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Small-molecule nociceptin receptor agonist ameliorates mast cell activation and pain in sickle mice

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ABSTRACT

Treatment of pain with morphine and its congeners in sickle cell anaemia is sub-optimal, warranting the need for analgesics devoid of side effects, addiction and tolerance liability. Small-molecule nociceptin opioid receptor ligands show analgesic efficacy in acute and chronic pain models. We show that AT-200, a high affinity nociceptin opioid receptor agonist with low efficacy at the mu opioid receptor, ameliorated chronic and hypoxia/reoxygenation-induced mechanical, thermal and deep tissue/musculoskeletal hyperalgesia in HbSS-BERK sickle mice. The antinociceptive effect of AT-200 was antagonized by SB-612111, a nociceptin opioid receptor antagonist, but not naloxone, a non-selective mu opioid receptor antagonist. Daily 7-d treatment with AT-200 did not cause tolerance and showed a sustained antinociceptive effect, which improved over time and led to reduced plasma serum amyloid protein, neuropeptides, inflammatory cytokines and mast cell activation in the periphery. These data suggest that AT-200 ameliorates pain in sickle mice via the nociceptin opioid receptor by reducing inflammation and mast cell activation without causing tolerance. Thus, nociceptin opioid receptor agonists are promising drugs for treating pain in sickle cell anaemia.
Introduction

Sickle cell anemia (SCA) is associated with unpredictable recurrent acute vaso-occlusive (VOC) pain episodes ("crises") and chronic pain, which can begin early and continue to increase, leading to poor quality of life and shortened survival.1, 2 Opioid treatment is widely used to treat pain in SCA, but remains a suboptimal approach due to side effects including somatic and psychiatric co-morbidities, dependence liability, and opioid hyperalgesia.3-5 Relatively higher doses of morphine are required to treat sickle pain as compared to other conditions, which may increase the risk of side effects. Morphine is also known to increase inflammation via the activation of toll-like receptor 4 (TLR4).6 We found that TLR4 expression is increased in mast cells and morphine stimulated the release of substance P (SP) from mast cells and promoted SP- and capsaicin-induced neurogenic inflammation in sickle mice.7 Therefore, while used for treating pain in SCA, morphine may exacerbate existing mast cell activation, neurogenic inflammation and pain.

Morphine and its congeners exert their anti-nociceptive effect via the mu opioid receptor (MOP/R). In addition to MOP/R, the opioid receptor family includes the nociceptin opioid receptor (NOP/R) which is involved in nociceptive signaling via its endogenous peptide ligand nociceptin/orphanin FQ (N/OFQ).8 NOP/Rs are found in the dorsal root ganglion (DRG), spinal cord and supraspinal regions of the brain involved in nociception.9-11 Agonists and antagonists of NOP/R have been demonstrated to influence pain at the peripheral, spinal and supraspinal level in rodent models of neuropathic and inflammatory pain.12-14

The antinociceptive role of spinal NOP/Rs is suggested to result from an inhibitory action on SP-mediated nociception via second-order neurons.15 SP levels are increased in the serum of patients with SCA and in sickle mice compared to healthy subjects and control mice, respectively.16, 17 The endogenous NOP/R peptide agonist N/OFQ has been shown to inhibit the release of SP and calcitonin gene-related peptide (CGRP) from sensory nerve endings.18 Chemically-induced release of neuropeptides from mast cells and capsaicin-sensitive afferent nerve terminals were also attenuated by N/OFQ.19 Mast cells are tissue-resident inflammatory cells, which can release pro-inflammatory cytokines, neuropeptides and proteases such as tryptase, upon degranulation/activation.20, 21 Tryptase released from mast cells activates protease activated receptor-2 (PAR-2) on peripheral nerve terminals stimulating the release of neuropeptides and pain.22, 23 Neuropeptide SP, in turn activates mast cells leading to a vicious cycle of mast cell and neural activation resulting in chronic pain. We found that sickle mice,
show mast cell activation and mast cell-dependent neurogenic inflammation and pain.\textsuperscript{7} Since, NOP/R agonists appear to block neuropeptide release from peripheral nerve endings and mast cells, we examined the possibility of using small-molecule NOP/R agonist AT-200 to treat pain in SCA.

We used transgenic homozygous HbSS-BERK mice, expressing sickle hemoglobin, which show characteristic features of experimental pain observed in human SCA.\textsuperscript{16, 24, 25} We demonstrate the antinociceptive activity of a small-molecule NOP/R agonist AT-200 (formerly SR14150)\textsuperscript{26}, and the mechanism of its action in sickle mice.

\section*{Methods}

\subsection*{Mice}

Experiments were performed following Institutional Animal Care and Use Committee's approvals. We used transgenic HbSS-BERK mice (designated ‘sickle’) that are homozygous for knockout of both $\alpha$ and $\beta$ murine globins, but carry the transgenes for human $\alpha$ and $\beta^\text{S}$ (hemoglobin S). Control HbAA-BERK mice (designated ‘control’) are also knockout for both $\alpha$ and $\beta$ murine globins and express normal human hemoglobin A. Sickle mice recapitulate the genetic, hematologic and pathological features of the human SCA.\textsuperscript{27} Sickle mice are challenging to breed, pups can die in utero or post-natally, resulting in few surviving adults and fewer male mice. Breeder mice and pups are fed “Sickle Cell Mouse Diet” (TestDiet, St Louis MO) up to 10-12 weeks and the regular Rodent Diet (Harlan Laboratories, Hayward, CA) thereafter. We and others found that sickle mice start developing hyperalgesia after 3 months of age, which continues to increase up to 10 months and also show central sensitization.\textsuperscript{16, 25, 28, 29} Features of experimental pain in sickle mice are similar to those observed clinically including, mechanical, thermal and deep tissue hyperalgesia.\textsuperscript{16, 24, 25, 28} In addition to hyperalgesia, sickle mice also show inflammation, endothelial dysfunction, mast cell activation and increased cutaneous SP and CGRP.\textsuperscript{7, 16, 30} Mice were geno-typed for the presence of human $\alpha$ and $\beta/\beta^\text{S}$ hemoglobins and absence of mouse $\alpha$ and $\beta$ hemoglobins (Transnetyx) and phenotyped for human HbA (control mice) or single band of HbS (homozygous sickle).\textsuperscript{7} Control and sickle mice show Hematocrit values of 46.64$\pm$2.8 and 32.5$\pm$6.3, $p=0.0005$; white blood cell counts of 5.3 K/uL$\pm$1.7 and 17.2 K/uL$\pm$7.9, $p=0.003$; and % reticulocyte levels of 5.7$\pm$1.9 and 65.6$\pm$6, $p=0.0000$, respectively.
**Drugs**

