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Analgesic effects of the GIRK Activator, VU0466551, alone and in combination with morphine in acute and persistent pain models

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Abstract

G protein-gated inwardly-rectifying potassium (GIRK) channels are potassium-selective ion channels. As their name suggests, GIRK channels are effectors of $G_{i/o}$ G protein-coupled receptors whereby activation of these GPCRs leads to increased GIRK channel activity resulting in decreased cellular excitability. In this way, GIRK channels play diverse roles in physiology as effectors of $G_{i/o}$ -coupled GPCRs: peacemaking in the heart rate, modulation of hormone secretion in endocrine tissues, as well as numerous CNS functions including learning, memory and addiction/reward. Notably, GIRK channels are widely expressed along the spinothalamic tract and are positioned to play roles in both ascending and descending pain pathways. More notably, GIRK channel knockout and knock-down studies have found that GIRK channels play a major role in the action of opioid analgesics which act predominantly through $G_{i/o}$ -coupled, opioid-activated GPCRs (e.g. μ -opioid receptors). Recent advances in GIRK channel pharmacology have led to the development of small molecules that directly and selectively activate GIRK channels. Based on research implicating the involvement of GIRK channels in pain pathways and as effectors of opioid analgesics, we conducted a study to determine whether direct pharmacological activation of GIRK channels could produce analgesic efficacy and/or augment the analgesic efficacy morphine,

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Supporting Information Available

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an opioid receptor agonist capable of activating μ -opioid receptors as well as other opioid receptor subtypes. In the present study, we demonstrate that the small-molecule GIRK activator, VU0466551, has analgesic effects when dosed alone or in combination with sub-maximally effective doses of morphine.

Keywords

GIRK; Activator; Morphine; Analgesia; Hot Plate; Formalin Assay

G protein-gated inwardly-rectifying potassium (GIRK) channels are potassium (K^+)-selective ion channels that are members of the inwardly-rectifying K^+ channels (K_{IR}) family.^{1–2} Activation of GIRK channels by pertussis toxin sensitive $G_{i/o}$ -coupled G protein-coupled receptors (GPCRs) result in decreased cellular excitability³. Both the $\beta\gamma$ subunits of heterotrimeric G proteins and phosphatidylinositol 4,5-bisphosphate are critical factors in the activation of GIRK channels while internal blockade by magnesium (Mg^{2+}) and/or polyamines are responsible for GIRK channels' characteristic inward rectification.^{1, 4–7}

GIRK channels form tetrameric complexes from four highly homologous subunits, GIRK1–GIRK4 (Kir3.1–3.4). While GIRK2 and GIRK4 channels can form functional homomeric channels, GIRK1 and GIRK3 subunits do not appear to form functional homomeric channel complexes. GIRK1 is able to form hetero-tetrameric channels with GIRK2, GIRK3, and GIRK4.⁸ GIRK channels made up of varying subunit compositions are differentially expressed throughout the body.⁹ For instance, channels composed of GIRK1 and GIRK4 subunits are expressed in the sinoatrial node of the heart where they play a crucial role in pace making. The GIRK1/2 channel subtype is chiefly expressed in the nervous system¹⁰, and notably along the spinal cord, the superficial layers of the dorsal horn, and the periaqueductal grey, an area associated with analgesia.¹¹ While GIRK1-containing GIRK channels dominate expression throughout the body, there are a few distinct cell types which appear to express non-GIRK1-containing GIRKs including the most dopaminergic cells in the ventral tegmental area and substantia nigra which are believed to express GIRK2/3 heterotetrameric and GIRK2 homotetrameric channels, respectively.^{12–13}

GIRK channels couple to the μ -opioid receptor (μ OR), a key target of opioid analgesics, in the superficial layers of the dorsal horn of the spinal cord^{14–16} and numerous lines of evidence indicate that opioid-based therapeutics, such as morphine and fentanyl, harness GIRK channel-mediated hyperpolarization to produce a significant portion of their analgesic effects in rodents through the action of $G_{i/o}$ -coupled opioid activated receptors.^{17–19} Furthermore, variations in the *Girk2* gene, *KCNJ6*, in humans is associated with decreased therapeutic efficacy of opioid analgesics and pain sensitivity.^{19–20}

