

Identification and Characterization of GAL-021 as a Novel Breathing Control Modulator

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ABSTRACT

Background: The authors describe the preclinical pharmacological properties of GAL-021, a novel peripheral chemoreceptor modulator.

Methods: The ventilatory effects of GAL-021 were characterized using tracheal pneumotachometry (n = 4 to 6), plethysmography (n = 5 to 6), arterial blood gas analyses (n = 6 to 11), and nasal capnography (n = 3 to 4) in naive animals and those subjected to morphine-induced respiratory depression. Morphine analgesia in rats was evaluated by tail-flick test (n = 6). Carotid body involvement in GAL-021 ventilatory effects was assessed by comparing responses in intact and carotid sinus nerve–transected rats. Hemodynamic effects of GAL-021 were evaluated in urethane-anesthetized rats (n = 7). The pharmacological profile of GAL-021 *in vitro* was investigated using radioligand binding, enzyme inhibition, and cellular electrophysiology assays.

Results: GAL-021 given intravenously stimulated ventilation and/or attenuated opiate-induced respiratory depression in rats, mice, and nonhuman primates, without decreasing morphine analgesia in rats. GAL-021 did not alter mean arterial pressure but produced a modest increase in heart rate. Ventilatory stimulation in rats was attenuated by carotid sinus nerve transection. GAL-021 inhibited $K_{Ca}1.1$ in GH3 cells, and the evoked ventilatory stimulation was attenuated in *Slo1*^{-/-} mice lacking the pore-forming α -subunit of the $K_{Ca}1.1$ channel.

Conclusions: GAL-021 behaved as a breathing control modulator in rodents and nonhuman primates and diminished opioid-induced respiratory depression without compromising opioid analgesia. It acted predominantly at the carotid body, in part by inhibiting $K_{Ca}1.1$ channels. Its preclinical profile qualified the compound to enter clinical trials to assess effects on breathing control disorders such as drug (opioid)-induced respiratory depression and sleep apnea. (**ANESTHESIOLOGY 2015; XXX:00-00**)

PERIOPERATIVE sleep apnea is an epidemic,¹ and a critical medical need exists for novel therapeutics to support breathing, maintain pain control, and lower hospital costs for surgical patients with sleep-disordered breathing. GAL-021 is being developed as a novel breathing control modulator to preserve respiratory drive and protect patients from respiratory impairment due to opioids and other modalities.

Only caffeine, doxapram, and almitrine are currently used clinically as breathing control modulators and, in particular, as respiratory stimulants. Caffeine, used primarily for apnea of prematurity, is an analeptic agent that stimulates breathing *via* adenosine receptor antagonism and/or phosphodiesterase inhibition at the level of the brainstem.² Doxapram, the most commonly prescribed respiratory stimulant, is used in the treatment of apnea of prematurity and acute respiratory depression in adults, but its use may be limited by cardiovascular adverse effects.³ The use of almitrine is severely

What We Already Know about This Topic

- Caffeine, doxapram, and almitrine are used clinically as respiratory stimulants, but use of the latter two may be limited by side effects
- GAL-021 is a novel breathing control modulator that is based on selective modification of the almitrine pharmacophore

What This Article Tells Us That Is New

- Intravenously administered GAL-021 attenuated opiate-induced respiratory depression in rats and nonhuman primates without affecting morphine analgesia in rats
- GAL-021 ventilatory stimulation in rats was attenuated by carotid sinus nerve transection
- GAL-021 ventilatory stimulation was attenuated in mice lacking the pore-forming α -subunit of the $K_{Ca}1.1$ channel

hampered by peripheral neuropathy,⁴ which develops pursuant to the actions of 4,4'-di fluorobenzhydrylpiperazine (DFBP), the major metabolite formed in humans.⁵

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Similar to acute hypoxia, almitrine and doxapram stimulate breathing by modulating the peripheral chemoreceptors in the carotid body,^{6,7} at least in part, by blocking the oxygen-sensitive K⁺ channels. These include voltage-dependent and Ca²⁺-dependent K_{Ca}1.1 channels (also known as Slo1, BK, BK_{Ca}, or maxi K and encoded by the gene KCNMA1) for almitrine and TASK-1 and/or TASK-3 channels for doxapram.^{3,8} We hypothesized that selective modifications to the almitrine pharmacophore would retain the desirable breathing control properties while minimizing the toxic liability. Accordingly, we synthesized and evaluated a series of alkylaminotriazines lacking the DFBP moiety using a phenotypic screening approach measuring ventilatory stimulation in anesthetized rats. Due to its magnitude of effect and rapid onset of action in this screen, *N*-(4,6-*bis-n*-propylamino-[1,3,5]-triazin-2-yl)-*N*, *O*-dimethylhydroxylamine (GAL-021) was selected for further evaluation. This report documents the preclinical profile of GAL-021, which has successfully completed phase 1 clinical trials, demonstrating its safety, pharmacokinetics, and pharmacodynamics as a ventilatory stimulant⁹ and ability to reverse opioid-induced respiratory depression in human volunteers.¹⁰

Materials and Methods

Animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committees at Galleon Pharmaceuticals, Inc., University of Pennsylvania, Philadelphia, Pennsylvania, or East Carolina University, Greenville, North Carolina. Animal husbandry was conducted under United States Department of Agriculture guidelines.

Rat and Mouse Spirometry

Male Sprague–Dawley rats (350 to 400 g; Harlan, USA) (n = 4) were anesthetized with urethane (1.8 g/kg IV) and instrumented as previously described.¹¹ In brief, PE-240 tubing was ligated into the cervical trachea and connected to a pneumotachometer (MLT1L; AD Instruments Inc., USA) and differential pressure transducer (FE141; AD Instruments) to measure respiratory airflow. Animals were permitted to breathe spontaneously on room air throughout the experiment. Femoral vessels were cannulated to permit monitoring of arterial blood pressure (transducer: SP844-28; Mems-cap Inc., USA; bridge amplifier: FE221; AD Instruments) and IV delivery of compounds. Airflow and blood pressure waveforms were digitized (PowerLab; AD Instruments) and continuously recorded (Lab-Chart-7 Pro software; AD Instruments). Respiratory flow was used to measure respiratory rate (RR) from cycle period and integrated to measure tidal volume (V_T). Minute volume (\dot{V}_E) was calculated as the product of RR and V_T. GAL-021 (stock 3.0 mg/ml in 0.9% saline) was administered IV as a slow bolus at 10-min intervals (0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg). The percent change in \dot{V}_E was plotted against the log dose of GAL-021 to calculate

an ED₅₀ value using a three-parameter nonlinear regression model ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{LogED}_{50} - X)})$) (Prism; GraphPad Software, Inc., USA). In a separate group of rats (n = 6), carotid bodies were denervated by transecting both carotid sinus nerves (CSNs) where they branch off the glossopharyngeal nerve. Sham-operated animals (n = 6) were included as controls.

The effects of GAL-021 on mean arterial pressure (MAP) and heart rate (HR) were evaluated using IV infusions, and not boluses, because preliminary assessment indicated that these cardiovascular parameters were highly variable after rapid IV injection. GAL-021 (0.125 mg kg⁻¹ min⁻¹ for 25 min, increasing to 0.20 mg kg⁻¹ min⁻¹ for an additional 25 min IV, n = 7) and vehicle (0.9% saline, for 50 min, n = 7) were administered at a constant infusion rate (6 ml kg⁻¹ h⁻¹). All rats received additional fluid support (50:50 mixture of lactated Ringer's solution and 6% hetastarch in 0.9% saline at 4 ml kg⁻¹ h⁻¹).