AT-200 [1-(1-cyclooctylpiperidin-4-yl)-indolin-2-one], formerly called SR14150, was synthesized as a hydrochloride salt using previously reported procedures by Zaveri et al.\(^\text{26}\) AT-200 is a high affinity NOP/R ligand, with >20-fold binding selectivity over MOP/R, and partial agonist activity at NOP/R, as determined by a functional assay measuring the stimulation of \(^{35}\text{S} \text{GTP}\gamma \text{S}.\) \(^\text{26}\) AT-200 also has low efficacy partial agonist activity at MOP/R.\(^\text{26}\) AT-200 was dissolved in 1–2% dimethylsulfoxide and 0.5% aqueous hydroxypropylcellulose, and administered subcutaneously (s.c.) at the dose of 5, 10 and 20 mg/kg. Morphine sulfate (Eli Lilly & Co., Indianapolis, IN) was administered s.c. at the dose of 20 mg/kg based on our previous study showing that 10 mg/Kg dose was not effective, but a 20 mg/Kg dose was effective in this model.\(^\text{16}\)

**Treatment of mice with AT-200**

*Chronic treatment with AT-200*: Mice were injected with 10 mg/kg s.c. AT-200 or vehicle daily for 7 days and tested for pain behaviors 30-min after drug administration, on Days 1, 3, 5 and 7 and on Day 8 (24 h after the last injection).

*Co-administration of Naloxone or SB-612111*: Mice were injected with 1 mg/kg s.c. Naloxone or 10 mg/kg s.c. SB-612111 (synthesized in the Zaveri laboratory\(^\text{31}\)) 10 minutes prior to injecting with 10 mg/kg s.c. AT-200. Hyperalgesia was measured at 30, 60, 90, 180, and 240 minutes, and 24 h after the administration of AT-200.

**Hypoxia/reoxygenation (H/R)**

Mice were exposed to hypoxia with 8% O\(_2\) and 92% N\(_2\) for 3 hours followed by re-oxygenation at room air for 1 h, as previously described by us.\(^\text{25}\)

Detailed descriptions of the following methods are in the Supplementary file.

**Somatosensory testing for pain behaviors**

Following behavioral measures were performed in a quiet, temperature-controlled room.\(^\text{16,25}\)

*Mechanical paw withdrawal threshold and frequency.*

*Grip force to determine deep tissue/musculoskeletal pain.*

*Withdrawal responses to heat stimuli.*
Withdrawal responses to cold stimuli.

Balance and motor coordination.

Cutaneous Blood Flow

Cutaneous Mast Cell Staining

Laser scanning confocal microscopy (LSCM) of skin

Statistical Analysis

Inferential test in Figure 1 is a repeated measures two-way ANOVA to determine the effect of time and/or dose of AT-200. For all other figures, two type of inferential tests were used: (1) For comparisons of baseline levels with other time points, one-way ANOVA followed by paired ‘t’ tests were used. (2) Comparisons of vehicle and AT-200 at individual time points were based on ANOVA followed by an unpaired ‘t’ test for individual comparisons along with additional comparisons as indicated between vehicle and other (morphine or NOP/OR antagonists) treatment and comparisons of pain measures at hypoxia/ reoxygenatioon and later time points. A two-way ANOVA is used in Figure 5 to determine the effect of genotype and/or treatment followed by an unpaired ‘t’ test within treatment category or within genotype. Bonferroni correction was used to adjust for multiple pairwise comparison in all the analyses. P < 0.05 was considered significant. Data were analyzed using Prism 5 software (GraphPad, San Diego, CA).

Results

AT-200 ameliorates hyperalgesia in sickle mice

Mechanical, deep tissue and thermal hyperalgesia increases with age in sickle mice similar to chronic pain in sickle patients. Therefore, older mice (~18.5 mo) showing increased sensitivity to different stimuli compared to young mice (~4.4 mo) were used to examine the effectiveness of AT-200 (Figure S1). The two-way repeated measures ANOVA shows no significance for comparison with control, whereas different doses of AT-200 in sickle mice show significant interaction over time for all behavioral measures (Table 1).

Overall, AT-200 at a dose of 10 mg/kg reduced sensitivity to all stimuli in sickle mice to levels observed in age-matched control mice and the effect of a single dose lasted up to 24 hours.
(Figure 1). A 5 mg/kg dose, on the other hand, was effective in reducing hyperalgesia significantly for only up to 30 min, whereas, the highest dose of 20 mg/kg led to lethality in sickle mice. Therefore, AT-200 demonstrates dose-dependence. Response to 10 mg/kg AT-200 appears to be optimum, and was therefore used in subsequent experiments.

**Mechanical hyperalgesia.** AT-200 treatment had no effect on mechanical sensitivity in control mice (Figures 1A, C). Mechanical withdrawal threshold in sickle mice was lower compared to control. AT-200 treatment increased withdrawal thresholds in sickle from approximately 0.4 g at baseline to 2.1 g over a 3 h period, which remained elevated to 1.1 g even after 24 h (sickle vehicle versus AT-200; p = 0.0108) (last period of observation) (Figure 1B). Similarly, mechanical PWF in sickle is higher compared to control but decreased after AT-200 treatment, and remained decreased for 24 h (sickle vehicle versus AT-200, p < 0.0001) (Figure 1D).

