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Inhibition of DAGLβ as a therapeutic target for pain in sickle cell disease

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Authorship

I Khasabova: designed and performed the biochemical and behavioral experiments, analyzed and interpreted data, and contributed to writing of the manuscript.

J Gable: performed behavioral experiments and read the manuscript.

M Johns: performed the siRNA knockdown experiments and contributed to the Western blot studies.

S Khasabov: performed behavioral experiments and edited the manuscript. SK has no competing financial interests.

A Kalyuzhny: performed the COX-2 immunoprecipitation studies.

M. Y. Golovko: performed the mass spectrometric assay and associated data analysis.

S. A. Golovko: performed mass spectrometric assay.

S. Kiven: bred and phenotyped sickle and control mice and performed quality control.

K. Gupta: designed the use of sickle mice, produced all mice and edited the manuscript.

V Seybold: contributed to design of experiments, interpretation of data, and writing of the manuscript.

D Simone: contributed to design of experiments, interpretation of data, and writing of the manuscript.

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Data sharing

The published methods and results of this study will be deposited to PubMed Central in accord with NIH policies.
Key Points

1. 2-arachidonoylglycerol, and the enzyme responsible for its synthesis, diacylglycerol lipase-β, were elevated in the blood cells of mice with sickle cell disease.

2. Blocking the activity of diacylglycerol lipase-β inhibited hyperalgesia in mice with sickle cell disease suggesting a new approach to treat pain in sickle cell disease.
Abstract

Sickle cell disease (SCD) is the most common inherited disease. Pain is a key morbidity of SCD and opioids are the main treatment but their side effects emphasize the need for new analgesic approaches. Humanized transgenic mouse models have been instructive in understanding the pathobiology of SCD and mechanisms of pain. Homozygous (HbSS) Berkley mice express >99% human sickle hemoglobin and several features of clinical SCD including hyperalgesia. Previously, we reported that the endocannabinoid 2-arachidonoylglycerol (2-AG) is a precursor of the pro-nociceptive mediator prostaglandin E2-glyceryl ester (PGE2-G) which contributes to hyperalgesia in SCD. We now demonstrate the causal role of 2-AG in hyperalgesia in sickle mice. Hyperalgesia in HbSS mice correlated with elevated levels of 2-AG in plasma, its synthesizing enzyme diacylglycerol lipase β (DAGLβ) in blood cells, and with elevated levels of PGE2 and PGE2-G, pro-nociceptive derivatives of 2-AG. A single intravenous injection of 2-AG produced hyperalgesia in non-hyperalgesic HbSS mice, but not in control (HbAA) mice expressing normal human HbA. JZL184, an inhibitor of 2-AG hydrolysis also produced hyperalgesia in non-hyperalgesic HbSS or hemizygous (HbAS) mice, but did not influence hyperalgesia in hyperalgesic HbSS mice. Systemic and intraplantar administration of KT109, an inhibitor of DAGLβ, decreased mechanical and heat hyperalgesia in HbSS mice. The decrease in hyperalgesia was accompanied by reductions in 2-AG, PGE2 and PGE2-G in the blood. These results indicate that maintaining the physiological level of 2-AG in the blood by targeting DAGLβ may be a novel and effective approach to treat pain in SCD.
Introduction

Pain is a characteristic feature of sickle cell disease (SCD).  

Patients experience acute and chronic pain that may be associated with hemolysis, vaso-occlusion, vasculopathy, ischemia-reperfusion injury, organ damage, neuropathy and persistent inflammation.  

Opioids are typically used to treat pain in SCD but are associated with increased symptom burden, depression and utilization of healthcare. New, effective and safe treatments are needed to manage pain in SCD.

Transgenic Berkley (BERK) and Townes mouse models of SCD expressing >99% human sickle hemoglobin exhibit hyperalgesia and have provided valuable information on mechanisms underlying pain in SCD.  

Inflammatory mediators such as prostaglandins (PGs), cytokines, interleukins and nerve growth factor are released from immune cells and endothelial cells and contribute to hyperalgesia by exciting and sensitizing primary afferent nociceptors. Importantly, many of these and other inflammatory mediators are increased in the blood of patients with SCD and in murine models of SCD. 

Targeting peripheral mechanisms that underlie nociceptor sensitization in SCD may provide a safe and effective approach for managing pain in these patients without the undesirable side effects of opiates.

