Oral administration of a liquid containing nitrous oxide abolishes vaso-occlusive pain in mice with sickle cell disease

Sickle cell disease (SCD), the most common inherited hemoglobinopathy worldwide, is characterized by acute vaso-occlusive crisis (VOC) accompanied by severe pain that often results in hospitalization. Non-steroidal anti-inflammatory drugs (NSAIDS) and opioids continue to be the primary treatments for VOC-related pain despite their inadequate control of VOC pain and well-known side effects.[1] Thus, new approaches for VOC-related pain are needed not only to effectively manage pain but also to decrease hospitalizations and opioid use.

N_2O is a colorless gas whose analgesic and anxiolytic properties have been widely used through inhaled dosing for over 150 years. The analgesic effect of inhaled N_2O (iN_2O) is achieved through the release of endogenous opioids and inhibition of NMDA receptors, while the anxiolytic effect of iN_2O is related to activation of GABA-A receptors.[2] iN_2O is used in a mixture with oxygen for short-term anesthesia and analgesia in minor surgical procedures and dental treatments in adults and children.[3] Emergency departments in Europe use iN_2O for short-term analgesia during VOC in patients with SCD.[4]

Although analgesic, there are a number of substantial barriers to mainstream use of iN_2O for patients with SCD, including inadvertent exposure, uncontrolled dosage and related toxicity and addiction.[5] A novel orally available N_2O-containing liquid product, HBI-201, was designed to eliminate these barriers. Here we determined the efficacy of HBI-201 in a mouse model of VOC-evoked pain and examined potential side effects.

Male and female Townes HbSS and HbAA mice aged 4-9 months that did not exhibit chronic pain, and naive C57 mice were used in this study. These HbSS mice were selected so that we could isolate pain from VOC. The colony of Townes mice was maintained at the University of Minnesota. Hemoglobin phenotypes of mice were determined by isoelectric focusing. All procedures were approved by the University of Minnesota's Institutional Animal Care and Use Committee. Because exposure to low ambient temperatures is a common trigger of VOC in patients with SCD[6,7] and induces VOC and hyperalgesia in HbSS mice,[8,9] exposure to cold was chosen to test the analgesic and anti-hyperalgesic properties of HBI-201. HBI-201 and vehicle were obtained from Hillhurst Bio and delivered to mice by gavage at different doses. For exposure to cold, mice were in a plastic cage without bedding and placed in a room maintained at 10°C (50°F) for 1 hour and then returned to room temperature (22°C/72°F) in their home cage.[8,9] Mechanical hyperalgesia was defined as a decrease in paw withdrawal threshold (PWT) determined using calibrated von Frey monofilaments applied to the plantar surface of each hind paw.[8,9] Heat hyperalgesia was defined as a decrease in paw withdrawal latency (PWL) in response to radiant heat applied to each paw.[8] Analgesia was defined as an increase in PWT and PWL. PWT and PWL were measured for both hind paws and averaged. Baseline (BL) measurements were taken over 3 consecutive days before each experiment. HbSS mice whose PWT and PWL values at BL were not less than the mean minus 2 standard deviations for HbAA mice were considered non-hyperalgesic[8] and were used for cold exposure. Spontaneous nocifensive behaviors (SNB), suggestive of ongoing pain, were assessed by ear position, orbital tightening and body eccentricity.[10] The position of the ears was determined by the angle of inclination relative to the head. Orbital tightening was defined as a decrease in the minor axis of the eye shape. Body eccentricity (decreased length and increased curvature) values were calculated according to the formula: $e=[\sqrt{(a2-b2)}]/a$, where a=the major axis and b=the minor axis of ellipses. All data are presented as mean ± standard error of the mean (SEM) and were analyzed by paired *t* test, Student's *t* test, two-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures followed by Bonferroni's *post hoc* test. $P<0.05$ was considered significant.

Consistent with our earlier studies,[8,9] cold exposure produced robust mechanical hyperalgesia lasting for the observation period of 210 minutes. Administration of HBI-201 after cold exposure abolished mechanical hyperalgesia dose-dependently (Figure 1A). The anti-hyperalgesic effects of HBI-201 occurred rapidly and lasted about 90 minutes. The highest dose of HBI-201 (20 mg/kg) also attenuated cold-induced heat hyperalgesia (Figure 1B) and produced analgesia to mechanical (Figure 1C) and heat (Figure 1D) stimuli in naive C57 mice.

