td>
<td>375.8 ± 8.3</td>
</tr>
<tr>
<td>Shifts between stages</td>
<td>109 ± 5.4</td>
<td>119.5 ± 4.6</td>
</tr>
<tr>
<td>Awakenings after sleep onset</td>
<td>27.5 ± 1.9</td>
<td>31.2 ± 1.8</td>
</tr>
<tr>
<td>Microarousal index (no. per h)</td>
<td>17.2 ± 1.5</td>
<td>17.3 ± 0.9</td>
</tr>
</tbody>
</table>

Differences between nelotanserin treatment groups and placebo were evaluated with an analysis of covariance. *,$p < 0.05$; **,$p < 0.01$; ***,$p < 0.001$ vs. placebo.
to 4 h after dosing. The EEG changes that were most prominent compared with placebo within this time window were observed with the 80-mg dose. Both in absolute and relative power analyses, this dose lowered the α-2 power, and enhanced the α-1 and theta powers. Consistently, the mean frequency was lower on 80 mg, indicating an overall slowing effect. The 40-mg dose lowered the relative α-2 power, had no effect on the α-1 power, but lowered the global alpha and enhanced the theta relative powers, although not significantly. This profile is in line with the other PD data, suggesting vigilance-lowering effects, which could be related to dose in a bell-shaped manner. These EEG changes were considered modest, because there were no clear signs of major sedation.

Changes in the scores of the Leeds Psychomotor test subscales (i.e., CFPT and MCRT) and the RAVLT memory test suggest minimal drug effects on overall cortical arousal and memory after a single dose of nelotanserin in the morning at either 20, 40, 80, or 160 mg (data not shown). The effects were small and occurred without impairing alertness level. No drug effects were observed in the DSST, the Bond and Lader VAS tests, and the Memory Consolidation Test. A slight impairment in long-term memory free recall was observed at 2.5 h after administration in the 160-mg nelotanserin dose group, which is consistent with peak plasma concentration of nelotanserin. On the basis of the LSEQ scores, nelotanserin taken in the morning enhanced subjective sleep at night. It improved both the “getting to sleep” and “sleep quality” scores without any detrimental effect on scores assessing next-morning functioning, suggesting that nelotanserin is devoid of next-day hangover effects. Subjective and psychomotor testing was also performed in the nelotanserin-002 study. The LSEQ did not show any improvement. The CFPT decreased slightly with 20 mg (−0.89 ± 1.42) and 40 mg (−1.09 ± 1.44) doses compared with placebo (−0.11 ± 1.48) (p < 0.05). No reaction time changes relative to baseline, or memory consolidation, or recall were observed for any dose of nelotanserin.

Safety Results. Administration of nelotanserin to healthy volunteers was well tolerated at all doses tested. There was no increase in CNS-related adverse events and no hangover effects from nelotanserin treatment. Moreover, no treatment-related serious adverse events or deaths were reported in either study.

Discussion

Nelotanserin, a highly selective 5HT2A inverse agonist, is a member of a novel therapeutic class under clinical investigation for the treatment of insomnia. Unlike the GABAa agonists, the 5HT2A inverse agonists are nonhypnotic and non-sedating. They promote sleep consolidation by increasing the amount of time spent in deep sleep and by decreasing the number of awakenings, sleep stage shifts, and arousals (Idzikowski et al., 1986; Landolt et al., 1999). Our preclinical and clinical results suggest that nelotanserin increases sleep consolidation and slow-wave deep sleep in both humans and rats after oral dosing. The inverse agonist efficacy of nelotanserin on human 5-HT2A receptor showed efficacy comparable with clozapine, a well-characterized inverse agonist on this receptor (Egan et al., 1998; Kenakin, 2004). Furthermore, functional IP accumulation assay data from other laboratories using clozapine as a benchmark to compare inverse agonist efficacy on the human 5-HT2A receptor suggest that nelotanserin is comparable in efficacy with other selective inverse agonists that are currently in clinical testing, including pimavanserin, volinanserin, and eplivanserin (Vanover et al., 2006).