The effects of GAL-021 on ventilation were also evaluated in age-matched male and female adult *Slo1*^{+/+} (20 to 22 g, n = 6) and *Slo1*^{-/-} (16 to 18 g, n = 5) mice. Animals were generated as previously reported¹² and were obtained from Dr. Andrea Meredith, University of Maryland, College Park, Maryland. The *Slo1*^{-/-} mice lack the pore-forming α -subunit of the K_{Ca}1.1 channel. *Slo1*^{+/+} and *Slo1*^{-/-} animals were littermates generated from heterozygote matings and were genotyped before study and instrumented as described for rats. Mice were anesthetized using 2 to 2.5% isoflurane in air (final F_{IO₂} = 0.21). As described in Material and Methods, Rat and Mouse Spirometry section, for rats, tracheal airflow was measured using flow spirometry before and after IV (femoral vein) bolus administration of GAL-021 (0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg) and vehicle (0.9% saline).

Morphine-induced Respiratory Depression in Rats

The effects of GAL-021 on morphine-induced respiratory depression were evaluated using whole-body plethysmography (n = 5 to 6 per group) and arterial blood gas (ABG) analysis (n = 6 to 11 per group). Plethysmography studies (PLY 3223; Buxco, Inc., USA) were conducted as described previously.¹³ Jugular vein cannulated rats were acclimated to plethysmography chambers for a minimum of 60 min before starting each experiment. The respiratory airflow waveform was derived from a pneumotachometer in the chamber wall. The airflow waveform, chamber temperature, and humidity were measured continuously and used in the calculation of V_T using the Epstein and Epstein algorithm.¹⁴ Body temperature was assumed to be 37°C throughout the study. RR was derived from the airflow waveform cycle period. Morphine sulfate (10 mg/ml; 10 mg/kg, IV) was administered as an IV bolus to elicit respiratory depression. GAL-021 (0.6, 1.5, and 6.0 mg/ml; 0.04, 0.1, and 0.4 mg kg⁻¹ min⁻¹) or vehicle (0.9% saline) was administered 5 min later as an IV infusion for 20 min.

For the ABG study, rats with femoral arterial and venous cannulas were acclimated to small rodent chambers for

60 min or longer before study. Morphine (10 mg/kg IV bolus) was administered followed 15 min later by IV infusion of GAL-021 (0.03, 0.1, 0.3, and 1.0 mg kg⁻¹ min⁻¹) for 30 min. ABG samples were collected and analyzed (ABL800 Flex; Radiometer America Inc., USA) for pH, PaCO₂, and PaO₂ before morphine administration (time = 0), 12-min post-morphine, and 25 min into the GAL-021 infusion.

Tail-flick Assay

The antinociceptive effects of morphine and GAL-021 were assessed by tail-flick latency test¹⁵ using a Tail-Flick Analgesia Meter (IITC Life Science Inc., USA). This involved minor manual restraint while positioning the tail to apply a thermal stimulus (instrument setting of 50 to 75% active intensity), sufficient to induce a latency of tail withdrawal of 2 to 3 s in control animals. Rats (n = 6 per group) with jugular vein cannulas received morphine (1.5 mg/kg IV bolus) followed immediately by either GAL-021 (0.3 mg kg⁻¹ min⁻¹, 60 min, IV infusion) or saline (IV infusion). An additional group received only GAL-021 (0.3 mg kg⁻¹ min⁻¹) without morphine.

Pharmacological Efficacy of GAL-021 in Cynomolgus Monkeys

The effects of GAL-021 on breathing and morphine-induced respiratory depression were evaluated in *Cynomolgus macaques* (*Macaca fascicularis*, 4 yr old, 2 to 5 kg; n = 3 to 4 per group). Anesthesia was induced with 5% isoflurane in oxygen (F_{IO₂} = 0.95) and maintained with 1.5 to 2% isoflurane in oxygen (F_{IO₂} = 0.98) using a tight-fitting facemask. Antebrachial veins were cannulated for drug delivery and blood sampling. Vivometrics Lifeshirts (VivoMetrics, Inc., USA) were fitted to the animals for monitoring respiratory function by inductance plethysmography, as previously described.¹⁶ Abdomen and rib cage deflections were calibrated using the qualitative diagnostic calibration procedure, and tidal volume was normalized to 15 ml, a typical value for these animals as previously measured using conventional techniques. To assess the effects of GAL-021 on breathing, cynomolgus monkeys remained in left lateral recumbency throughout the experiment. End-tidal carbon dioxide (ETCO₂) was measured *via* a neonatal nasal cannula connected to a microstream carbon dioxide sensor (Cardell monitor, Model 9405; Midmark Corporation, USA). Bolus doses of GAL-021 (0.01, 0.03, and 0.1 mg/kg dissolved in 20% hydroxypropyl β-cyclodextrin or 0.9% saline) were delivered over 20 s to animals while anesthetized, with 5 min between the start of each dose. To investigate whether the anesthetic *per se* produced changes in airways resistance that could confound the interpretation of GAL-021 effects, we monitored or considered the presence of eupneic breathing pattern, the phase-angle relation for expansion of the thoracic and abdominal bands, the small change in upper airway resistance predicted for varying inhaled dose with the range used for this study (1.5 to 2%), and the magnitude and

direction of change for ETCO₂ during periods of increased respiratory effort.

To assess the effects of GAL-021 on morphine-induced respiratory depression, cynomolgus monkeys were anesthetized and instrumented as described in the preceding paragraph and then moved from left lateral recumbency to a sitting position in a primate chair to recover from isoflurane anesthesia. The body position change precluded use of inductance plethysmography to accurately measure ventilation after anesthesia. Therefore, changes in ETCO₂ were used to indicate morphine-induced and GAL-021-induced changes in ventilation. Upon recovery from anesthesia, morphine (3 to 4 mg/kg IV) was administered followed 15 min later by vehicle or GAL-021 infusion at one of two paradigms: (1) a 5-min load at 0.2 mg kg⁻¹ min⁻¹ IV, with maintenance infusion of 0.1 mg kg⁻¹ min⁻¹ for the remainder of the experiment; and (2) a 5-min load at 0.1 mg kg⁻¹ min⁻¹ IV, with maintenance infusion of 0.05 mg kg⁻¹ min⁻¹ for the remainder of the experiment. Immediately before terminating the GAL-021 infusion, a 0.5-ml venous blood sample was collected for plasma drug concentration analysis.

Methods of Analysis

GAL-021 was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Each plasma sample was mixed with three volumes of acetonitrile containing an internal standard, followed by vortexing and centrifugation at 3,000g for 10 min. The resulting supernatant was then analyzed by LC-MS/MS (LC-20AD pumps, SIL-5000 autosampler; Shimadzu Scientific Instruments, USA, and an API-4000 LC-MS/MS system; AB/Sciex, USA) using calibration standards ranging from 2 to 5,000 ng/ml. Quality control samples at three concentration levels (10, 100, and 1,000 ng/ml) were also included in each run. Samples were separated on an Atlantis T3 column (4.6 × 50 mm; Waters Corp., USA) at a flow rate of 1.0 ml/min, using 3-min gradient elution from 95% mobile phase A (0.1% formic acid in water) to 100% mobile phase B (0.1% formic acid in acetonitrile) with an injection volume of 10 μl. All analytes were ionized in the positive electrospray ionization mode and detected using selected reaction monitoring at mass-to-charge ratio 255.1 to 224.1 for GAL-021. All ion source and tandem mass spectrometry instrument parameters for the analytes were optimized for high sensitivity and selectivity.

In Vitro Specificity Assays

In these studies, GAL-021 was dissolved in dimethyl sulfoxide, and final assay concentration of dimethyl sulfoxide was 0.1% or less with one exception (γ-aminobutyric acid [GABA] electrophysiology; ≤0.3%).