Recently, development of the direct-acting, selective small-molecule GIRK channel activator, ML297 has opened the door to evaluating the *in vivo* effects of GIRK channel activation.²² Previously we have shown that ML297 can produce anxiolytic effects²³ and reduced seizure-like activity in mouse models.²² Further medicinal chemistry efforts resulted in the development of VU0466551.²⁴ This compound is approximately 2-fold more potent than ML297^{22,24} (Fig 1) and 20% more efficacious at activating GIRK1/2 channels

(Table 1s) while retaining the specificity for GIRK1-containing GIRK channels observed for ML297. Due to the superior potency and efficacy of VU0466551 compared to ML297, we chose this compound to further explore the effects of GIRK channel activation on physiological behaviors, particularly with respect to their role in analgesia. To this end, we investigated the *in vivo* effects of VU0466551 alone or in combination with opioid receptor agonist, morphine, in two preclinical mouse models of acute supraspinal-mediated nociception and persistent pain, specifically the hotplate and formalin tests.^{25–27} Our data demonstrate the ability of pharmacological GIRK activation to produce analgesia and to enhance the analgesic efficacy of sub-maximally effective doses of morphine. These observations suggest the potential for GIRK channels as a target for analgesia and opioid-sparing therapeutic approaches which are badly needed in response to the present opioid abuse crisis.

Results and Discussion

Although GIRK channels have been the target of intense research interest for well over a decade, it was only recently that potent and selective small-molecule activators have become available to the research community.^{28–29} In particular the prototype GIRK channel activator, ML297, is being used by a growing number of investigators to explore the role of GIRK channels in physiology.^{30–31} While ML297 remains a useful tool to study GIRK channels in a variety of settings, for this present study we chose to use VU0466551 (Fig 1). Notably, this compound displays an approximately 2-fold improved potency compared to ML297, while retaining selectivity for only GIRK1-containing channels²⁹. In addition, we explored the selectivity of VU0466551 for a closely related K_{ir} family member, $K_{ir}6.1$, other ion channels targeted for analgesia, as well as the μ OR itself. We found VU0466551 to be inactive at homomeric GIRK2 channels, $K_{ir}6.1$ -containing channels, $K_v7.2$ channels, TRPV1 channels, and μ OR (Table 1s). We did, however, find that a 10 μ M concentration of VU0466551 inhibited the activity of $Na_v1.7$ channels by approximately 50%. However, VU0466551 still displayed a greater than 140-fold higher potency for GIRK1/2 channels compared to $Na_v1.7$ channels. Thus, we believe it unlikely that inhibition of $Na_v1.7$ channels plays a role in the analgesic efficacy observed with VU0466551 alone or in combination with morphine.

In order to further evaluate the potential of VU0466551 as an *in vivo* tool, we first investigated the pharmacokinetic (PK) properties of this compound *in vivo* after systemic administration in C57bl/J6 mice. VU0466551 possessed suitable physiochemical properties to allow formulation in uniform suspensions utilizing 20% (w/v) hydroxypropyl- β -cyclodextrin (BCD)/water vehicle (1.5 mg/mL) facilitating dosing up to 30 mg/kg i.p. As shown in Table 1s, VU0466551 displayed a T_{max} of 15 min and was cleared with a $T_{1/2}$ of ~1 hour (Table 2s). The total C_{max} plasma and brain exposure levels of VU0466551 were 554 ($\pm$ 79) ng/mL and 209 ($\pm$ 44) ng/g, respectively (Table 3s). Taking into account *in vitro* measurements of plasma protein binding $f_{u, plasma}$ 0.03 and brain homogenate binding $f_{u, brain}$ 0.011, the calculated $K_{p,uu}$ for VU0466551 was 0.14. Thus, at T_{max} the estimated free brain concentration of VU0466551 was ~7 nM. Based on the enhanced potency and suitable PK properties of VU0466551 for *in vivo* dosing, we first systematically evaluated the effects of this compound administered i.p. across a broad range of autonomic and

somatomotor nervous systems functions^{32–33} using a modified Irwin neurological behavior battery in mice.