**Deep/musculoskeletal pain.** AT-200 showed no significant increase in grip force in control after treatment (Figure 1E). Lower grip force in sickle mice reflects musculoskeletal pain in SCA. AT-200 increased grip force exerted by sickle mice by 25 g over a 3 h period (sickle vehicle versus AT-200, p = 0.0021), but its effects were not sustained for 24 h (Figure 1F).

**Thermal sensitivity to heat and cold.** sickle mice showed a significant increase in thermal sensitivity compared to control (Fig 1G-L). AT-200 reduced thermal sensitivity in sickle mice (compared to baseline, $F_{4,156} = 53.48$, $p < 0.0001$ (heat), $F_{4,72} = 5.446$, $p = 0.0007$ (cold)) but had no effect on thermal sensitivity in control mice (Figures 1G-L). Over a 3 h period, AT-200 noticeably doubled paw withdrawal latency (PWL) to heat and to cold in sickle mice (vehicle versus AT-200, p= 0.0001 (cold) and $p < 0.0001$ (heat); Figure 1H and L). Further confirming a reduction to cold sensitivity by AT-200, PWF to cold decreased significantly over a 3 h period in sickle mice (vehicle versus AT-200, $p = 0.0002$; Figure 1J). Remarkably, 10mg/kg AT-200 reduced heat sensitivity up to 24 h in sickle mice (sickle vehicle versus AT-200, $p < 0.0001$; Figure 1L).

**AT-200 attenuates hypoxia/reoxygenation-evoked pain.**

H/R injury leads to increased sensitivity in all pain measures in sickle mice and recapitulates VOC in SCA. Mechanical threshold ($t_9 = 3.4$, $p = 0.0078$) and PWL to heat ($t_9 = 5.7$, $p = 0.0003$) markedly decreased and mechanical PWF ($t_9 = 6.2$, $p = 0.0002$) increased after incitement of H/R in sickle mice (Figure 2A-B and D). Pain evoked by H/R was responsive to
AT-200 and to a high dose of morphine in sickle mice. Morphine attenuated mechanical threshold and heat sensitivity up to 4 h and PWF up to 90 minutes. Both threshold and heat sensitivity returned to pre-H/R levels after 24 h and PWF after 4 h in sickle mice. AT-200, on the other hand, provided sustained mechanical antinociceptive response up to 24 hours ($t_{11} = 2.4$, $p = 0.035$) (Figure 2A-B). AT-200 treatment showed a remarkable increase in PWL, more than 2-fold that of morphine at 30 minutes and sustained antinociception up to 24 hours (baseline versus 24 h: $t_9 = 1.08$, $p = 0.307$ for morphine and $t_{11} = 5.1$, $p = 0.0003$ for AT200; Figure 2D). AT-200 was notably superior to morphine and showed a sustained effect for up to 24 h.

Deep tissue pain (compared to baseline: $F_{5, 24} = 9.12$, $p < 0.0001$ for morphine, $F_{5, 30} = 16.15$, $p < 0.0001$ for AT-200 and cold sensitivity increased following H/R, but both AT-200 and morphine similarly attenuated these features of pain in sickle mice (Figures 2C, E and F). Morphine and AT-200 increased grip force up to 30 minutes, but returned to baseline levels by 90 minutes (Figure 2C). Cold sensitivity returned to baseline levels by 30 minutes following morphine treatment, but AT-200 markedly reduced cold sensitivity up to 90 minutes ($t_5 = 7.6$, $p = 0.0006$) before returning to baseline levels by 240 minutes (Figure 2E-F). Importantly, sickle mice, but not control mice, exhibited sustained hyperalgesia up to 24 h with vehicle treatment following H/R.

**Chronic treatment shows improved antinociceptive response over time without causing tolerance.**

Sickle mice were treated with AT-200, morphine and vehicle daily, and behavioral measures were obtained at baseline and 30 min after injection of drugs every other day (Figures 3A-C). A significant and consistent decrease in PWF response to mechanical stimuli, an increase in grip force and in PWL to heat occurred with both morphine and AT-200 treatment up to 7 days (last period of drug injections) of testing compared to vehicle-treated sickle mice (vehicle versus morphine versus AT200 $F_{2, 14} = 87.6$, $p < 0.0001$ (PWF), $F_{2, 14} = 16.02$, $p = 0.0002$ (grip force) and $F_{2, 14} = 129.1$, $p < 0.0001$ (PWL heat) (Figure 3A-C). The antinociceptive effect of AT-200 was significantly higher than morphine on each day of testing. A significant decrease in hyperalgesia was also observed with AT-200 on day 8, 24 hours after the last injection on day 7 (vehicle versus AT-200 $t_9 = 6.2$, $p = 0.0002$ (PWF), $t_9 = 4.4$, $p = 0.0016$ (grip force) and $t_9 = 8.3$, $p < 0.0001$ (PWL heat)) (Figures 3A-C). Morphine did not show a significant reduction in hyperalgesia compared to baseline on day 8. On the contrary, 24 hours after the last injection, morphine-treated mice showed an increase in PWF in response to von Frey fiber application,
suggestive of an increase in mechanical hyperalgesia compared to baseline (Figures 3A-C). Together these data demonstrate that daily treatment with AT-200 maintains its antinociceptive potency and shows a sustained effect even 24h following the last injection.

**Chronic AT-200 treatment reduces cutaneous blood flow and enhances motor coordination.**

The dorsal cutaneous blood flow is drastically reduced in sickle mice chronically treated with AT-200 compared to control mice (vehicle versus AT-200 $t_{14} = 5.7, p < 0.0001$ and $t_8 = 4.2, p = 0.0028$; sickle and control, respectively) (Figure 3D). In addition, the latency to fall increased in sickle mice treated with AT-200, suggesting that motor coordination and balance were significantly enhanced on day 8 (baseline versus day 8 $t_7 = 2.4, p = 0.04$) (Figure 3E). Overall, vehicle-treated mice showed no changes in any parameters tested.