Oral doses of nelotanserin provided sufficient central exposure to prevent DOI (5-HT2A agonist)-induced hypolocomotion in rats. PK analysis suggests that a 10 mg/kg nelotanserin dose provides maximum plasma exposure of approximately 1 μg/ml, with ~40% brain exposure. These values correspond to MRTs in plasma and brain of 5.6 and 4.4 h, respectively (Table 2), which represent maximally efficacious exposures in rat sleep. Indeed, nelotanserin induced dose-dependent increases in sleep consolidation, marked by decreases in sleep bout number with corresponding increases in sleep bout duration. Furthermore, nelotanserin treatment increased total NREM sleep time and deep sleep, the latter marked by increases in EEG delta power. These findings are in agreement with previously published reports of the effects of selective 5-HT2A inverse agonists on rat sleep consolidation and delta power (Fish et al., 2005; Monti and Jantos, 2006; Morarity et al., 2008).

Contrary to our findings with nelotanserin, evidence from a recent report comparing the effects of volinanserin (a 5-HT2A selective inverse agonist) with zolpidem (a GABA hypnotic) on rat sleep suggests that volinanserin significantly decreased sleep onset latency compared with vehicle-treated control animals, although these effects were modest compared with zolpidem and were not dose-dependent (Morarity et al., 2008). Similar studies with two other selective 5-HT2A inverse agonists, namely eplivanserin and pruvanserin, showed no changes in sleep onset latency when tested in rats (Monti and Jantos, 2006; Francon et al., 2007). Furthermore, our data in healthy volunteers, which are in agreement with other reports of 5-HT2A inverse agonist activity in humans, suggest that this mechanism does not affect sleep onset latency (Landolt et al., 1999); in fact, nelotanserin induced a delay in sleep onset latency when dosed at 40 mg in healthy volunteers. The primary benefits derived from this mechanism are observed in measures of sleep maintenance. The most significant acute effects of nelotanserin on rat sleep parameters, namely, sleep consolidation and EEG delta power, were maintained through repeated dosing. Nelotanserin (10 mg/kg) was administered for six consecutive days, during which statistically significant increases in NREM sleep consolidation, and EEG delta power were maintained (as determined by PSG analysis on days 1, 3, and 5 of dosing). These findings suggest that the efficacy of nelotanserin does not wane after repeated daily exposures. Furthermore, there were no statistically significant changes in any baseline sleep parameters after cessation of treatment as measured on day 7. The absence of both tolerance with repeated dosing and rebound after dosing cessation suggests that nelotanserin and possibly the 5-HT2A mechanism are suitable for long-term use in the clinic.

In healthy human volunteers, nelotanserin was rapidly absorbed after oral administration and achieved maximum concentrations 1 h later. Within the dose range tested (10–160 mg), nelotanserin had dose-limited absorption beginning at the 80-mg dose. The most significant EEG effects after daytime administration of nelotanserin occurred within 2 to
4 h after dosing. Although the effects were mild, their pattern was consistent with vigilance-lowering. Further assessment of nelotanserin activity was evaluated with self-rating psychometrics scales (ARCI-49, Bond and Lader VAS), vigilance and cognitive tasks (CFCT, DSST, and RAVLT), and the LSEQ. Results from these scales suggest a decrease in vigilance and cortical arousal with doses of 40 mg or greater. These decreases occurred without impairment of alertness level, and are clearly of a smaller magnitude than those observed with benzodiazepines or related compounds given at therapeutic doses.

On the basis of the LSEQ scores, nelotanserin taken in the morning enhanced subjective sleep the same night. It improved both the “getting to sleep” and “sleep quality” scores without any detrimental effect on scores assessing next-morning functioning, which suggests that nelotanserin is devoid of next-day hangover effects.