As shown in Table 3s, we observed several dose-related changes in the different measurements of the Irwin battery. For example, VU0466551 decreased core body temperature at both the 10 mg/kg and 30 mg/kg doses (Table 4s), with sustained effects observed for over 8 hours at the 30 mg/kg dose. Interestingly, we also observed a dose-related increase in tail erection or “Straub-tail” similar to effects previously reported with μ OR agonists.³⁴ In addition, modest decreases in pinna reflex, general locomotor output, grasping loss, leg weakness, placing loss, righting loss, and induction of catalepsy were observed at the 30 mg/kg dose (Table 4s). However, most of these effects resolved after 1 hour and none were evident at the end of the 8 hour test period, with the exception of decreases tail pinch response and escape loss.

Based on these initial behavioral observations with VU0466551, we next conducted studies to further investigate the effects of this compound on locomotor output and measures of acute nociception. Previous studies reported that ML297 produced a substantial decrease in general locomotor activity.²² Indeed, we also observed a decrease in motor output when VU0466551 was assessed in the Irwin battery. To more quantitatively evaluate VU0466551 effects on locomotor function and coordination, we utilized the open-field activity and rotarod tests. After pretreatment with 10 or 30 mg/kg of VU0466551, mice were placed into open field chambers and changes in locomotion were evaluated for a duration of 90 min. As shown in Figure 1s, the 10 mg/kg dose of VU0466551 did not affect locomotion, while the 30 mg/kg dose produced a marked increase in locomotor activity in the initial 30 min of exploratory activity. While the underlying mechanisms for the observed increases in locomotor activity with VU0466551 remain unclear; it is interesting to note that μ OR agonists³⁴ such as morphine also have been shown to induce similar effects on the motor activity of mice, suggesting that GIRK channel activation may, at least in part, mediate this effect.³⁵ To further assess the effects of VU0466551 on motor coordination and balance, we employed the accelerated rotarod test. Following injection of VU0466551 at dosages of 1,3,10, and 30 mg/kg, we did not observe a decrease in rotarod performance (Figure 2s). Thus, within the dose range tested, VU0466551 did not have a significant effect coordinated balance and motor functions.

Because of the preponderance of data implicating a role for GIRK in the efficacy of opioids^{14–21, 36} and the decreased sensitivity we observed in the tail-pinch portion of the Irwin battery, we were interested in determining whether VU0466551 had any effects on acute nociception utilizing the mouse hot plate assay. The mouse hotplate assay is a preclinical model of acute supraspinally mediated nociception in which latency to lick the front or hind paws exposed to a 55° C hot plate is recorded. As shown in Figure 2, a 30 mg/kg i.p. dose of VU0466551 had no effect on response latency to the hotplate. Whereas, a 30 mg/kg s.c. dose of morphine produced significant increases in response latency.

We next investigated whether VU0466551 could augment the analgesic efficacy of a sub-maximally effective dose of morphine in the hotplate assay. First, we evaluated the full dose-related analgesic effects of morphine on latencies to respond in the mouse hot plate assay.

As shown in Figure 2, morphine produced robust dose-dependent analgesic effects, significant at 10, 18 and 30 mg/kg s.c.

In Figure 2, we observed significant increases in analgesic response for sub-maximally effective doses of morphine in the presence of 30 mg/kg VU0466551 compared to morphine alone, significant at the morphine doses of 10 and 18 mg/kg. (Figure 2). Thus, although VU0466551 had no effect on acute supraspinal nociception when administered alone, it did appear to augment the analgesic efficacy of sub-maximally effective doses of morphine in the hot plate assay. Next, we evaluated the effects of VU0466551 in a mouse formalin model of persistent pain alone and in combination with sub-maximally effective doses of morphine. Persistent pain states can arise from alterations in sensitivity within both ascending and descending pain pathways in the brain and the spinal cord.^{25–26} The formalin model of persistent pain involves induction of moderate, continuous pain generated by tissue injury from a subcutaneous injection of formalin into the hindpaw of the mouse, resulting in an early and late phase of paw-licking behavior where the late phase is considered to be an index of persistent pain mechanisms including central sensitization.^{37–39} In particular, the resultant state of central sensitization can produce an ongoing condition of spontaneous, persistent pain as well as an increased sensitivity to painful stimuli (hyperalgesia) or to normally nonpainful mechanical or thermal stimuli (allodynia).⁴⁰