**AT-200 exerts antinociceptive effect via NOP/R in sickle mice**

To determine whether the antinociceptive effect of AT-200 is via NOP/R and/or MOP/R, mice were pre-treated with SB-612111, a NOP/R antagonist, or naloxone, an opioid receptor antagonist. Pretreatment of sickle mice with SB-612111 inhibited the antinociceptive effects of AT-200 on mechanical PWF, musculoskeletal pain, and heat sensitivity, whereas pretreatment with naloxone did not influence the antinociceptive effect of AT-200 (Figure 4A-C). Importantly, the antinociceptive effect of AT-200 was sustained up to 24 h in sickle mice pretreated with naloxone (vehicle versus Naloxone + AT-200 $t_{28} = 3.7, p = 0.0009$, $t_{13} = 5.18, p = 0.0002$ and $t_{28} = 6.8, p < 0.0001$; PWF, grip force and PWL (heat), respectively). *Naloxone alone had no effect compared to baseline.*

**AT-200 inhibits cutaneous mast cell degranulation**

Mast cell density/mm$^2$ and percent degranulating mast cells were nearly 2- and 1.7-fold higher respectively in sickle mouse skin, compared to control mice (control and sickle vehicle versus AT-200 $F_{3,22} = 12.39, p < 0.0001$ for density and $F_{3,22} = 26.02, p < 0.0001$ for degranulating cells; Figures 5A-F). Interestingly, AT-200 showed a robust 1.6-fold decrease in the percentage of degranulating mast cells in skin (vehicle versus AT-200 $t_{14} = 7.4, p < 0.0001$) (Figure 5F) but did not decrease mast cell density significantly (Figure 5E). Importantly, AT-200 treatment significantly reduced plasma tryptase in sickle mice (vehicle versus AT-200 $t_6 = 3.3, p = 0.015$) (Figure 5G), suggesting reduced mast cell degranulation. In addition, laser scanning confocal microscopy (LSCM) revealed a strong immunoreactivity of mast cell activation markers FcεRI
(green), CD117 (red), and tryptase (turquoise) in the skin of sickle mice, which was reduced appreciably upon AT-200 treatment (Figure S2).

**AT-200 inhibits neuropeptides and inflammatory cytokines.**

Plasma of sickle mice showed a nearly 1.4-fold and a 1.8-fold increase in SP and CGRP respectively, compared to control mice ($t_{12} = 4.0, p = 0.0016$ and $t_{11} = 6.7, p < 0.0001$ respectively) (Figures 5H, I). Treatment with AT-200 significantly reduced plasma SP and CGRP in sickle mice. Plasma SAP, an acute-phase protein and a marker of inflammation, was elevated 3-fold in sickle mice compared to control mice. AT-200 reduced plasma SAP in sickle mice by 2.25-fold (vehicle versus AT-200 $t_6 = 7.5, p = 0.0003$) (Figure 5J). Complementary to the increase in SAP, plasma IL-6, TNF-α, and RANTES were significantly higher in sickle mice compared to control mice (Figure 5K–M). Two-way ANOVA suggests that there is interaction between the treatment and genotype in almost all of the parameters tested. AT-200 treatment led to a significant decrease in these inflammatory cytokines in sickle mice. Together, AT-200 reduces neuroinflammation and the inflammatory response in sickle mice.

**Discussion**

Pain in SCA is unique, often spanning the entire lifespan of an individual, and requiring long-term treatment with opioids. Due to the liabilities of dependence, tolerance, and side effects such as constipation and hyperalgesia, opioid analgesia remains a sub-optimal approach. The observed effectiveness of AT-200, a NOP/R agonist, to treat hyperalgesia and VOC-associated nociception in sickle mice suggests a promising new approach for treating pain in SCA. Since chronic AT-200 treatment does not result in diminished analgesic efficacy but reduces mast cell activation and neuroinflammation, it may have distinct advantages over opioids for a long-term use in ameliorating pain in SCA.

Pain in SCA displays enormous phenotypic variability characterized by deep tissue pain and increased sensitivity to mechanical, heat and cold stimuli. Sickle mice show these features of pain, which are further enhanced with increasing age and upon H/R, recapitulating VOC. A relatively small dose (10 mg/kg) of AT-200 was able to ameliorate characteristic features of sickle pain in sickle mice. AT-200 induced dose-dependent antinociception, while a starting high dose above the therapeutic range caused lethality, which could be at least in part due to the fragility of sickle mice. The 10 mg/kg dose of AT-200 was determined to be the optimum dose.
showing a potent antinociceptive effect which was sustained for 24 hours. Chronic pain is reported in about 50% of patients at all times, and increases with age. A significant number of these patients do not respond to opioid analgesia. AT-200 was effective in reducing pain measures in older mice, which could be similar to chronic pain in patients with SCA. Small-molecule NOP/R agonists as well as bifunctional NOP-MOP/R agonists show anti-nociceptive effects in several rodent models of chronic and neuropathic pain. Therefore, AT-200 may be an alternative to opioids when pain is chronic and/or non-responsive to opioids.

Acute pain due to VOC can be recurrent, lasting several days and requiring hospitalization. Treatment at the primordial stage of pain is suggested to be more effective in SCA. Opioid treatment poses a major challenge due to its dependence liability and Schedule II classification leading to delays in treatment. Given the lack of rewarding effects of AT-200, availability of NOP/R-based analgesics such as AT-200 may provide an advantage in treating SCA pain at an early stage, possibly without hospitalization. Indeed, our data show that AT-200 remained moderately effective for up to 24 hours following the H/R-evoked nociception in sickle mice. Therefore, NOP/R agonists represent an innovative class of non-addicting analgesics effective in several sickle pain modalities.

AT-200 binds to both NOP/R and MOP/R, but its affinity for NOP/R is about 20-fold higher than for the MOP/R receptor. Opioid antagonist naloxone did not inhibit the antinociceptive effect of AT-200, but NOP/R antagonist SB-612111 completely attenuated the antinociceptive effect of AT-200 on mechanical and heat stimuli and deep tissue/musculoskeletal pain in sickle mice. Thus, AT-200 likely exhibits its antinociceptive effect predominantly via NOP/R. Studies with NOP/R-targeted drugs in rodents and primates suggest that the anti-nociceptive effects of NOP/R agonists may be devoid of abuse liability, tolerance, respiratory depression, constipation and pruritis. Moreover, NOP/R agonists also show anxiolytic-like effects. Pain in SCA is also associated with depression and anxiety. NOP/R agonists may therefore provide a therapeutic profile suitable for the management of pain in SCA.