The effects of GAL-021 (30 μM) on a panel of 55 receptors, transporters, and ion channels were evaluated using radioligand binding analyses (Express Profile; Eurofins CEREP, USA). Potential kinase inhibition by GAL-021 (10 μM) was assessed using the Kinase HotSpot Screen

(Reaction Biology Corp., USA), where activity of 50 kinases was measured in the presence of adenosine triphosphate (10 μ M). Effects of GAL-021 (30 μ M) on 12 different human cardiac ion channels ($Ca_v1.2$, $Ca_v3.2$, HCN2, HCN4, hERG, $K_{ir}2.1$, $K_{ir}3.1/K_{ir}3.4$, $K_{ir}6.2/SUR2A$, $K_v1.5$, $K_v4.3$, $K_vLQT1/minK$, and $Na_v1.5$) were evaluated using an automated parallel patch clamp system (ChanTest, USA).

The effects of GAL-021 (1 to 10 μ M) on single $K_{Ca}1.1$ channel activity in rat pituitary tumor GH3 cells were measured using conventional (nonautomated) patch clamp electrophysiology (ChanTest) assay. Patch pipettes were made from glass capillary tubing using a P 97 micropipette puller (Sutter Instruments, USA), and the amplifier was Axopatch 200B (Axon Instruments; Molecular Devices, USA). Single-channel currents were recorded in inside-out patches in symmetrical KCl solutions at a holding potential of -60 mV. The bath (intracellular) solution comprised 125 mM KCl, 10 mM HEPES, 11.25 mM EGTA, pH adjusted to 7.2 with potassium hydroxide, and was Ca^{2+} free. The pipette (extracellular) solution comprised 125 mM KCl, 8.5 mM $CaCl_2$, 10 mM HEPES, 11.25 mM EGTA, and pH adjusted to 7.2 with potassium hydroxide. Under these conditions, the strong driving force for Ca^{2+} influx activated single $K_{Ca}1.1$ channels. Test compounds were added to the bath solution (intracellular).

The effects of GAL-021 on GABA receptor type A ($GABA_A$) channel electrophysiology were evaluated using cloned human $GABA_A$ channels ($\alpha 1/\beta 2/\gamma 2$) expressed in *Xenopus* oocytes. Individual oocytes were injected with 5 to 50 ng of each subunit mRNA, and two-electrode voltage clamp recordings were made 3 to 14 days after mRNA injections at a holding voltage of -70 mV. GAL-021 was evaluated for effects on submaximal GABA-evoked control currents that were approximately 10% (" EC_{10} ") of maximal GABA currents.

Statistical Evaluation

Sample sizes were selected based on previous experience quantifying ventilation and respiratory motor drive. Blinding methods were not performed. Although formal randomization methods were not used, animals were not assigned to any group based on *a priori* selection criteria. Minute volume was expressed as the percent of baseline above baseline. A three-parameter nonlinear regression model was used to create best-fit lines and calculate ED_{50} values ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{Log}ED_{50} - X)})$). The effects of GAL-021 in wild-type and *Slo1*^{-/-} mice, effects of CSN transection, effects of morphine and GAL-021 on breathing and ABG, and effects of GAL-021 and morphine in the tail-flick assay were all evaluated using two-way ANOVA with repeat measures on time. When differences were detected with ANOVA, Tukey, Sidak, or Bonferroni multiple comparisons tests were used to detect differences between means. Differences between means were considered significant when *P* value was less than 0.05. Statistical analyses were performed using Prism (GraphPad Software, Inc.). Values are expressed as means \pm SD.

Results

Ventilatory Stimulant Effects of GAL-021

Phenotypic screening of test compounds IV in anesthetized rats using direct tracheal spirometry identified GAL-021 (fig. 1) as a breathing control modulator. When administered as an IV bolus, GAL-021 produced large (maximum approximately 170%), dose-dependent ($ED_{50} = 0.1$ mg/kg) increases in \dot{V}_E , arising from effects on V_T and RR (fig. 1). Ventilatory stimulation evoked by GAL-021 was also evident in mice (\dot{V}_E $ED_{50} = 0.5$ mg/kg) (fig. 2) and cynomolgus monkeys (fig. 3). In the anesthetized cynomolgus monkey studies, we observed no evidence of meaningful changes in airway resistance related to varying degrees of anesthesia before or during GAL-021 administration as indicated by absence of paradoxical chest movement, plethysmography phase shift, stridor, or prolongation of inspiratory time.

Pharmacological Profile of GAL-021

Pharmacological activity and specificity of GAL-021 were assessed initially using commercially available radioligand binding assays (panels of receptors, ion channels, and transporters), enzymology assays (kinase inhibition in presence of 10 μ M adenosine triphosphate), and electrophysiology assays (GH3 cells and cardiac ion channel panel). Using inside-out patches in GH3 cells, GAL-021 exerted concentration-dependent inhibition of single-channel $K_{Ca}1.1$ activity (22% inhibition [I] at 1 μ M = 254 ng/ml; 42% I at 10 μ M), a target inhibited by almitrine (90% I at 1 μ M).⁶ When evaluated against 12 different cardiac ion channels, inhibition was 35% or less at 30 μ M. No significant kinase inhibition was observed at 10 μ M. At 30 μ M in the radioligand binding assays, interactions (defined as $>50\%$ radioligand displacement) were detected at adenosine A_1 (65% I), A_{2A} (79% I, IC_{50} approximately 5 μ M), and A_3 (93% I; IC_{50} approximately 1 μ M) receptors, at 5-HT_{2B} receptors (60% I; IC_{50} approximately 30 μ M), and at $GABA_A$ receptors (picrotoxin-binding site; 61% I). Follow-up cellular and tissue functional assays for adenosine A_1 , A_{2A} , and A_3 receptors indicated weak nonspecific effects. The interaction of GAL-021 with $GABA_A$ receptors was evaluated electrophysiologically (data not shown) and was characterized as low-potency positive allosteric modulation. Because $GABA_A$ receptors are inhibitory toward carotid body activation¹⁷ and because there are no reports of modulation of ventilation by 5-HT_{2B} agonists or antagonists, we concluded that interactions with A_1 , A_{2A} , A_3 , 5-HT_{2B}, and $GABA_A$ receptors would not be relevant to the pharmacology of GAL-021 at plasma concentrations associated with ventilatory responses.

Carotid Body Involvement in the Ventilatory Stimulant Effects of GAL-021 in Rats

Almitrine and related compounds mimic some of the effects of hypoxia in provoking carotid body chemotransduction to increase ventilation. To investigate whether the carotid body