In this study, unlike in the effects observed in the hotplate assay, VU0466551 produced a dose-dependent decrease in paw-licking responses consistent with increased analgesia, significance at the 30 mg/kg doses in both the acute (Fig 3a) and tonic (Fig 3b) phases of the formalin assay. These effects observed with VU0466551 were similar to the dose-related effects of morphine on both the acute and tonic phases of the formalin assay (Fig 3). In addition, enhanced analgesic efficacy was observed during the acute and tonic phases when a 30 mg/kg dose of VU0466551 was administered in combination with morphine (Fig 3).

In the acute phase, 3 mg/kg morphine in combination with 30 mg/kg VU0466551, produced a similar degree of efficacy as the dose of 10 mg/kg morphine alone. While in the second phase, the addition of 30 mg/kg VU0466551 to the 1 mg/kg dose of morphine produced efficacy comparable to the maximally effective dose of 3 mg/kg morphine when given alone in the tonic phase of the formalin assay.

Because it is critical to be aware of potential of pharmacokinetic drug-drug interactions that might confound the interpretations of the efficacy studies conducted in the hotplate and formalin tests with VU0466551 and morphine in combination, we performed an *in vivo* exposure study wherein we dosed VU0466551 alone, morphine alone, or VU0466551 in combination with morphine. In this study, we did not observe a significant difference in plasma or brain exposure levels for morphine (18 mg/kg) when given with VU0466551 (30 mg/kg) in comparison with exposure levels detected for each compound alone (Table 2s).

Herein we report the investigation of the effects of VU0466551, an ML297 analog, on gross neurological function using a modified Irwin behavioral battery, tests of effects on motor function, and tests in two models of pain. While we observed only modest effects on most neurological functions, we did observe an acute decreased locomotor activity in the Irwin

evaluation. This loss of activity is consistent with what we previously observed with ML297. Also, as with ML297, at doses resulting in decreased locomotor activity the animals were fully capable of coordinated movement during rotarod assay thus demonstrating that the loss of spontaneous locomotor activity is not the result of an inability to produce coordinated movement. However, with a more careful evaluation of spontaneous locomotor effects using an open field test we found that, at a 30 mg/kg dose of VU0466551 produced an increase in locomotor activity reminiscent of what has been observed with morphine. While it is intriguing to speculate about potential reasons for the observed increase in spontaneous locomotor activity, further study is needed to gain a broader understanding of the physiological consequences of GIRK channel activation.

In addition to the observed effects on locomotor function, we observed that direct activation of GIRK channel produced analgesic efficacy in two murine models of nociception either alone (formalin assay) or in conjunction with low-dose morphine (hotplate and formalin assays). It is unclear at present why VU0466551 was effective on acute nociception when dosed in the absence of morphine in the formalin assay and not in the hotplate assay. However, these differences may reflect differential sensitivity to thermal versus chemically induced nociception and/or the potential for enhanced effects to supraspinal versus spinal-mediated mechanisms of nociception by GIRK1/2 channel activators. As noted previously, GIRK channels are expressed in many types of neurons along the spinothalamic tract. Future studies will focus on gaining a greater understanding of the mechanistic basis of the analgesic efficacy of VU0466551 alone as well as in the presence of morphine. It will also be of great interest to determine the effects of GIRK channel activation in animal models of chronic pain including nerve injury models as well as how GIRK channel activation affects chronic morphine dosing.

While much remains to be investigated regarding the mechanism by which activation of GIRK channels promotes analgesia in the absence or presence of opioids, these findings do suggest that GIRK channel activators may offer an opportunity for non-opioid or opioid-sparing, adjuvant therapies.