Pain was consistently ameliorated over a period of time by daily treatment of mice with AT-200 without decrease in efficacy that normally accompanies tolerance development. Morphine at 20 mg/Kg dose was significantly less effective than AT-200 upon daily treatment throughout the 7-day period. Moreover, 24 hours after the last injection, morphine appeared to lose its antinociceptive efficacy, whereas AT-200-treated mice still showed a significant anti-hyperalgesic effect. In another study we found that daily subcutaneous administration of AT-200
(10 mg/kg) to ICR (normal) mice over 9-days showed no decrease in its antinociceptive efficacy in the tail-flick assay, unlike with morphine (3 mg/kg) (Zaveri et al., manuscript under preparation). Further, we observed a similar lack of tolerance development with another bifunctional NOP/mu agonist compound AT-201 (SR16435) in an acute tail-flick assay \(^{41}\) and after repeated intrathecal administration of SR16435 (3 mg) in mice with neuropathic pain. \(^{14}\) Together, these findings suggest that AT-200 maintains its antinociceptive efficacy upon repeated administration over an extended period of time.

Reduced cutaneous blood flow following 7 days of treatment in sickle mice is suggestive of reduced inflammation. Indeed AT-200 reduced cutaneous mast cell degranulation and decreased circulating inflammatory cytokines, neuropeptides, mast cell tryptase and SAP. We have previously shown that treatment of sickle mice with mast cell inhibitors imatinib or Cromolyn sodium for 5 days reduced, plasma CGRP and SP, inflammation marker SAP, and markers of mast cell activation, viz. tryptase and \(\beta\)-hexosaminidase. \(^{7}\) Release of inflammatory cytokines TNF-\(\alpha\), IL6 and RANTES from the skin of sickle mice was also significantly reduced upon treatment with mast cell inhibitors, suggesting that activated mast cells contribute to inflammation in SCA. \(^{7}\) Mast cell activation has also been strongly implicated in the development of pain. \(^{42-45}\) Therefore, inhibition of mast cell-associated tryptase, inflammatory cytokines and neuropeptides with corresponding decrease in hyperalgesia in sickle mice treated with AT-200, is suggestive of an anti-inflammatory effect of AT-200. Moreover, the inhibitory effect of AT-200 on peripheral mast cell activity observed herein is consistent with dose-dependent inhibition of mucosal mast cell density for up to 52-hours upon s.c. infusion of N/OFQ into the distal colon. \(^{46}\)

Increased SP and CGRP in sickle mouse skin and plasma are accompanied by neurogenic inflammation and TRPV1 activation in the peripheral nerve terminals. \(^{7,28}\) N/OFQ reduces local axon reflex-mediated neurogenic inflammation by reducing the release of vasoactive neuropeptides CGRP and SP from sensory afferent nerve terminals in the periphery. \(^{18}\) In the chronically denervated rat model, an intraperitoneal injection of N/OFQ inhibited the release of neuropeptides SP, CGRP and somatostatin from isolated rat trachea in response to capsaicin or bradykinin. \(^{19}\) The concomitant decrease in mast cell degranulation and serum SP, SAP and cytokines in sickle mice following AT-200 treatment is suggestive of reduced nociceptor excitation and decreased inflammation. Since SP is elevated in SCA patients ranging from 2-18 years of age compared to age-matched normal subjects, \(^{17}\) mast cell activation may start early in age and contribute to elevated SP and pain observed in SCA. Our results suggest that AT-200 may have direct peripheral anti-inflammatory effects, possibly by inhibiting the release of...
chemical mediators from mast cells and capsaicin-sensitive afferent nerve terminals.

NOP/R agonist AT-200 significantly ameliorates tonic and H/R evoked hyperalgesia and inhibits peripheral neuroinflammation in sickle mice. The antinociceptive effect of AT-200 lasts for more than 24 h when administered immediately following the incitement of H/R. Small-molecule NOP/R agonists such as AT-200 are a novel class of analgesics, which directly target mechanisms underlying pain in SCA. Thus, NOP/R agonists are promising analgesics for sickle pain, without the liabilities associated with opioids.

**Authorship and Disclosures**

DV, Sensory behavior testing, physiological measures, confocal microscopy, data analysis and wrote the manuscript; JAP, statistical analysis, behavioral testing and manuscript writing; JN, Mast cell analysis and ELISA; HT, behavioral and hematocrit analysis; DY, synthesis of NOP compounds; NTZ, Drug design, synthesis and writing the manuscript; KG, Experimental design, supervision of entire study, data analysis, interpretation of data, writing the manuscript. The authors declare no competing financial interests.
References


Table 1. Statistical analysis of two-way repeated measures ANOVA for comparing behavioral response to AT200 treatment under normoxia.