In addition, HBI-201 (20 mg/kg) reversed SNB produced by cold exposure in HbSS mice. SNB was expressed as a decrease in ear angle relative to the head (Figure 1E), orbital tightening (Figure 1F) and a decrease in body eccentricity (Figure 1G). Collectively, these data show that HBI-201 reversed hyperalgesia and SNB associated with cold-evoked VOC.

Naïve C57 mice were used to determine if HBI-201 exhibited abuse potential, analgesic tolerance, and sedation, common side effects associated with iN_2O. We utilized the conditioned place preference (CPP) test[11] to assess

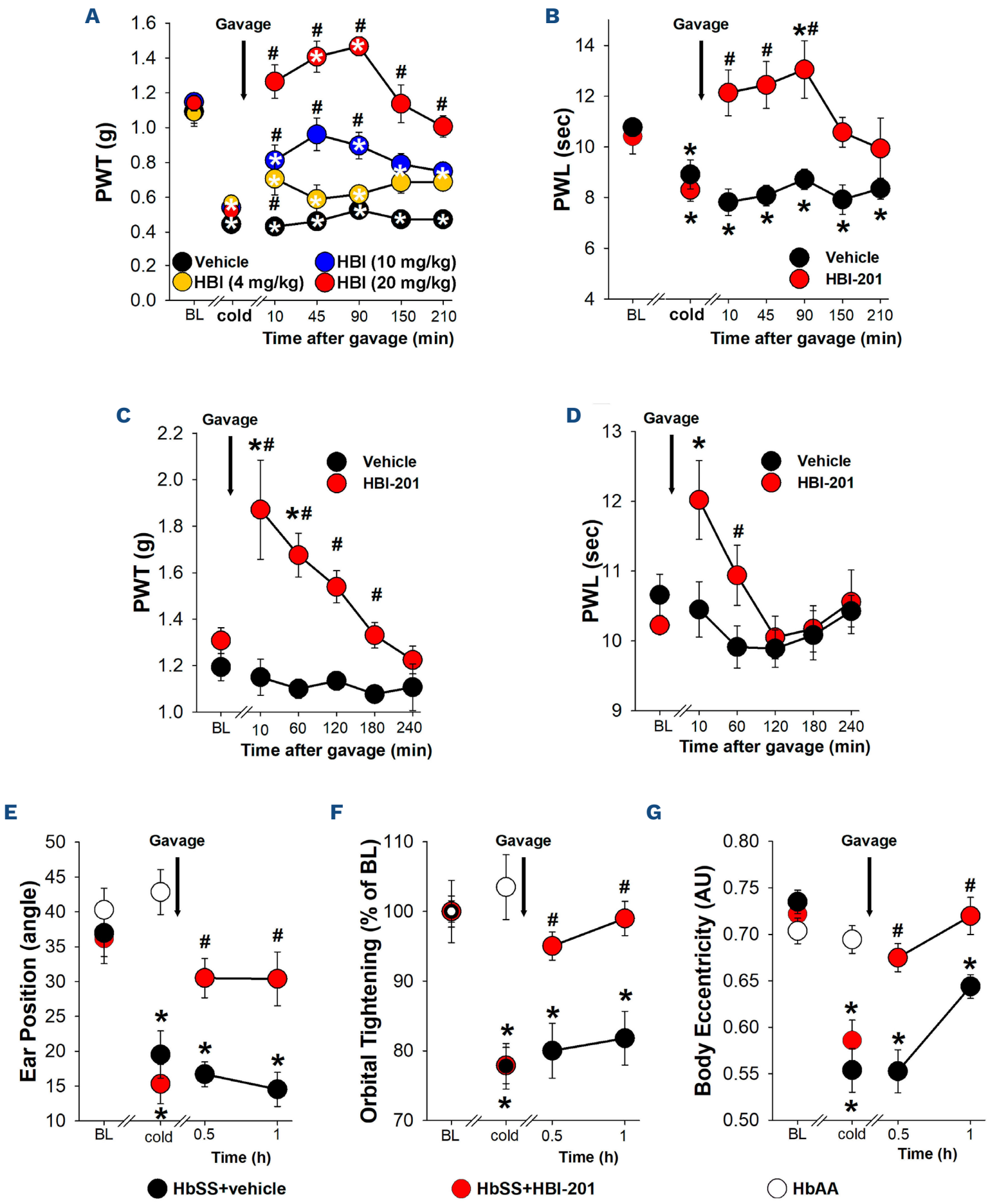
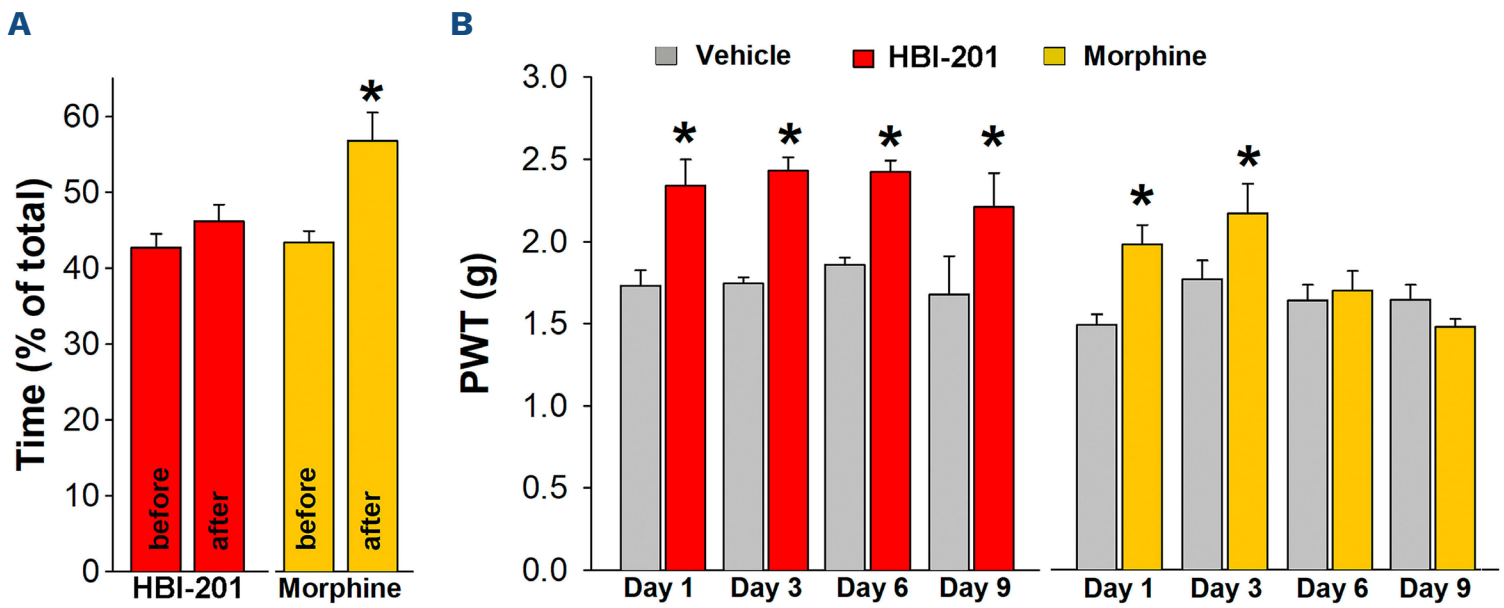