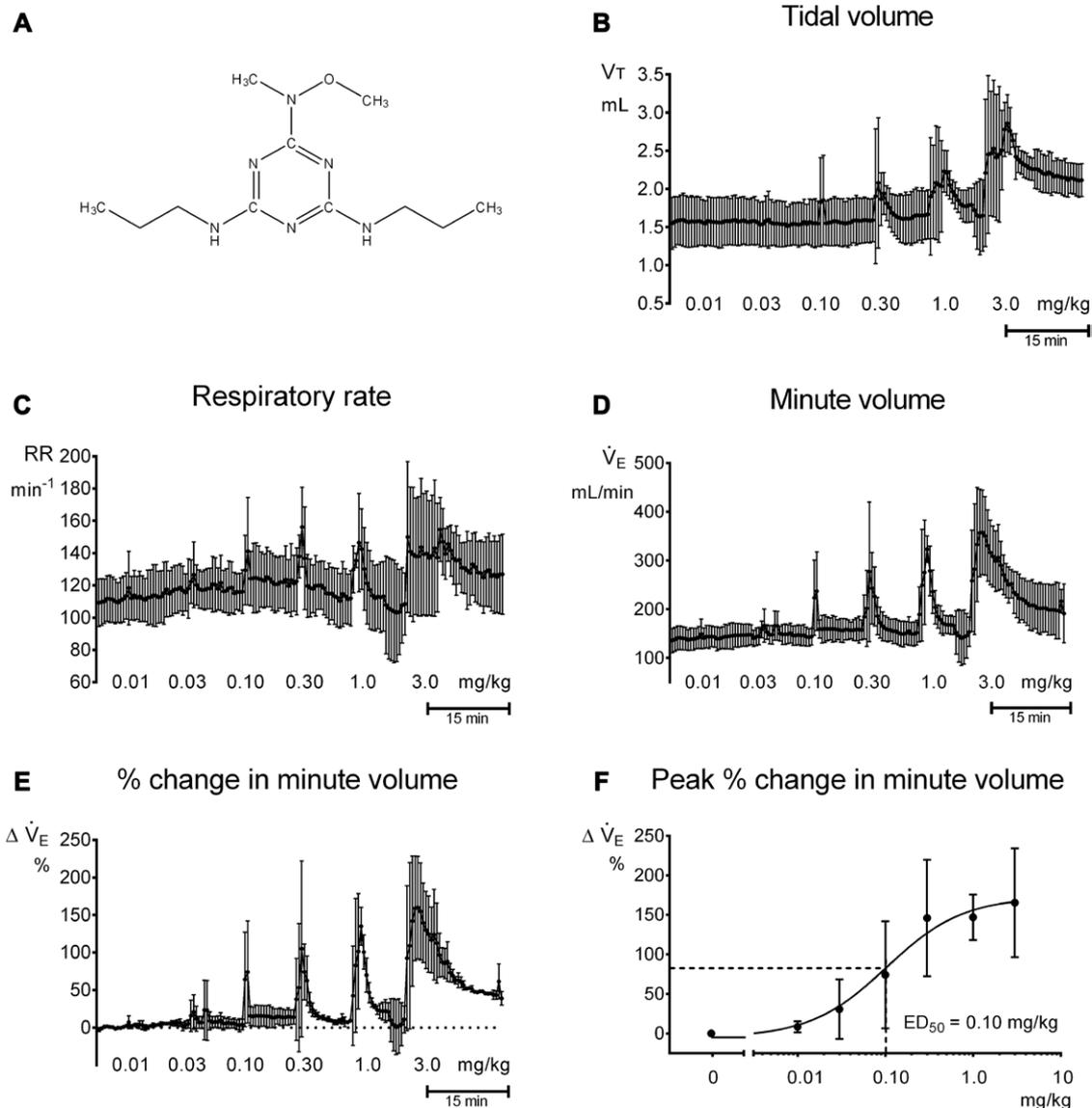


Fig. 1. The effects of GAL-021 on breathing in urethane-anesthetized rats. Ventilatory parameters were measured using direct tracheal spirometry. GAL-021 (0 to 3 mg/kg) was administered intravenously as boluses using a cumulative dose paradigm. GAL-021 (A) dose dependently increased (B) tidal volume (V_T), (C) respiratory rate (RR), and (D) their product minute volume (\dot{V}_E) above baseline values. The effects of GAL-021 on breathing were characterized as fast onset and short duration. The duration of effect increased with increasing dose. (E) Normalization of the changes in minute volume expressed as the percent of baseline above baseline. (F) A three-parameter nonlinear regression model was used to identify an ED_{50} of 0.10 mg/kg. Values are means \pm SD, $n = 4$.

was the primary site of action of GAL-021 in rats, we evaluated the effects of acute CSN transection on its ventilatory effects. When administered to anesthetized rats immediately after bilateral CSN transection or sham surgery, the ventilatory effects of GAL-021 (0.3 mg/kg IV bolus) were markedly diminished (approximately 80% reduction) in the CSN-transected rats compared with sham-operated controls (fig. 4). These data suggest that GAL-021 stimulates breathing predominantly by effects on the carotid body. Functional carotid body denervation was confirmed using exposure to acute hypoxia before and after CSN transections (fig. 4). In preliminary studies, we established that GAL-021-induced ventilatory stimulation in

anesthetized rats was only modestly reduced when breathing pure oxygen ($F_{IO_2} = 1.0$; \dot{V}_E $E_{max} = 113\%$) compared with room air ($F_{IO_2} = 0.21$; \dot{V}_E $E_{max} = 148\%$), suggesting that ventilatory stimulant efficacy can be maintained under conditions of oxygen supplementation.

Involvement of $K_{Ca}1.1$ in the Ventilatory Stimulant Effects of GAL-021: Studies Using $Slo1^{-/-}$ Mice

To further probe $K_{Ca}1.1$ involvement in ventilatory stimulation evoked by GAL-021, dose-response curves for GAL-021-induced increases in \dot{V}_E were compared in wild-type ($Slo1^{+/+}$) mice and in $Slo1^{-/-}$ mice lacking the pore-forming α -subunit