Finally, because GIRK channels may also be effectors of morphine's addictive properties, it will be important to investigate whether direct GIRK activation alone has any addictive potential. Although our previous studies showing that ML297 was not active in conditioned place preference tests²³, a more thorough assessment of the effects of GIRK activation on reward and addiction is needed. These studies should assess small molecule-mediate GIRK activation alone as well as in conjunction with morphine and potentially other addictive agents to determine whether direct GIRK1-containing GIRK activation has any effect on their addictive properties.

Methods

Thallium Flux Assay

HEK-293 cells (ATCC) stably transfected with GIRK1, 2, were used for thallium (Tl⁺) flux assays, GIRK1/2 channel-expressing cells were dislodged from T-175 tissue culture flasks using TrypLE Express (Thermo-Fisher, Waltham, MA), then transferred into a 50 mL

conical tube, and centrifuged at 500 g for 2 min. The supernatant solution was removed and cells were resuspended at a concentration of ~1000 cell/ μ L in α -MEM containing 10% (v/v) fetal bovine serum (Thermo-Fisher, Waltham, AM) and 1% (v/v) GlutaGro (Corning, Corning, NY). Twenty μ L of the cell suspension were plated into amine-coated, clear-bottom, and black-walled 384-well plate (cat# 354719, BD, Franklin Lakes, NJ) using an electronic multichannel pipette. Plates were then cultured overnight at 5% CO₂ in a cell-culture incubator at 37°C. Following overnight incubation, cell-culture medium was removed and replaced with dye-loading assay buffer (Hank's balanced salt solution, 20 mM HEPES containing 0.03% (w/v) Pluronic F-127 (Sigma-Aldrich, St. Louis, MO) and 1 μ M Tl⁺-sensitive dye, Thallos AM (TEFlabs, Austin TX) and incubated for 1 hour at room temperature. After incubation, dye loading solution was removed and replaced with 20 μ L/well of assay buffer. The plates were then loaded into a WaveFront Panoptic (Franklin, TN). The data were acquired at 1Hz (excitation 480 $\pm$ 20nm and emission 540 $\pm$ 20nm) for 10 seconds, then 20 μ L/well of GIRK modulators were added (prepared as described below) and incubated for 2 minutes. Following incubation, 10 μ L/well of Tl⁺-stimulus buffer (125 mM NaHCO₃, 1.8 mM CaSO₄, 1 mM MgSO₄, 5 mM glucose, 2 mM Tl₂SO₄, 10 mM HEPES pH 7.4 all 5-fold higher than their final concentration in the assay) was added and data collection continued for 2 additional minutes. Data were analyzed using Microsoft Excel and potencies were obtained using a four-parameter logistic equation in the Excel add-in, XLfit (IDBS, Surrey, UK). Test compounds were solubilized in DMSO to 30mM, then plated on ECHO-qualified 384-well plates. A 10-point 3-fold serial dilution was achieved using a Echo 555 liquid handler (Labcyte, San Jose, CA) in conjunction with Labcyte's Dose-Response software. Data represent at least 8 independent experiments and all DMSO concentration were 0.3% (v/v).

Animal Care and Housing

All *in vivo* studies were carried out using adult male wild-type C57BL/6 mice (Jackson Labs) Animals were group-housed under a 12/12-h light-dark cycle (lights on at 6 AM) with food and water available *ad libitum*. All animal experiments were approved by the Vanderbilt University Animal Care and Use Committee, and experimental procedures conformed to guidelines established by the National Research Council's *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering and the number of animals used.

Hot Plate Assay

The effects of VU0466551 on nociception was tested using a hot plate apparatus (Ugo Basile, Gemonia, Italy) set to a constant temperature of 55°C. The heating plate (20 cm diameter) was surrounded by a restraining cylinder made of Plexiglass. Baseline reactivity to the thermal stimulus was assessed in a pre-test by placing mice on the hot plate and determining the latency to the nociceptive response, such as front or hind paw licking or jumping. If an animal did not exhibit a nociceptive response within 30 s, the animal was removed from the hot plate to avoid tissue damage and returned to its cage. One hour after the baseline test, mice were injected with vehicle or morphine (1 – 30 mg/kg, s.c.) followed 15 minutes later by administration of VU0466551 (30 mg/kg, i.p.) or vehicle. Another 15 minutes later, mice were tested on the hot plate again to determine antinociceptive treatment