Figure 1. HBI-201 decreased cold-evoked hyperalgesia and spontaneous nocifensive behaviours in HbSS mice. (A) A novel orally available N_2O-containing liquid product (HBI-201) decreased cold-evoked mechanical hyperalgesia. The anti-hyperalgesic effect of HBI-201 was dose-dependent. *Different from baseline (BL) at P=0.002 and #different from vehicle at P<0.001 (F[18,146]=8.5), two-way ANOVA with Bonferroni t test, N=6-8 mice/group. (B) HBI-201 at the highest dose (20 mg/kg) reduced cold-evoked heat hyperalgesia. *Different from BL at P<0.01 and #different from vehicle at P<0.001 (F[18,146]=8.5), two-way ANOVA with Bonferroni t test, N=6-8 mice/group. The highest dose of HBI-201 completely reversed mechanical and heat hyperalgesia. The highest dose of HBI-201 (20 mg/kg) also produced analgesia to mechanical (C) and heat (D) stimuli in naïve C57 mice, defined as an increase in paw withdrawal threshold (PWT) and paw withdrawal latency (PWL), respectively. *Different from BL at P<0.01 and #different from vehicle at P<0.05 (F[5,78]=3.1), 2-way ANOVA with Bonferroni t test, N=8-9 mice/group. (E) Exposure to cold produced signs of spontaneous nocifensive behaviors (SNB) in HbSS mice including decreased ear angle, orbital tightening (F), and decreased body eccentricity (decreased length and increased curvature) (G), in HbSS mice, but not in HbAA mice (P=0.534, Student's t test, N=6 mice). Unlike the vehicle, HBI-201 (20 mg/kg) reduced all cold-induced SNB parameters. *Different from BL at P=0.002 and #different from HbSS + vehicle at P=0.003 (F[3,121]=5), two-way ANOVA with Bonferroni t test, N=6-8 mice/group.