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<td>F&lt;sub&gt;4,156&lt;/sub&gt; = 35.94, p &lt; 0.0001</td>
<td>F&lt;sub&gt;2,39&lt;/sub&gt; = 83.98, p &lt; 0.0001</td>
</tr>
<tr>
<td>1F</td>
<td>F&lt;sub&gt;8,72&lt;/sub&gt; = 7.330, p &lt; 0.0001</td>
<td>F&lt;sub&gt;4,72&lt;/sub&gt; = 18.91, p &lt; 0.0001</td>
<td>F&lt;sub&gt;2,18&lt;/sub&gt; = 5.161, p = 0.0169</td>
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<tr>
<td>1H</td>
<td>F&lt;sub&gt;8,72&lt;/sub&gt; = 4.905, p &lt; 0.0001</td>
<td>F&lt;sub&gt;4,72&lt;/sub&gt; = 5.446, p = 0.0007</td>
<td>F&lt;sub&gt;2,18&lt;/sub&gt; = 20.26, p &lt; 0.0001</td>
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<tr>
<td>1J</td>
<td>F&lt;sub&gt;8,72&lt;/sub&gt; = 3.417, p = 0.0022</td>
<td>F&lt;sub&gt;4,72&lt;/sub&gt; = 8.757, p &lt; 0.0001</td>
<td>F&lt;sub&gt;2,18&lt;/sub&gt; = 11.94, p = 0.0005</td>
</tr>
<tr>
<td>1L</td>
<td>F&lt;sub&gt;8,156&lt;/sub&gt; = 32.79, p &lt; 0.0001</td>
<td>F&lt;sub&gt;4,156&lt;/sub&gt; = 53.48, p &lt; 0.0001</td>
<td>F&lt;sub&gt;2,39&lt;/sub&gt; = 111.6, p &lt; 0.0001</td>
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Two-way repeated measures ANOVA with Bonferroni’s correction for multiple comparisons was used to analyze the effect of dose (AT-200), time, and the interaction of dose and time in the control and sickle mice separately. Analysis shows significant interaction between the doses tested over time, and between the different doses and the time points independently, only in the sickle mice.
Figure Legends

**Figure 1. AT-200 ameliorates chronic mechanical, deep tissue and thermal hyperalgesia.**
Sickle mice were injected subcutaneously with 10 mg/kg AT200, 5 mg/kg AT200 or vehicle, while control mice were injected subcutaneously with 10 mg/kg AT-200 or vehicle. The sensitivity to mechanical stimuli (A-D), deep tissue hyperalgesia (E,F), and thermal hyperalgesia (G-L) were measured over the indicated time period. (A,B) Mechanical withdrawal threshold using von Frey monofilaments, a measure of cutaneous nociception is shown. Lower thresholds are indicative of increased sensitivity to cutaneous nociception. (C,D) PWF in response to 10 applications of a 9.8 mN (1.0 g) von Frey monofilament suggestive of mechanical hyperalgesia. A higher PWF indicates increased nociception. (E,F) Grip force measurements indicating deep tissue/musculoskeletal pain are shown. Lower grip force suggests increased deep tissue hyperalgesia. (G,H) PWL and (I,J) PWF on a cold plate maintained at 4 ± 1°C. A lower PWL and higher PWF in a 2-minute period on a cold plate are indicative of increased sensitivity to cold-induced nociception. (K,L) PWL to a heat stimulus. Shorter PWL (in seconds) in response to heat stimulus is indicative of increased heat sensitivity. Sickle mice treated with 10 mg/kg of AT200 showed significant analgesic response at all the time points tested compared to control mice. However, sickle mice treated with 5 mg/kg of AT200 did not show sustained anti-nociceptive effect. Statistical significance was calculated by comparing each value to BL (*) and between vehicle and AT-200 (†). *P < 0.05 and **P < 0.005 compared to BL; and † P < 0.05 and †† P < 0.005 compared to vehicle for that time point. Mean age of mice ± SEM in months were, HbAA-BERK vehicle (n=12) and control AT200 (n=12), 17.2 ± 1.6; HbSS-BERK vehicle (n=16), 21.2 ± 1.2, and HbSS-BERK AT-200, 20.7 ± 1.4 (10 mg/kg; n=16); 10.8 ± 1.9 (5 mg/kg; n=10). BL, baseline; PWF, paw withdrawal frequency; PWL, paw withdrawal frequency; Veh, vehicle.

**Figure 2. AT-200 attenuates hypoxia-reoxygenation evoked pain.** Sickle mice were exposed to 3 h of hypoxia and 1 h of reoxygenation at room air and treated with vehicle, AT-200, or morphine. Sensory testing was performed at baseline, immediately following H/R and after drug treatments at indicated times (A,B) mechanical threshold, and supra-threshold; (C) deep tissue hyperalgesia, and (D-F) thermal sensitivity to heat and cold. Open triangles with solid lines, crosses with dashed lines, and solid circles with dashed lines represent vehicle, AT-200, and morphine treatment, respectively. Statistical significance was calculated by comparing each value with BL (*) or with H/R (¶); and between vehicle and AT-200 (†). *P < 0.05 and **P <
0.005 compared to BL; †P < 0.05 and ††P < 0.005 compared to H/R; and ‡P < 0.05 and ‡‡P < 0.005 compared to vehicle for that timepoint. Mean age of mice ± SEM in months were, HbSS-BERK Vehicle (n=10), 20.9 ± 1.7, and HbSS-BERK AT-200 (n=12), 21.2 ± 1.7, and sickle MS (n=10), 21.4 ± 1.6. BL, baseline; H/R, hypoxia reoxygenation; Veh, vehicle; MS, morphine sulfate; PWF, paw withdrawal frequency; PWL, paw withdrawal latency. BL, baseline; H/R, hypoxia reoxygenation; Veh, vehicle; MS, morphine sulfate; PWF, paw withdrawal frequency; PWL, paw withdrawal latency.

Figure 3. Sustained anti-nociceptive effect and lack of tolerance with chronic AT-200 treatment. Mice were treated daily with vehicle, morphine 20 mg/kg/day or AT-200 10 mg/kg for 7 days. Sensory testing was performed at baseline before drug treatment and 30 minutes following the drug treatment on days 1, 3, 5 and 7 and on d8 (24h following the last injection). Responses of sickle mice with each pain stimulus are shown (A-C). A separate set of control and sickle mice were treated daily for 7 days with AT-200 or vehicle and tested before (baseline) and after 8 days of starting the treatments, i.e. 24h after the last daily drug injection (given on Day 7, for dorsal cutaneous blood flow measured by laser Doppler velocimetry (D) and performance on an accelerating rotarod (0-72 rpm) over a period of 5 minutes to measure balance and motor coordination (E). Statistical significance was calculated by comparing each value to BL (*), and with vehicle (†) for each time point. *P < 0.05 and **P < 0.005 compared to BL; and ††P < 0.005 compared to vehicle for that time point. Mean age of mice ± SEM in months were, HbSS-BERK Vehicle, 4.53 ± 0.22 (n=6); HbSS-BERK Morphine, 4.6± 0.25 and HbSS-BERK AT-200 (n=6), 4.6 ± 0.20. For the analysis of physiological parameters 5 HbAA-BERK mice and 8 HbSS-BERK mice were used in each group. Veh, vehicle; PWF, paw withdrawal frequency; PWL, paw withdrawal latency.