The endocannabinoid 2-arachidonoylglycerol (2-AG) is an important pain modulator that has both anti- and pro-nociceptive effects. The reduction in pain has been attributed to suppression of inflammation as well as direct effects on nociceptors and targets within the central nervous system. In some pathological conditions, inhibitors of monoacylglycerol lipase (MGL), the enzyme that hydrolyzes 2-AG to arachidonic acid (AA) and glycerol, increased the level of 2-AG and reduced hyperalgesia through CB1 and CB2 receptor-dependent mechanisms. Although increasing endogenous 2-AG may seem attractive as a strategy for managing pathological pain, 2-AG is also an intermediate in the production of pro-nociceptive lipids. 2-AG is a substrate for cyclooxygenase-2 (COX-2). This enzyme is induced in certain inflammatory conditions including SCD. Oxidation of 2-AG produces prostaglandin E2-glycerol (PGE2-G), a highly potent pro-nociceptive lipid. Hydrolysis of 2-AG by MGL also contributes to the metabolic pool of arachidonic acid, a precursor of multiple prostaglandins.

In the present study, we used a humanized transgenic murine model of SCD, the homozygous BERK mouse, to investigate whether hyperalgesia in SCD is associated with increased levels of circulating 2-AG and the enzyme most closely associated with its generation. In the periphery, is 2-AG synthesized from diacylglycerides by the β-isofrom of diacylglycerol lipase (DAGLβ).
Because DAGLβ is upstream from MGL and COX-2 in the production of PGE2 and PGE2-G, we determined whether inhibition of DAGLβ reduces hyperalgesia in HbSS mice and whether the decrease is associated with a reduction of 2-AG and its related metabolites, PGE2 and PGE2-G. Our results show that 2-AG is an important intermediate in the synthesis of PGE2 and PGE2-G. The accumulation of 2-AG as a result of increased synthesis leads to an increase in the levels of pro-nociceptive lipids involved in the sensitization of nociceptors and pain in SCD. Targeting 2-AG synthesis may block pain at its source, thus contributing to prevention of hyperalgesia.

Methods

Mice

Male (5-9 months old), homozygous HbSS-BERK, HbAA-BERK and hemizygous HbAS mice were used (Supplement 1). All protocols were approved by the Institutional Animal Care and Use Committee.

Drugs

2-AG, anandamide (AEA), PGE2-G, PGE2, and their deuterated analogs 2-AG-d5, AEA-d8, PGE2-G-d5, and PGE2-d4 were purchased from Cayman Chemical; stock solutions were prepared in ethanol (10 mg/mL). JZL184, a selective inhibitor of MGL and KT182, an inhibitor of ABHD6 were purchased from Cayman Chemical. KT109, an inhibitor of DAGLβ and KT195, an inhibitor of serine hydrolase ABHD6 were purchased from Sigma-Aldrich. Stock solutions of enzyme inhibitors were prepared in dimethyl sulfoxide (DMSO, 10 mg/mL) and diluted to their final concentration in sterile saline with Tween 80.

Blood collection and analysis

Whole blood (0.5 ml) was collected into MiniCollect® EDTA Tubes (Greiner Bio-One). Blood cells were isolated from plasma by centrifugation for 10 minutes at 2,000 x g at 4°C. The pellet containing blood cells was used for Western blot; the supernatant (i.e., plasma) was used for measurement of lipids. Samples were frozen in liquid nitrogen and stored at –80°C until processing.
amount of DAGLβ and COX-2 proteins in blood cell lysates were determined from Western blot. Levels of 2-AG, AEA, PGE2-G and PGE2 were analyzed by LC-NSI-MS/MS. Specificity of the DAGLβ antibody was tested by knocking down DAGLβ in mouse fibrosarcoma cells clone NCTC 247237 with small interfering RNA (siRNA) specific for the DAGLβ gene. Specificity of the COX-2 antibody was tested by pre-incubation of the antibody with nickel resin (GE Healthcare) coated with a 10-fold molar excess of COX-2 His-tag protein purchased from R&D systems (Supplement 1).

**Behavioral Measures of Hyperalgesia**

*Mechanical hyperalgesia* was defined as a decrease in paw withdrawal threshold measured by the up-down method or an increase in the frequency of paw withdrawal evoked by 10 stimulations with a von Frey monofilament (Stoelting) with a bending force of 3.9 mN applied to each plantar hind paw (Supplement 1). *Heat hyperalgesia* was defined as a decrease in the latency of paw withdrawal from radiant heat applied to each plantar hind paw. Baseline measurements were taken over three days prior to each experiment. The withdrawal threshold, frequency of withdrawal responses and latency were averaged for both paws.