the rewarding properties of HBI-201. Mice were placed in a plexiglass box with each half lined with either vertical or horizontal black and white stripes and allowed to move freely for 20 minutes. The mouse location and the time spent in each half of the box were recorded by computer for 3 consecutive days to obtain baseline (BL) values before drug administration. During the 5-day training, treatment with HBI-201 (20 mg/kg) or morphine (5 mg/kg, subcutaneously [s.c.]) twice a day (morning and afternoon) was paired with a 20-minute placement in isolated halves of the box (either vertical or horizontal stripes). On day 6, the mice were again given free access to both halves of the box for 20 minutes. The time spent in each half of the box was recorded and compared to BL for each treatment. Unlike morphine, HBI-201 did not produce conditioned place preference, suggesting low abuse liability (Figure 2A). This is consistent with studies indicating that substance use disorder was not a significant problem for the majority of sickle patients that received iN_2O.[4]

We also determined if HBI-201 produced analgesic tolerance. Separate groups of mice received HBI-201 (20 mg/kg) or morphine twice per day (morning and afternoon separated by approximately 6 hours) for 9 days. Morphine (5 mg/kg, s.c,) was used as a positive control. PWT was determined before (BL) and on days 1, 3, 6 and 9 at 1 hour after the morning administration of the drug. Whereas morphine