Figure 4. Nociceptin receptor mediates the analgesic effect of AT-200. HbSS-BERK mice were injected with naloxone (1 mg/kg), a non-selective opioid receptor antagonist or SB-612111 (10 mg/kg), a selective NOP receptor antagonist,10 minutes before injecting AT-200 (10 mg/kg) or were administered vehicle, AT-200 or naloxone alone. Measures of (A) PWF, (B) Grip Force, and (C) PWL to heat are shown. SB-612111 antagonized AT-200–induced anti-nociceptive effect, but naloxone did not. Data are shown as mean ± SEM from 5-7 HbSS-BERK mice per treatment. Statistical significance was calculated by comparing each value to BL values (*), between vehicle and treatment groups (†).* P < 0.05 and **P < 0.005 compared to BL; †P < 0.05 and ††P< 0.005 compared to vehicle for that time point. Mean age of mice ± SEM in months
were, HbSS-BERK Naloxone (n=6), 6.2 ± 0.08; HbSS-BERK SB-612111 + AT-200 (n=7), 5.42 ± 0.06; and HbSS-BERK Naloxone + AT-200 (n=7), 5.5 ± 0.09.

**Figure 5. AT-200 treatment reduces mast cell degranulation and inflammation.** Control and sickle mice were treated with vehicle or 10 mg/kg AT-200/day for 7 days as described in Fig 3D-K, and their dorsal skin and serum/plasma were analyzed 24h following the last injection on day 7 (A-D). **Toluidine blue staining for mast cells in dorsal skin:** Inset in (C) shows magnified degranulating mast cells. Original magnification X900; scale bar, 20 μm. Each image represents 12-16 reproducible images per mouse from 10-12 mice per treatment. Image acquisition information: Olympus IX70 microscope, 60x/0.45 objective lens, DP70 digital camera and DP70 Manager Software (Olympus) and Adobe Photoshop. (E) Mast cell density shown as means ± SEM per square millimeter of skin sections stained with Toluidine blue. (F) Percentage of degranulating mast cells shown as means ± SEM degranulating mast cells to the total number of mast cells per square millimeter. Plasma concentrations are shown in (G) Tryptase, (H) Substance P, (I) CGRP, (J) SAP, (K) IL-6, (L) TNF-α, and (M) RANTES. Statistical significance was calculated by comparing each value to control HbAA-BERK mice (*) and between vehicle and AT-200 (†). * P < 0.05 and ** P < 0.005 compared to vehicle-treated HbAA-BERK; and † P < 0.05 and †† P < 0.005 compared to vehicle for that time point. Mean age of mice ± SEM in months were, HbAA-BERK Vehicle (n=4), 4.06 ± 0.29, HbAA-BERK AT-200 (n=4), 4.04 ± 0.29, HbSS-BERK Vehicle (n=4-7), 4.38 ± 0.32, and HbSS-BERK AT-200 (n=4-7), 4.41 ± 0.34. Veh, vehicle; CGRP, Calcitonin Gene-Related Protein; SAP, Serum Amyloid Protein; IL-6, Interleukin 6; TNF-α, Tumor Necrosis Factor alpha; RANTES, Regulated on Activation, Normal T-Cell Expressed and Secreted.
Material and Methods

Somatosensory testing for pain behaviors

Behavioral measures were performed in a quiet, temperature-controlled room dedicated
to behavioral testing of mice, as previously described. All mice were acclimated to
the room and to the tests before collecting data.

*Mechanical paw withdrawal threshold and frequency.* Mechanical allodynia was
measured as paw withdrawal threshold using Von Frey filaments. Mice were placed on
an elevated wire mesh table. Testing was initiated with the 5.9 mN (0.6 g) von Frey
filament (Stoelting Co, Wood Dale, IL, USA), using the up-down method to stimulate the
mid-plantar surface of the hind paw until the mouse withdrew its paw. If no paw
withdrawal was noted, the next higher weight filament was used. If paw withdrawal was
observed, the next lower weight filament was used. The resulting pattern of 6 responses
was tabulated and the 50% paw withdrawal threshold was calculated as described
previously by us.  

The paw withdrawal frequency (PWF) to a standard von Frey monofilament with 9.8 mN
(1.0 g) applied 10 times at an inter-stimulus interval of approximately 5 seconds to the
plantar surface of the hind paw was also recorded.

*Grip force to determine deep tissue/musculoskeletal pain.* Peak forepaw grip force was
measured using a computerized grip force meter (SA Maier Co., Milwaukee, WI) as
previously described. Mice were held by the tail and allowed to grasp a wire-mesh grid with their forepaws. Grip force increases as mice are gradually pulled away from the mesh by the tail. The peak force exerted (up to 1 kg) was recorded and presented as the mean from three trials. Deep tissue hyperalgesia was defined as a decrease in grip force. \textsuperscript{47,48}

*Withdrawal responses to heat stimuli.* This was carried out using a Hargreaves apparatus. Radiant heat, from a heat source placed below, was delivered to the plantar surface of the hindpaw and paw withdrawal latency (PWL) was recorded in seconds. \textsuperscript{48}

*Withdrawal responses to cold stimuli.* Mice were placed on a cold plate at 4°C. PWL and PWF responses for each hind paw over a 2 minute period were recorded. \textsuperscript{47,48}

*Balance and motor coordination.* Mice were placed on a (0-72 rpm) accelerating rotarod apparatus (Model 7650, Ugo Basile, Verese, Italy). The latency to fall within a period of 5 minutes was recorded. Mice were subjected to 3 conditioning trials of 2 minutes, at intervals of 2 hours and required to remain on the bar for >60 seconds to be included in the experiment. \textsuperscript{47}

**Cutaneous Blood Flow**

Cutaneous blood flow was measured non-invasively and in real time with a laser Doppler blood perfusion monitor (Laserflo\textsuperscript{R} Model BPM 403, Vasamedics, Inc., St. Paul, Minnesota) as described previously. Hair on the dorsal skin over the thoracolumbar region was gently shaved with electric clippers one day before blood flow measurement. The head of the Laserflo\textsuperscript{R} probe (model PD-434, Vasamedics, St. Paul, MN) was placed on the shaved skin and blood flow values were obtained from the midpoint of the stable portion of the recordings, as described. \textsuperscript{49} The built-in micro-processor calculates the blood flow in ml/min/100 g tissue as the product of velocity and volume values.