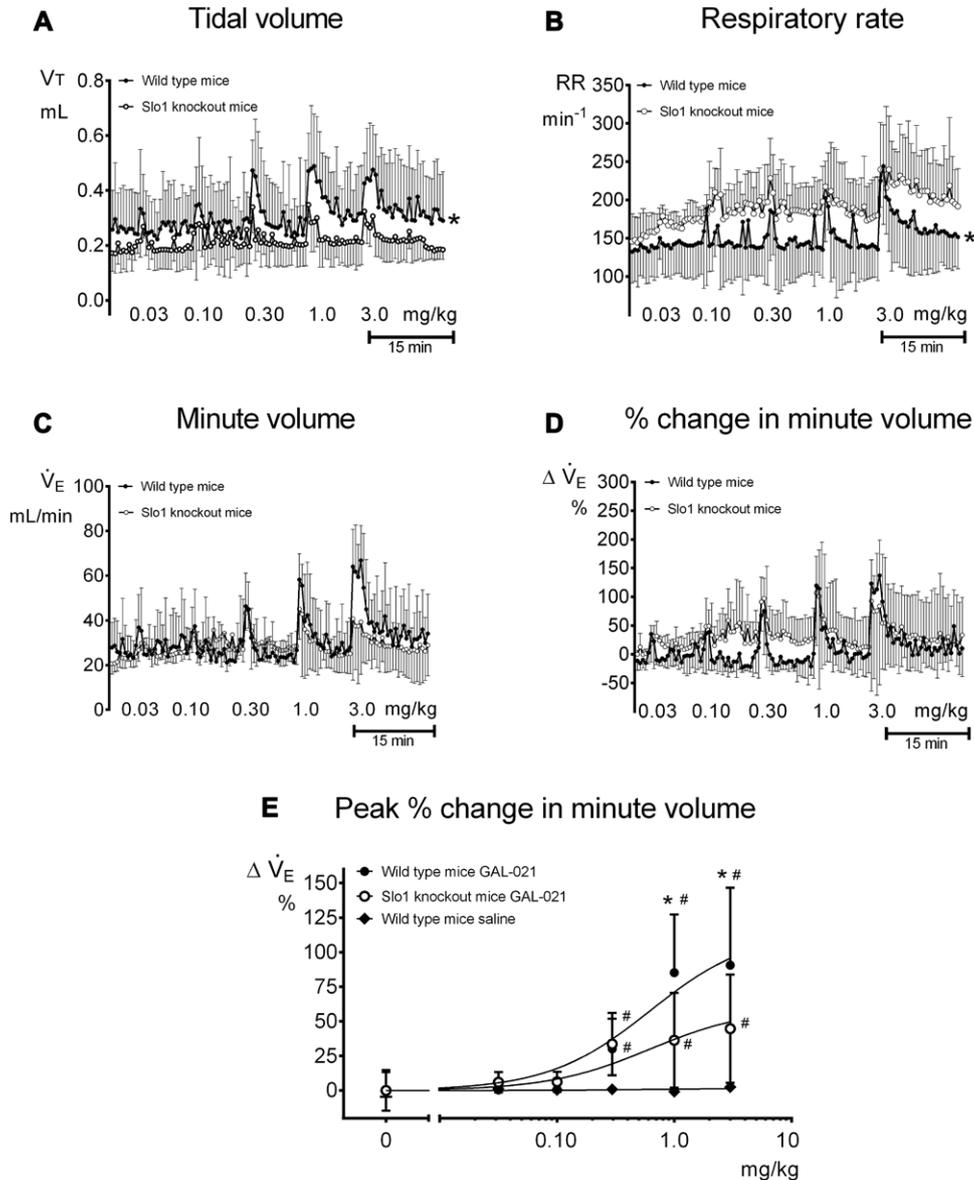


Fig. 2. The effects of GAL-021 on breathing in isoflurane-anesthetized wild-type and pore-forming BK_{Ca}1.1 α subunit (*Slo1*^{-/-}) knockout mice. Ventilatory parameters were measured using direct tracheal spirometry. GAL-021 (0 to 3 mg/kg) was administered intravenously as boluses using a cumulative dose paradigm. GAL-021 dose dependently increased (A) tidal volume (V_T), (B) respiratory rate (RR), and (C) their product minute volume (\dot{V}_E) above baseline values. (D) The change in \dot{V}_E expressed as percentage of baseline above baseline. (E) The peak percent change in \dot{V}_E was normalized to the mean maximal increase in \dot{V}_E at 3.0 mg/kg GAL-021 in the wild-type group and then a three-parameter nonlinear regression model applied to identify the best-fit curves. An equivalent volume of saline administered intravenously had no effect on minute volume. *Different from the knockout mouse GAL-021 group; #different from the wild-type saline group (two-way ANOVA, with Tukey multiple comparison tests; $P < 0.05$). Values are means \pm SD, $n = 6$ for wild-type GAL-021 and wild-type saline groups, and $n = 5$ for the *Slo1*^{-/-} group.

of K_{Ca}1.1. The dose–response curve for GAL-021-induced ventilatory stimulation was apparently right shifted in *Slo1*^{-/-} mice ($ED_{50} = 2.8$ mg/kg) compared with *Slo1*^{+/+} mice ($ED_{50} = 0.5$ mg/kg) (fig. 2). Furthermore, the peak ventilatory response to 1 and 3 mg/kg GAL-021 was significantly larger in wild-type mice than in knockout mice (fig. 2). Although baseline minute volume was not different between the groups (fig. 2, C and D), the knockout mouse maintained

minute volume using a lower V_T (fig. 2A) and higher RR (fig. 2B) than the wild-type group. This rapid shallow breathing pattern in knockout mice was not influenced by time or dose of GAL-021 administered. Preliminary findings (unpublished data; Hoshi T., Ph.D., Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania, and Baby S., Ph.D., Galleon Pharmaceuticals, Inc., Horsham, Pennsylvania, February 2011) using whole-body

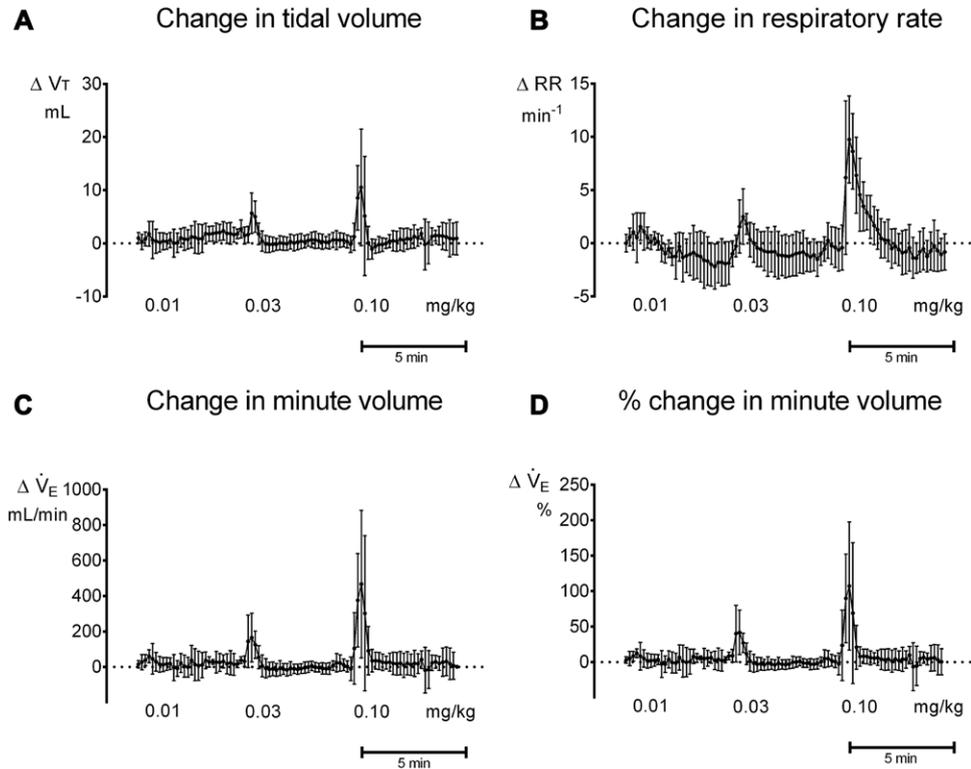


Fig. 3. The effects of GAL-021 on breathing in isoflurane-anesthetized cynomolgus monkeys. Ventilatory parameters were measured using inductance plethysmography. GAL-021 (0.01 to 0.10 mg/kg) was administered intravenously as boluses using a cumulative dose paradigm. GAL-021 dose dependently increased (A) tidal volume (V_T), (B) respiratory rate (RR), and (C and D) their product minute volume (\dot{V}_E) above baseline values. The effects of GAL-021 on breathing were characterized as fast onset after injection and short duration. Values are means \pm SD, $n = 4$.

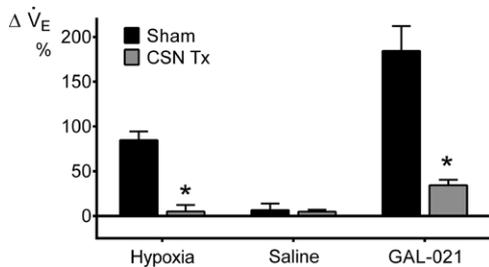


Fig. 4. The effects of GAL-021 on the peak change in minute volume ($\Delta\dot{V}_E$) in urethane-anesthetized rats with intact (sham operated) and bilaterally transected carotid sinus nerves (CSN). CSN transection (Tx) abolished the ventilatory response to acute hypoxia ($F_{iO_2} = 0.12$ during 3 min), confirming the functional completeness of the carotid body denervation. There was no effect on breathing of administering vehicle (saline) before or after carotid body denervation. The effects of GAL-021 (0.3 mg/kg IV bolus) on breathing were markedly diminished in rats with carotid body denervation compared with sham-operated controls. *Different from sham-operated rats (two-way ANOVA with Sidak multiple comparisons tests; $P < 0.05$). Values are means \pm SD, $n = 6$.

plethysmography indicate that ventilatory responses to normoxic, hypoxic, hyperoxic, and hypercapnic gas mixtures are similar in *Slo1^{+/+}* and *Slo1^{-/-}* mice. Thus, reduced efficacy of GAL-021 in *Slo1^{-/-}* mice is unlikely to be due to an altered pattern of breathing.