effects. Using the response latency from the two tests, the % maximum possible effect (%MPE) was calculated as follows: $\%MPE = (\text{postdrug latency} - \text{predrug latency}) / (\text{maximum latency} - \text{predrug latency}) \times 100$. The %MPE for each treatment was then normalized to the %MPE obtained with the most efficacious morphine dose (30 mg/kg). Data were analyzed in GraphPad Prism using ANOVA with post-hoc Dunnett's multiple comparison test. Hotplate assays were conducted in a blinded fashion whereby the experimenter scoring the assay did not know if the animal had been treated with vehicle or, if not, what dose of VU0466551 had been administered.

Formalin Assay

Mice were placed into clear plastic cylinders for 30 min before being injected with vehicle or morphine (1 – 30 mg/kg, s.c.) followed 15 minutes later by administration of VU0466551 (30 mg/kg, i.p.) or vehicle. Another 15 minutes later, animals were confined in a restraining tube and 2.5% formalin solution in 0.9% saline was injected into the foot pad of the right hind paw. The animals' behavior was video recorded for a total of 60 minutes for subsequent manual scoring of licking responses of the injected hind paw. Licking behavior of the affected paw was scored during the acute phase (0–5 minutes) and the tonic phase (20–30 minutes) of the nociceptive response. Inflammation of the injected paw was confirmed visually, and any animals that did not show inflammation of the injected paw were excluded from the study. Animals were sacrificed immediately after the conclusion of the assay. Data are presented as the duration of licking (mean $\pm$ S.E.M.) and were analyzed in GraphPad Prism using ANOVA with post-hoc Dunnett's multiple comparison test. Formalin assays were conducted in a blinded fashion whereby the experimenter scoring the assay did not know if the animal had been treated with vehicle or, if not, what dose of VU0466551 had been administered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Concentration-dependent efficacy of VU0466551.

Shown are the concentration-dependent effects of VU0466551 (inset) on GIRK1 + GIRK2 expressed in HEK293 cells as measured using fluorescence-based Ti^+ flux assay. $N = 8$ independent determinations. The error bars depicted the standard error of the mean (SEM). The potency range expressed is the 95% confidence interval for the fit to the data. The efficacy values are normalized to a maximally effective concentration of ML297.

- △ Vehicle s.c. + Vehicle i.p.
- ▼ Vehicle s.c. + VU551 30 mg/kg i.p.
- Morphine s.c. + Vehicle i.p. ($ED_{50} = 18.3$ mg/kg)
- Morphine s.c. + VU551 30 mg/kg i.p. ($ED_{50} = 11.7$ mg/kg)

Figure 2. Analgesic efficacy of VU0466551 and morphine in the hotplate assay.

Shown are the VU0466551 (i.p.) alone, morphine (s.c.) alone, or VU0466551 plus morphine on latency of mice to respond to being placed on a 55° C hotplate. Data are shown are mean latencies normalized to maximum possible effect (MPE) as defined in the Methods $\pm$ SEM for 10–12 animals per group. Asterisks (*) represent comparison of test compounds (morphine and/or VU0466551) to the vehicle + vehicle control data. Hash (#) represent comparison of the effect of morphine + vehicle to morphine + VU0466551 ($P < 0.001$, ***; $P < 0.05$, #).

Figure 3. Efficacy of morphine and VU0466551 alone or in combination in the formalin assay. Shown are the effects in the (a) Acute phase (0–5 minutes) and (b) Tonic phase (20–30 minutes), measured 0 – 5 min and 20 – 30 min after injection of formalin, respectively. Data are shown as the duration of licking (mean $\pm$ SEM) of 10–12 (a) and 8 – 14 (b) animals per group. Asterisks (*) represent comparison of test compounds (morphine and/or VU0466551) to the vehicle + vehicle control data. Hash (#) represent comparison of the effect of morphine + vehicle to morphine + VU0466551 (P 0.05, *; P 0.01, **; P 0.001, ***; P 0.01, ##; P 0.001, ###).