**Cutaneous Mast Cell Staining**

Sections approximately 6 microns thick were stained with Toluidine blue. Skin sections were dipped in a solution (pH 2.3) containing 0.025 g Toluidine blue O (Allied Chemical, Morristown, NJ), 2.5 ml 70% ethanol, and 47.5 ml of 1% sodium chloride solution at
room temperature. Sections were washed with 12 changes of distilled water and allowed to air dry before mounting with DPX (Electron Microscopy Sciences, Hatfield, PA). Mast cells were counted in 12-16 reproducible and similar fields (900 x magnification) per mouse using an Olympus IX70 inverted microscope (Olympus Corporation, Center Valley, PA). Mast cells were counted and expressed as cells per square millimeter (mm$^2$). Degranulated mast cells with $\geq$ 8 granules outside the cell membrane were counted and quantified as a percentage of degranulating mast cells to all mast cells counted.$^{50}$

**Enzyme-linked Immunosorbent Assays**

Plasma/serum were analyzed for tryptase (Antibodies-Online Inc., Atlanta, GA), calcitonin gene-related peptide (CGRP; Life Sciences Advanced Technologies, Inc., St. Petersburg, FL), substance P, serum amyloid P (SAP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and regulated upon activation, normal T cell expressed and secreted (RANTES); all from R&D systems, Minneapolis, MN, according to manufacturer instructions, using a microplate reader (Synergy HT, Biotek, Winooski, VT) and the plate reader Gen5™ 1.0 data analysis software (Biotek, Winooski, VT). All analyses and calibrations were performed in duplicate.$^{50}$

**Laser scanning confocal microscopy (LSCM) of skin**

Mice were euthanized with compressed CO$_2$ and dorsal skin was fixed in Zamboni’s solution (0.03% w/v picric acid and 2% paraformaldehyde) for 24 or 48 hours at 4°C, and then transferred to 20% sucrose with 0.05% sodium azide in PBS.$^{47}$ Approximately 6 µM thick cryosections were stained with goat anti-tryptase (1:100; Santa Cruz Biotechnology, Inc., Dallas, TX), rabbit anti-FcεR1 (1:100; eBioscience, San Diego, CA), rat anti-CD117 (1:100; BD Pharmingen, Inc., San Jose, CA) and species-specific secondary antibodies conjugated with FITC, Cy3, and Alexa649 (Jackson ImmunoResearch Laboratories, West Grove, PA) as previously described.$^{50}$ In parallel, control staining was performed with isotype-matched IgG. LSCM data sets of 2-µ each of z-stack image sets / field of view (FOV) were captured using 40X immersion oil objective with an Olympus FluoView 1000 system (Olympus Corporation, Center Valley,
PA). Co-stained images from the same FOV were overlaid as required using Adobe Photoshop (Adobe, San Jose, CA).
Results

Figure S1. Pain behaviors in young and old control (HbAA-BERK) and sickle HbSS-BERK mice. (A) Mechanical withdrawal threshold using von Frey monofilaments. Lower thresholds are indicative of increased sensitivity to cutaneous nociception. (B) Paw withdrawal frequency (PWF) in response to 10 applications of a 9.8 mN (1.0 g) von Frey monofilament in the same mice. (C) Grip Force measurements normalized for body weights indicating deep tissue/musculoskeletal pain are shown. PWL to heat (D), to cold (E) and PWF on a cold plate (F) in the same young and old mice. Hollow white
bars represent young HbAA-BERK mice and hollow white bars with black horizontal lines represent old HbAA-BERK mice. Solid black bars represent young HbSS-BERK mice and black bars with white horizontal lines represent old HbSS-BERK mice. Data are shown as mean ± SEM from 10-12 mice in HbAA-BERK and from 16 mice in HbSS-BERK per age group. Statistical significance is denoted by * $P < 0.05$ and ** $P < 0.005$. Mean age of mice ± SEM in months were, young HbAA-BERK, 4.05 ± 0.2, old HbAA-BERK, 18.5 ± 1.1, young HbSS-BERK, 4.4 ± 0.3, and old HbSS-BERK, 20.9 ± 0.2.
Figure S2. Mast cell activation markers in the skin. A panel of LSCM images of MCs from sickle and control mice skin treated with vehicle or AT-200. Images show FcεRI- (first row, pseudo-colored green), c-kit/CD117- (second row, pseudo-colored red), and tryptase-immunoreactive (third row, pseudo-colored turquoise) mast cells. The fourth row is an overlay of the three colors. HbSS-BERK vehicle-treated mice (third column)
showed increased immunoreactivity as compared to MCs of control mice (first column) and AT-200 treated HbSS-BERK mice (fourth column). Original magnification X900; white scale bar represents 20 µm. Each figure is representative of images from 3 sections of skins of 10-12 different mice per treatment group. Image acquisition information: Fluoview FV1000 Laser Scanning Confocal BX61 Microscope (Olympus), 60X/1.42 oil objective lens, In-built image acquisition system, Adobe Photoshop. Mean age of mice ± SEM in months were, HbAA-BERK Vehicle, 4.06 ± 0.29, HbAA-BERK AT-200, 4.04 ± 0.29, HbSS-BERK Vehicle, 4.38 ± 0.32, and HbSS-BERK AT-200, 4.41 ± 0.34.

References