Effects of GAL-021 in Models of Opiate-induced Respiratory Depression in Rats and Cynomolgus Monkeys

The effects of GAL-021 under conditions of impaired ventilation were assessed in conscious rat and cynomolgus monkey models of respiratory depression induced by opiates (opiate-induced respiratory depression [OIRD]). In the rat model, consistent with literature reports,¹⁸ morphine (10 mg/kg IV) caused a decrease in \dot{V}_E due to decreases in RR and V_T as well as an increase in P_{aCO_2} and decreases in pH and P_{aO_2} . In ABG studies, when administered as an IV infusion post-morphine, GAL-021 (0.03 to 1 mg kg^{-1} min^{-1} for 20 min) caused a dose-dependent reduction in P_{aCO_2} and increases in pH and P_{aO_2} , indicating a reversal of OIRD (fig. 5). In whole-body plethysmography studies, and in comparison with baseline values, morphine produced a 25 to 50% reduction in \dot{V}_E . Under these conditions, no overt periodicity or long apneas were present. GAL-021 (0.04 to 0.4 mg kg^{-1} min^{-1} for 20 min IV) post-morphine produced dose-dependent reversal of the morphine suppression of \dot{V}_E (fig. 6). In this paradigm, GAL-021 increased predominantly V_T with little effect on RR. Overall, the beneficial profile of GAL-021 in the OIRD models was comparable using plethysmography and ABG parameters as indices of efficacy.

In the cynomolgus monkey model of OIRD, conscious animals were given IV morphine to induce respiratory

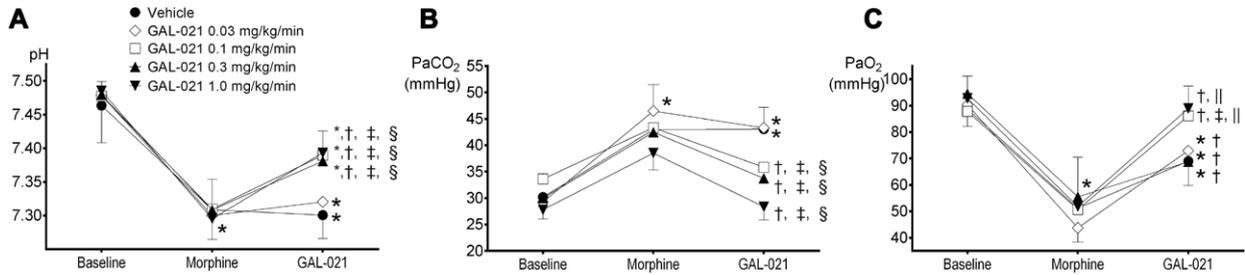


Fig. 5. The effects of GAL-021 on morphine-induced respiratory acidosis and hypoxia in conscious rats. Respiratory depression was assessed using arterial pH and blood gas analysis. After baseline arterial blood samples had been collected, morphine (10 mg/kg) was administered intravenously to induce respiratory depression. Fifteen to 18 min after morphine administration, GAL-021 (0.03, 0.10, 0.3, and 1.0 mg kg⁻¹ min⁻¹) or vehicle was administered as an intravenous infusion for 20 min. Morphine elicited respiratory depression as evidenced by (A and B) respiratory acidosis (decreased pH and increased PaCO₂) and (C) hypoxia (decreased PaO₂) compared with baseline values. Vehicle infusion and the lowest doses of GAL-021 (0.04 mg kg⁻¹ min⁻¹) had no effect on morphine-induced respiratory depression. The three highest doses of GAL-021 (0.1, 0.3, and 1.0 mg kg⁻¹ min⁻¹) partially restored arterial pH and returned PaCO₂ to baseline values. Some of the effect of morphine on PaO₂ appeared to be transient: PaO₂ increased by the end of the vehicle infusion but remained below the baseline value. GAL-021 (0.1 and 1.0 mg kg⁻¹ min⁻¹) increased PaO₂ further so that PaO₂ was no longer different to baseline values. *Different from baseline values; †different from morphine; ‡different from vehicle; §different from GAL-021 0.04 mg kg⁻¹ min⁻¹; ||different from GAL-021 0.3 mg kg⁻¹ min⁻¹ (two-way ANOVA with Bonferroni multiple comparisons test, *P* < 0.05). Values are means ± SD, *n* = 6 to 11 per group.

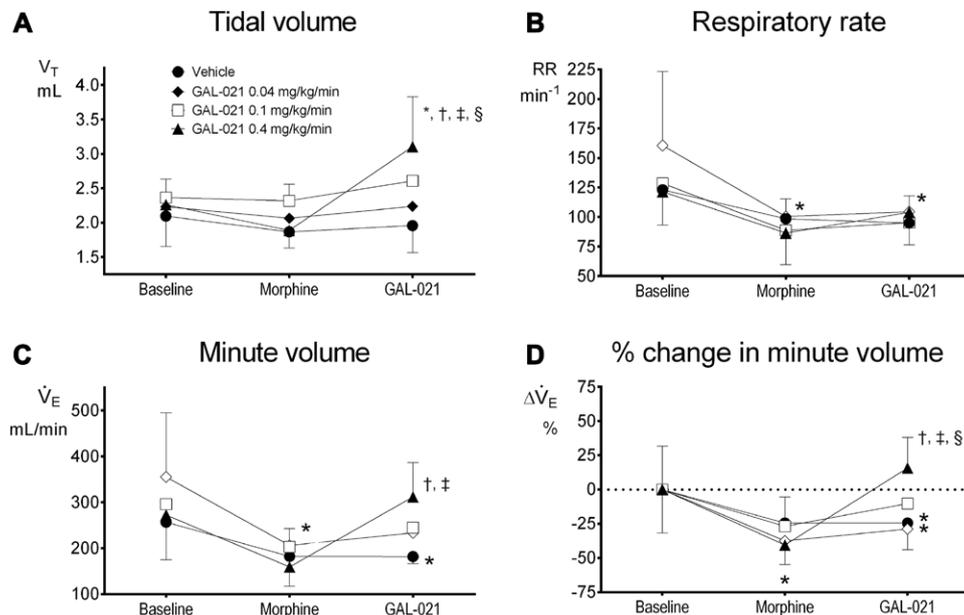


Fig. 6. The effects of GAL-021 on morphine-induced respiratory depression in conscious rats. Ventilatory parameters were measured using whole-body plethysmography and animals were permitted to move freely within the chambers. After baseline ventilatory recordings had been completed, morphine (10 mg/kg) was administered intravenously to induce respiratory depression. Five minutes after morphine administration, GAL-021 (0.04, 0.10, and 0.4 mg kg⁻¹ min⁻¹) or vehicle was administered as an intravenous infusion for 20 min. Morphine had minimal effects on (A) tidal volume (V_T), but decreased (B) respiratory rate (RR) and (C and D) minute volume (V_E). Vehicle infusion and the two lowest doses of GAL-021 had no effect on morphine-induced respiratory depression. The highest dose of GAL-021 (0.4 mg kg⁻¹ min⁻¹) completely reversed the respiratory depression by increasing V_T but with no effect on RR. *Different from baseline values; †different from morphine; ‡different from vehicle; §different from GAL-021 0.1 mg kg⁻¹ min⁻¹ (two-way ANOVA with Bonferroni multiple comparisons test, *P* < 0.05). Values are means ± SD, *n* = 5 to 6 per group.

depression followed by IV infusion of GAL-021 to reverse OIRD. In each animal, IV bolus doses of morphine were titrated (mean = 3.4 mg/kg) to evoke an approximately 10 mmHg increase in ET_{CO₂}. In comparison with vehicle-treated control animals, GAL-021 caused dose-dependent

reductions in ET_{CO₂}, which were rapid in onset and sustained and indicative of reversal of OIRD (fig. 7). The higher dose infusion regimen produced approximately 90% reversal of the morphine-induced increase in ET_{CO₂}, where the GAL-021 plasma level was approximately 700 ng/ml (=2.76 μM).