In the second clinical trial, all three doses of nelotanserin significantly improved measures of sleep consolidation, including decreases in the number of stage shifts, number of awakenings after sleep onset, microarousal index, and number of sleep bouts, concomitant with increases in sleep bout duration. Nelotanserin did not affect total sleep time or sleep onset latency. Subjective pharmacodynamic effects observed the day after dosing were minimal and had no functional consequences on psychomotor skills (i.e., reaction time) or memory.

Studies with nelotanserin highlight important mechanistic differences between 5-HT2A inverse agonists and GABA agonists and their effects on sleep. The former selectively inhibit serotonergic pathways within arousal circuits of the reticular-activating system (Abrams et al., 2005). This effect promotes sleep consolidation and deep SWS without affecting sleep onset latency or total sleep time. The latter activate inhibitory circuits within the CNS and induce a more global suppression of central activity. This inhibition promotes a decrease in sleep onset latency and an increase in total sleep time (Da Settimio et al., 2007). Based on these differences, drugs from the two classes might be ideally suited for treatment of different forms of insomnia, the first for sleep maintenance and the second for sleep onset insomnia (Roth et al., 2007).

At present, the most commonly used prescription medications for insomnia are nonbenzodiazepine hypnotics, which bind the GABA_A receptor and produce strong sedative and hypnotic effects. These medications include zolpidem (Ambien), zaleplon (Sonata), and eszopiclone (Lunesta). These agents decrease sleep onset latency, and increase stage 2 and slow-wave sleep (Nicholson and Pascoe, 1986; Scharf et al., 1994; Walsh et al., 2000; Krystal et al., 2003; Zammit et al., 2004; Roth et al., 2005). The efficacy of these compounds is tied to their exposure, i.e., agents with longer half-lives might be better suited to address sleep maintenance insomnias, but they also tend to have more significant next-day hangover effects that are associated with psychomotor and memory impairments (Drover, 2004). Agents like zaleplon, which have a very short half-life of approximately 1 h, are very useful for sleep-onset insomnia and have no hangover effects, but they are not ideal for sleep maintenance (Troy et al., 2000; Walsh et al., 2000). Inverse agonism of the 5HT2A receptor seems to produce sleep maintenance without residual psychomotor impairment upon wakening. Nelotanserin administered at 10 and 40 mg has a half-life of 3.9 and 10.7 h, respectively. These longer half-lives seem to be of little consequence with respect to residual effects for this class of drug. As shown in the phase 1 studies, nelotanserin showed no clinically important psychomotor impairment, even at peak blood levels.

To date, no 5-HT2A inverse agonist has been approved for treatment of insomnia, although at least two are in late-stage clinical testing, namely volinanserin and eplivanserin (information obtained from www.clinicaltrials.gov). Based on our studies, nelotanserin, which promotes sleep consolidation and slow-wave deep sleep, might be ideal for the treatment of sleep maintenance insomnias, in particular, because these effects are devoid of the psychomotor and memory impairments associated with the GABA hypnotics. Indeed, polled data compiled by the National Sleep Foundation suggest that the more common symptoms of insomnia are associated with sleep maintenance, including waking up feeling unfreshened and waking up several times during the night. Less common symptoms include difficulty falling asleep, and/or waking up too early and not being able to return to sleep (2005 Sleep in America Poll, National Sleep Foundation, Washington, DC). These findings suggest a medical need that might be better met with 5-HT2A inverse agonists like nelotanserin that hold the promise of improvement in both efficacy and safety profiles compared with traditional GABA hypnotics. This promise rests on the outcomes of ongoing and future clinical trials.

Acknowledgments

We thank James M. Willis for editorial assistance.

References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 per cent inhibition (IC50) of an enzymatic reaction. Biochem Pharmacol 22:2699–2708.


Landolt HP, Meier V, Burgess HD, Finelli LA, Cattelin P, Aechmann P, and Borbely