**Statistical Analyses**

Data are presented as the mean ± standard error of the mean and were analyzed by one- and two-way ANOVAs with repeated measures followed by Bonferroni t-tests when normally distributed. Data are presented as median and 95% confidence interval and compared using a nonparametric test when they did not meet the requirement of normality. The effective dose for 50% of the population (ED50) was determined by non-linear regression analysis in Prism (GraphPad Software). Behavioral dose response data were initially converted to the percent of maximum possible effect (%MPE) that was calculated using the average response in the vehicle (V)-treated mice and the post-drug (PD) response in each KT109-treated HbSS mouse in the equation:

\[
\%\text{MPE} = \frac{(V \text{ HbSS} - PD \text{ HbSS})}{(V \text{ HbSS} - V \text{ HbAA})} \times 100\%
\]
Results

Hyperalgesia in HbSS mice was accompanied by an increase in 2-AG in plasma

Consistent with our previous reports, 9-11,17,20,36-38 the majority of HbSS mice exhibited robust mechanical and heat hyperalgesia, and this was accompanied by an increase of 2-AG in plasma (Figure 1A, B, C). HbAS or HbSS-BERK sickle mice that exhibited baseline withdrawal frequencies less than 50% and withdrawal latencies to heat ≤ the mean minus 2 S.D. for the HbAA group, were considered non-hyperalgesic (~ 15%). The plasma level of 2-AG in non-hyperalgesic HbSS mice was similar to that of HbAA mice.

To determine if 2-AG contributes directly to hyperalgesia in SCD, 2-AG (18 μg/100 μl) was administered intravenously (i.v.) into the lateral tail vein of non-hyperalgesic HbSS mice in a vehicle of ethanol:saline (20:80, v:v). Mechanical hyperalgesia developed rapidly following a single injection of 2-AG in non-hyperalgesic HbSS mice and persisted for 24 h. No effect was observed in response to the vehicle in non-hyperalgesic HbSS mice, and 2-AG had no effect in HbAA mice (Figure 2A). Importantly, this dose of 2-AG administered by intraplantar route suppressed mechanical hyperalgesia by ~68% in a mouse model of bone cancer pain and had no effect on naive mice.

JZL184, an inhibitor of 2-AG hydrolysis, increased 2-AG and decreased hyperalgesia in models of neuropathic and bone cancer pain, 25. Therefore, a single intraperitoneal injection of JZL184 (0.33 mg/kg) or vehicle consisting of DMSO:Tween-80:saline (12:1:87, v:v:v) were used to test the effect of elevating the level of endogenous 2-AG in non-hyperalgesic HbSS mice. A single injection of JZL184 transformed the silent state of non-hyperalgesic hemizygous mice (HbAS), causing mechanical hyperalgesia in these mice and inducing heat hyperalgesia in non-hyperalgesic HbSS mice (Figure 2B, C). Injection of the vehicle in both cases had no effect. Administration of the same dose of JZL184 to hyperalgesic HbSS mice did not increase the hyperalgesia, which most likely reflects maximum hyperalgesia in these mice. JZL184 had no effect in HbAA mice.

Although DAGLβ is upstream of COX-2 in the synthesis of nociceptive derivatives of 2-AG, elevated levels of COX-2 in tissues from SCD contribute to systemic increases in pro-nociceptive products of 2-AG. COX-2 protein was significantly elevated in blood cells of both hyperalgesic and non-hyperalgesic HbSS mice compared to samples from HbAA mice (Figure 3).
KT109 reduced mechanical and heat hyperalgesia in HbSS mice

The higher level of 2-AG in plasma of hyperalgesic HbSS may reflect an increase in 2-AG synthesis or a decrease in its hydrolysis. Initially we determined whether the increase in 2-AG in plasma was associated with an increase in its biosynthesis. The β-isoform of DAGL contributes to the synthesis of 2-AG in the periphery. Indeed, hyperalgesia in HbSS mice was accompanied by an increase in DAGLβ protein in blood cells (Figure 4). It is noteworthy that the amount of DAGLβ protein in blood cells of non-hyperalgesic HbSS mice did not differ from that of HbAA mice.