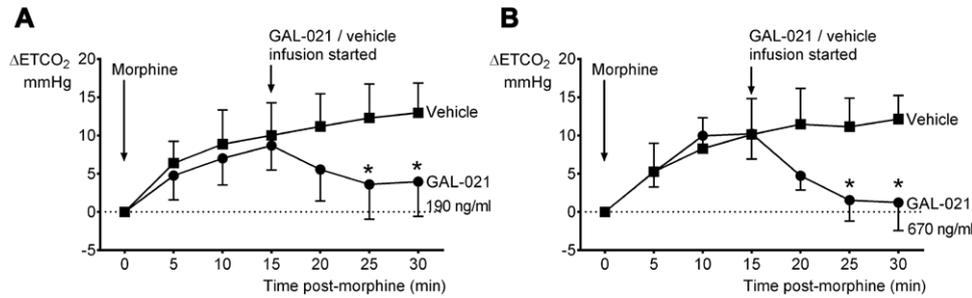


Fig. 7. The effects of GAL-021 on morphine-induced respiratory depression in conscious cynomolgus monkeys. End-tidal carbon dioxide (ETCO_2) was measured using a nasal cannula and side-stream capnography. Morphine (approximately 3 mg/kg) was administered as an intravenous bolus. (A and B) Morphine elicited respiratory depression as evidenced by an increase in ETCO_2 . At 15 min after morphine injection, one of two doses of GAL-021 was initiated as a loading and maintenance intravenous infusion: (A) Loading = 0.1 mg kg⁻¹ min⁻¹ for 5 min, then maintenance = 0.05 mg kg⁻¹ min⁻¹ for 10 min; (B) Loading = 0.2 mg kg⁻¹ min⁻¹ for 5 min, then maintenance = 0.1 mg kg⁻¹ min⁻¹ for 10 min. GAL-021 infusion diminished the magnitude of morphine-induced respiratory depression as evidenced by decreased ETCO_2 toward the end of the infusion period. Mean GAL-021 plasma concentrations for each dose at the end of the infusions are listed on the graphs. *Significantly different from vehicle infusion (two-way ANOVA with Bonferroni multiple comparisons test, $P < 0.05$). Data points are connected by straight lines for an illustrative purpose only. Values are means \pm SD, $n = 3$ to 4 per group.

The lower-dose infusion regimen yielding plasma concentrations of approximately 200 ng/ml (=0.79 μM) was associated with approximately 70% reversal of morphine-induced increases in ETCO_2 .

Effects of GAL-021 on Morphine-induced Thermal Nociception in Rats

A critical consideration for treating OIRD with a respiratory stimulant is that, unlike naloxone, the therapy should neither compromise opioid analgesia nor induce opioid withdrawal.^{19,20} To investigate the potential modulation of opioid analgesia, GAL-021 was infused at 0.3 mg kg⁻¹ min⁻¹ IV for 1 h, and morphine-induced analgesia was assessed using a standard rat tail-flick assay, which reports thermal nociceptive thresholds (fig. 8). In vehicle-treated groups, 1.5 mg/kg IV morphine produced strong analgesia at the 15- and 30-min time points, with tail-withdrawal latencies returning to baseline levels by 75 min. GAL-021 alone did not alter nociception. However, GAL-021 prolonged the duration of action of morphine, with full analgesia evident at 120 min. Tail-withdrawal latencies returned to baseline by 180 min. There was no evidence that GAL-021 reduced or otherwise compromised morphine analgesia.

Hemodynamic Effects of GAL-021 in Rats

As clinical use of doxapram can be limited by hemodynamic effects, we evaluated effects of GAL-021 or vehicle (saline) infusion on MAP and HR in urethane-anesthetized rats (fig. 9). GAL-021 infused under ventilatory stimulant regimens (0.125 mg kg⁻¹ min⁻¹ IV for 25 min, then increased to 0.2 mg kg⁻¹ min⁻¹ for an additional 25 min) had no effect on MAP compared with baseline or vehicle treatment. In contrast, GAL-021 progressively and significantly increased HR during the first 25-min infusion with no further increase during the second 25-min infusion.

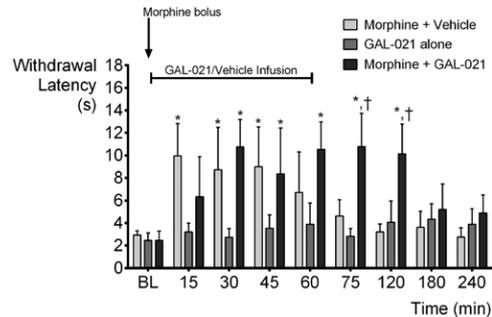


Fig. 8. The effects of GAL-021 infusion on morphine-induced analgesia in rats. The analgesic effects of morphine and GAL-021 were assessed using the tail-flick latency test. The latency to flick the tail in response to a thermal stimulus was measured before and at fixed time points after starting morphine (1.5 mg/kg IV, bolus) and vehicle, GAL-021 (0.3 mg kg⁻¹ min⁻¹, 60 min, IV), and simultaneous morphine (1.5 mg/kg IV, bolus) + GAL-021 (0.3 mg kg⁻¹ min⁻¹, 60 min, IV) infusion. Morphine increased tail-flick latencies, and this effect persisted for 45-min post-injection. GAL-021 infusion alone had no effect on tail-flick latencies. However, in combination, GAL-021 and morphine elicited analgesia of longer duration compared with morphine alone. *Different from GAL-021 alone; †different from morphine and vehicle infusion (two-way ANOVA with Bonferroni multiple comparisons test, $P < 0.05$). Values are means \pm SD, $n = 6$ per group.

Discussion

GAL-021 administered IV exerted potent, dose-dependent stimulation of ventilation in anesthetized and/or conscious mice, rats, and cynomolgus monkeys. Effects on ventilation were manifest as increased \dot{V}_E deriving from evoked increases in V_T and RR and as increased pH and PaO_2 and decreased PaCO_2 . Excitation of carotid bodies initiates a range of cardiovascular reflexes, the sequelae of which are context dependent and vary across species.²¹ After IV infusion in rats using ventilatory stimulant regimens, there was no

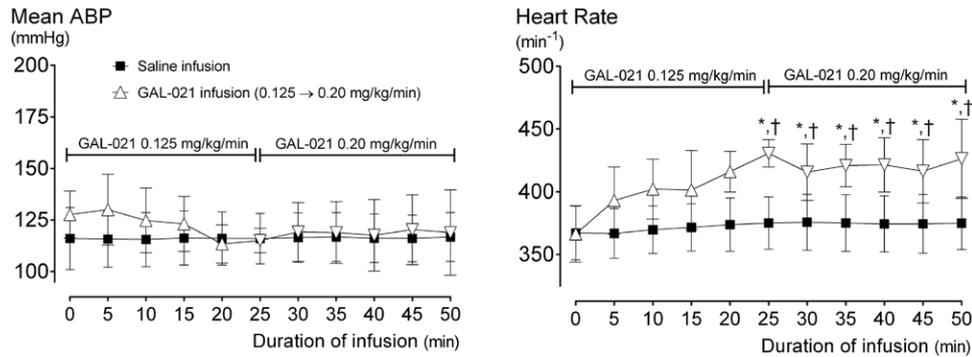


Fig. 9. The effects of GAL-021 infusion on mean arterial blood pressure (ABP) and heart rate in urethane-anesthetized rats. GAL-021 was infused at a dose of 0.125 mg kg⁻¹ min⁻¹ IV for 25 min and then increased to 0.20 mg kg⁻¹ min⁻¹ for an additional 25 min. GAL-021 had no effect on mean ABP compared with baseline (0 min) and with saline control. In contrast, GAL-021 progressively increased heart rate during the first 25 min infusion with no further increase during the second 25-min infusion. Heart rate was significantly higher than baseline and saline control at the 25-min time point and beyond. *Different from baseline (0 min); †different from saline infusion (two-way ANOVA with Bonferroni multiple comparisons test, $P < 0.05$). Values are means \pm SD, $n = 7$ per group.

accompanying change in MAP, but HR increased modestly at the higher dose. Infusion of GAL-021 in humans did not evoke significant changes in MAP or clinically meaningful changes in HR.⁹

We established that the carotid body, the primary peripheral organ responsible for sensing changes in oxygen and carbon dioxide²² is a major locus of action, at least in rats, as demonstrated by marked attenuation of ventilatory stimulation after acute transection of the CSN. In these respects, GAL-021 is similar to almitrine,²³ doxapram,²⁴ and hypoxia.²⁵ A minor component of GAL-021-induced ventilatory stimulation may occur at the level of the aortic bodies²⁶ or other vagally innervated glomus tissue. Furthermore, GAL-021 is brain penetrant and may access respiratory centers in the brainstem to stimulate breathing.