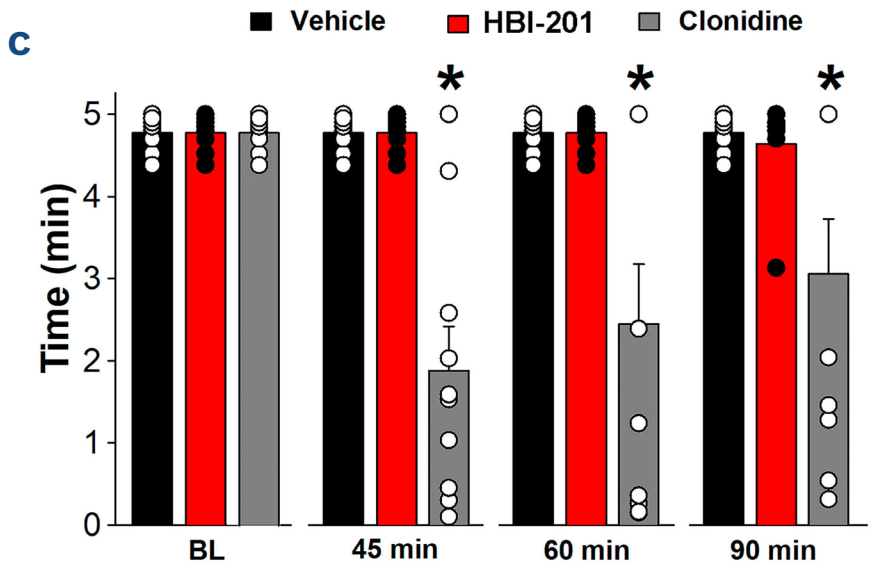


Figure 2. HBI-201 did not produce place preference, analgesic tolerance or sedation. C57 mice were used to determine if the novel orally available N_2O-containing liquid product (HBI-201) produced side effects that are associated with inhaled N_2O (iN_2O) and morphine. (A) Unlike morphine (5 mg/kg, subcutaneoulsy [s.c.]), mice treated with HBI-201 did not exhibit place preference, suggesting that HBI-201 is not rewarding (P=0.347). The time spent in the drug-paired halves of the box is represented as a percentage of the total testing time. *Different from before morphine treatment at P=0.001, paired t test, N=10 mice/group. (B) Prolonged administration of morphine (5 mg/kg, s.c.), but not HBI-201 (20 mg/kg), was accompanied by the development of analgesic tolerance, defined as a decrease in the analgesic effect. *Different from the baseline (BL) on the same day at P=0.045, ** at P=0.002 and *** at P<0.001, paired t test, N=10 mice/group. (C) HBI-201 (20 mg/kg) did not produce sedation, defined as a decrease in time spent on the treadmill. In contrast, clonidine (5 mg/kg, s.c.), a positive control, produced sedation as indicated by less time spent on the treadmill. *Different from the BL at P<0.001 ($F[6,96]=5.6$), two-way repeated-measures ANOVA with Bonferroni t test, N=10 mice/group.

produced analgesic tolerance following 9 days of repeated administration, tolerance was not observed following HBI-201 administration (Figure 2B).

Since reduced withdrawal responses could be due to sedation rather than analgesia, we used the rotarod test,[12] a measure of motor coordination, to determine if HBI-201 produced sedation. Mice were placed on an elevated treadmill which was gradually accelerated from 3.75 to 5 rpm, with a maximum cutoff time of 300 sesonds. Mice were trained to remain on the treadmill for at least 4 minutes. On the test day, mice received HBI-201 (20 mg/kg) or the equivalent volume of the vehicle by gavage. Clonidine, a well-known sedative (5 mg/kg, s.c.), served as a positive control. Testing was done before and at 45, 60 and 90 minutes after drug administration and the time when the mouse fell off the treadmill was recorded and compared between groups. HBI-201 did not reduce the time spent on the treadmill, while mice that received clonidine were unable to remain on the treadmill (Figure 2C). These data demonstrate that the analgesic effects of HBI-201 at the doses used were not attributed to sedation or motor impairment.

Finally, we measured blood levels of N_2O following a single dose of HBI-201 (20 mg/kg) or inhalation of 70% N_2O for 30 minutes (2 L/minute) in adult Sprague-Dawley rats (N=3/group) using gas chromatography with an electron capture detector. The mean (±SEM) maximum blood concentration following HBI-201 (0.448±0.13 mg/L) occurred at 15 minutes after administration and was much lower than that following inhaled N_2O (60.4±5.1 mg/L).

Our results demonstrate high analgesic efficacy of HBI-201 without side effects inherent to iN_2O, making HBI-201 an attractive tool for early relief of VOC-related acute pain in patients with SCD. Although studies of side effects were done in C57 mice, we expect similar results in HbSS mice given the low blood level of N_2O following HBI-201, which most likely accounts for the lack of side effects. Since it's unclear if iN_2O is effective for chronic pain[13,14] and therefore may not require long-term use, most of our results are based on a single dose of HBI-201 to relieve acute VOC-related pain. Given its relatively short duration of analgesia, repeated or higher dosing might be needed clinically. Additional studies of side effects following repeated administration of HBI-201 are needed. In addition, future clinical trials should include monitoring vitamin B12-related metabolism, which may be impaired with repeated exposure to iN_2O, including in sickle patients,[15] and can result in myeloneuropathy.

In summary, oral administration of N_2O-containing HBI-201 reduced acute pain and hyperalgesia associated with VOC in mice with SCD. Importantly, HBI-201 given over a relatively short time course did not produce side effects that are commonly associated with iN_2O or opioids. These preliminary studies are encouraging and suggest that HBI-201 may be a safe and effective approach to manage VOC pain in patients.

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Contributions

AO was involved in experimental design, data collection and editing the manuscript. FA was involved in breeding and gavaging the mice, experimental design and data collection. IAK and DAS were involved in experimental design, preparation of figures, data analyses and writing the manuscript. SGK contributed to experimental design, data collection and editing the manuscript. JDB and GMV were involved in experimental design and editing the manuscript. VV-K participated in data collection, data analyses and edited the manuscript. MY provided the HBI-201 and edited the manuscript. AH and KT were involved in data collection.

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Data-sharing statement

For original data, please contact the corresponding author.

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