We next determined if inhibition of DAGLβ would reduce hyperalgesia in HbSS mice. KT109, an inhibitor of DAGLβ with no activity against DAGLα, and KT195, a control for the inhibition of serine hydrolase ABHD6 by KT109 were used to selectively inhibit DAGLβ. Mice were injected intraperitoneal (i.p.) with 50 µl of KT109 or the vehicle for the highest dose of KT109 (300 mg in DMSO:Tween 80:Saline, 30:1:69). Systemic (i.p.) administration of KT109 reduced mechanical hyperalgesia (Figure 5A). A dose of 30 µg eliminated mechanical hyperalgesia in HbSS mice by 60 min after injection; frequency of withdrawal from the mechanical stimulus was not different from that of HbAA mice treated with vehicle at this time point (31.4±4.7% and 27.5±5.2%, respectively).

Although the reduction in hyperalgesia in HbSS mice treated with KT109 persisted at 3 h post-administration compared to HbSS mice treated with vehicle, the effect of the drug was diminished: after 3 h the withdrawal frequency in HbSS mice treated with KT109 was greater than that of HbAA mice treated with vehicle at that time point. The responses of HbAA mice treated with KT109 were not different from baseline nor responses to vehicle through the 3 h testing period (F[1,50]= 0.59, p=0.46 for treatment, n=4-6 mice/group, 2-way repeated measures ANOVA). Because higher doses had a similar time course, the anti-hyperalgesic effect of doses ranging from 3-300 µg are shown at 90 min post injection (Figure 5B). A dose-response effect was determined on the percent of the maximum possible effect (%MPE). The minimally effective dose was 30 µg and the ED50 was 13.1 µg (0.61-283 µg, 95% confidence interval) (GraphPad Prism).

In order to determine if the systemic effect of KT109 on mechanical hyperalgesia was due to a peripheral site of action, mice received one intraplantar (i.pl.) injection of vehicle (DMSO:Tween80:Saline, 13:05:86.5) or KT109 at doses of 1, 3 and 10 µg into
one hind paw (10 μl). Following injection of vehicle, HbSS mice exhibited mechanical hyperalgesia compared to vehicle-treated HbAA mice throughout the 48 hour testing period (Figure 5C). Whereas 3 μg i.pl. KT109 had no effect in HbAA mice, this dose blocked mechanical hyperalgesia in HbSS mice from 30 min through 24 hours post injection. Importantly, responses to the mechanical stimulus were also inhibited in the contralateral paw following injection of 3 μg KT109 (Figure 5D). The reduction in mechanical hyperalgesia in the contralateral paw did not occur until 90 min after injection, and mechanical hyperalgesia was blocked on both hind paws through 24 hours after injection. Administration of 1, 3 and 10 μg (i.pl.) KT109 confirmed that 3 μg was the minimally effective dose to reduce mechanical hyperalgesia in the paw ipsilateral to the injection in HbSS mice (Figure 5E).

KT109 also inhibits ABHD6, but no other serine hydrolases. KT195 is a structural analog of KT109 and a more potent inhibitor of ABHD6 but is inactive against DAGLβ and other serine hydrolases. Therefore, we tested the effect of KT195 in HbSS mice (Figure 5F). Consistent with the previous experiment, KT109 (3 μg, i.pl.) reduced mechanical hyperalgesia through the 3 hour observation period after treatment. In contrast, intraplantar injection of KT195 did not alter mechanical sensitivity at any time nor have an effect in HbAA mice (P=0.34, 1-way repeated measures ANOVA, N=4 mice/group; data not shown). A more potent derivative of KT195, KT182, at the same dose was also without effect (P=1.0 for KT182 compared to vehicle, 2-way repeated measures ANOVA with Bonferroni t-test; data not shown). Together these data support the conclusion that the effect of KT109 was specific to the inhibition of DAGLβ.