The molecular mechanisms whereby carotid body chemoreceptors, the glomus (type I) cells, transduce decreased P_{aO_2} into increased CSN activity remain unresolved. It is proposed that hypoxic inhibition of oxygen-sensitive K⁺ channels causes depolarization, opening of voltage-dependent Ca²⁺ channels, and Ca²⁺ influx with resultant release of neurotransmitters.²⁷ The identity of the carotid body K⁺ channel(s) inhibited by hypoxia remains a matter of debate.²⁸⁻³⁰ Indeed, available data suggest that oxygen-sensitive K⁺ channels vary between species and even within a given species. In the rat carotid body, evidence supports the presence and functional importance of K_{Ca}1.1 and TASK-like channels in chemotransduction. There is little information relating to chemotransduction components within the human carotid body although a recent report indicates that the human carotid body transcriptome also contains K_{Ca}1.1 and TASK-1 channels.³¹

Specificity profiling using radioligand binding analyses failed to identify a pharmacology that would preclude the development of GAL-021 and revealed a number of potential molecular targets. Functional assays indicated that effects

on binding to adenosine A₁, A_{2A}, and A₃ receptors; to 5-HT_{2B} receptors; and to GABA_A receptors were nonspecific and/or weak and unlikely to contribute to the ventilatory stimulant effects. Electrophysiology studies in GH3 cells identified inhibitory effects of GAL-021 on K_{Ca}1.1 channels although less than that exhibited by almitrine, indicating greater potency of the latter. As inhibition of K_{Ca}1.1 by GAL-021 was much greater than inhibition of cardiac ion channels, interaction with K_{Ca}1.1 remains a potential molecular target for the evoked ventilatory stimulation. A comparison of responses in wild-type mice and in mice lacking the pore-forming α -subunit (*Slo1*) of K_{Ca}1.1 also implicates K_{Ca}1.1 in the ventilatory stimulant effects of GAL-021. The effects of GAL-021 on drug-induced respiratory depression in cynomolgus monkeys are evident at plasma concentrations in the low micromolar range (*i.e.*, from approximately 200 ng/ml = 0.79 μ M to approximately 700 ng/ml = 2.76 μ M). The higher concentrations of GAL-021 required for inhibition of K_{Ca}1.1 channels in GH3 cells may reflect the fact that K_{Ca}1.1 channel pharmacology is complex and influenced by the subunit composition of the channel and its phosphorylation status, which vary in a tissue-specific manner.³² Thus, relations between extent of inhibition of channels expressed in GH3 cells (and putatively in cynomolgus carotid body glomus cells) and the attendant evoked biological response in these cell types are unknown. The detailed effects of GAL-021 on rat glomus cell ion channels as measured by whole cell electrophysiology will be the subject of a separate report.

Drug therapy for OIRD is predicated on maintaining analgesia while minimizing respiratory depression. One approach to this involves modifying the properties of the opioid ligand to favor analgesia over respiratory depression and a peripherally restricted agonist with marked selectivity for $\kappa \gg \mu$ receptors (*e.g.*, CR845) offers promise.³³ Another approach involves coadministering (with opioids) agents that selectively impair the respiratory depressant effects of

opioids without compromising analgesia. Several ventilatory stimulant agents that increase respiratory drive by various mechanisms demonstrate an acceptable preclinical profile and have been evaluated clinically. The ampakine CX717, a modulator of glutamatergic neurotransmission, impaired alfentanil-induced respiratory depression in human volunteers without affecting analgesia, but at the expense of increased sedation.³⁴ In contrast, the 5HT₄ agonist mosapride and the 5-HT_{1a} partial agonist buspirone were not clinically effective against OIRD in humans,²⁰ emphasizing that not all agents effective against OIRD in animals are clinically efficacious. As shown in this report, GAL-021 does not adversely affect opiate-induced analgesia at least as reported by the rat tail-flick assay but attenuates morphine-induced respiratory depression both in rodents and nonhuman primates.

The apparent prolongation of the acute analgesic effects of morphine in the tail-flick study in theory could result from effects on morphine pharmacokinetics or pharmacological actions. With respect to the former, the main metabolic pathway of morphine in mammals is glucuronidation catalyzed by the uridine diphosphate-glucuronyltransferase isozyme UGT2B7. The human enzyme is not inhibited by GAL-021, but effects on rat UGT2B7 have not been evaluated. Although unlikely, species differences in the inhibitory effects of GAL-021 on UGT2B7 may exist and in the rat may manifest as prolonged analgesia. A more likely explanation is that the respiratory stimulant properties of GAL-021 and subsequent alkalosis enhance morphine-induced analgesia by increasing morphine entry into the central nervous system. Morphine uptake into brain is two- to three-fold greater in alkalotic rats (pH = 7.62) than in acidotic rats (pH = 7.16) consistent with the pH partition hypothesis for morphine (pK_a 8.0) entry into the brain.³⁵ This magnitude of respiratory alkalosis is evident in rats receiving GAL-021 alone at doses used in the tail-flick assay experiments. Potential pharmacodynamic explanations for augmentation of morphine analgesia by GAL-021 include direct activation of opiate receptors, enhancement of opiate receptor signaling, interference with opiate-induced receptor desensitization or internalization,^{36,37} and functional impairment of so-called “opponent processes” such as glial activation known to compromise opiate analgesia.^{38,39} GAL-021 alone is devoid of analgesic activity in the tail-flick assay and does not interact with opiate receptors (using radioligand binding analyses). Additional studies are required to evaluate GAL-021 influences on opiate receptor signal transduction or internalization or on indices of glial cell activation. If replicated in humans, this finding offers the potentially desirable effect of decreasing opiate dosage required to maintain analgesia under conditions where the propensity for OIRD is reduced, partly due to the actions of GAL-021 on respiratory depression and partly to the decreased dose of opiate. Potential negative consequences include augmentation of opiate-induced sedation.

In aggregate, the available data suggest that GAL-021 is a novel, potent ventilatory stimulant in rodents and nonhuman primates, where it is effective to prevent or reverse OIRD. In rats, effects on OIRD are not accompanied by attenuation of morphine-induced antinociception at least as reported by the tail-flick assay. The major locus of action appears to be at the carotid body (in the rat) where phenotypically it mimics some of the effects of acute hypoxia. The detailed molecular mechanism of action of GAL-021 remains to be elucidated, but a component of its action appears to involve blockade of K_{Ca}1.1 channels implicated in carotid body chemotransduction. It remains possible, however, that GAL-021 acts, at least in part, through targets other than K_{Ca}1.1. Its overall preclinical pharmacological and tolerability profile qualified GAL-021 as suitable for IV administration to humans to evaluate its tolerability and effectiveness in drug-induced respiratory depression as a prelude to assessing its effects on sleep-related breathing disorders. Testament to the translational aspects of the preclinical studies documented here, GAL-021 has successfully completed phase 1 clinical trials, demonstrating its safety, pharmacokinetics, and pharmacodynamics (ventilatory stimulation) and its effectiveness in reversing opioid-induced respiratory depression in normal volunteers.^{9,10}

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Competing Interests

The authors declare no competing interests.

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