Intraplantar administration of KT109 (3 μg) also reduced sensitivity to noxious heat (Figure 6A), but the effect had a longer latency and a shorter duration compared to the change in mechanical sensitivity. KT109 did not reduce the level of heat hyperalgesia in HbSS mice until 120 min after injection and the effect was no longer present at 24 hr. Similar to the data for mechanical hyperalgesia, the effect of i.pl. injection of KT109 on the paw contralateral to the injection was consistent with its effect on the paw ipsilateral to the injection (Figure 6B). Neither KT109 nor its vehicle had an effect in HbAA mice. Administration of 1, 3 and 10 μg (i.pl.) KT109 confirmed that 3 μg was the minimally effective dose to reduce thermal hyperalgesia in the paw ipsilateral to the injection in HbSS mice (Figure 6C).
KT109 reduced the level of 2-AG and its downstream products in HbSS mice

To assess the role of DAGLβ and the effect of KT109 on the production of 2-AG, PGE2 and PGE2-G, these lipids were measured in plasma after intraperitoneal administration of 30 µg of KT109, the smallest dose that reduced mechanical and heat hyperalgesia. Blood was collected at 60 min after injection, a time that coincided with the maximum systemic anti-hyperalgesic effect. PGE2 was measured because of its pro-nociceptive activity and because hydrolysis of 2-AG by monoacylglycerol lipase produces arachidonic acid, a precursor for PGE2 (Table 1). The endocannabinoid AEA was also measured because of its importance in endogenous analgesia. Consistent with their roles in contributing to hyperalgesia, the levels of PGE2 and PGE2-G were elevated in the plasma of HbSS mice compared to that of HbAA mice following intraperitoneal administration of vehicle (note the difference in units: pmol for PGE2 and fmol for PGE2-G). The level of AEA was lower in the samples of plasma of HbSS mice. KT109 reduced the level of 2-AG in HbSS mice to a level that was also lower than that in HbAA mice. The levels of PGE2-G and PGE2 in HbSS mice treated with KT109 were reduced to the levels measured in HbAA mice, however, KT109 had no effect on the level of AEA in plasma of HbSS mice. These effects of KT109 are consistent with its role in blocking the production of 2-AG. The recovery of hyperalgesia 24 hours after administration of KT109 in HbSS mice was associated with an increase in 2-AG in plasma to the level before administration (153.3 ± 19.3 pmol/ml, P=0.57).

Discussion

These data demonstrate for the first time the exceptional contribution of DAGLβ in blood and the associated accumulation of 2-AG, its synthetic product, to hyperalgesia in mice with SCD. A high level of DAGLβ in blood cells distinguished HbSS mice with hyperalgesia from HbAA and non-hyperalgesic HbSS mice. Moreover, the simultaneous increase in both DAGLβ and COX-2 in blood cells ensures the accumulation of 2-AG and the formation of its pro-nociceptive derivatives that are sufficient for hyperalgesia. Two strategies were used to increase 2-AG: injection of exogenous 2-AG (i.v.) and injection of JZL184 to inhibit hydrolysis of endogenous 2-AG. Whereas administration of 2-AG did not promote hyperalgesia in HbAA mice with low levels of DAGLβ and COX-2 in blood cells, the same dose of 2-AG caused hyperalgesia in non-hyperalgesic HbSS mice with a low level of DAGLβ but high
level of COX-2. Although these two enzymes may be regulated independently, functionally they act in concert to achieve maximum hyperalgesia. The hyperalgesic effect of an increase in endogenous 2-AG in response to the administration of JZL184 in non-hyperalgesic HbAS mice, which may represent SCD trait (Supplement 1), emphasizes the importance of the proposed mechanism.

Several factors may contribute to the accumulation of 2-AG in HbSS mice. Since SCD in patients and mice is associated with an increase in the number of immune cells, most of which express DAGLβ, an increase in the level of DAGLβ protein in hyperalgesic HbSS mice may be associated with an overall increase in immune cells, although an increase in the activity of the enzyme cannot be excluded as well. Posttranslational modifications, including phosphorylation and cysteine palmitoylation may contribute to an increase in DAGLβ activity. In addition, increased production of 2-AG may reflect increased availability of substrate. Intracellular mobilization of Ca²⁺ through Gq/11 protein-dependent activation of phospholipase Cβ promotes the hydrolysis of phosphatidylinositol and the formation of DAG, the precursor of 2-AG. Enriched levels of DAGLβ, 2-AG and downstream arachidonic acid and PGE2 in white blood cells are associated with hyperalgesia in mouse models of inflammation.

KT109 and its analog KT195 were initially screened for selective binding to serine hydrolases using activity-based protein profiling (ABPP). In this assay KT109 bound to DAGLβ and ABHD6, exhibited partial binding to isoforms of phospholipase-A2 (PLA2), but did not bind to DAGLα or COX-2. KT195 bound to ABHD6 and PLA2 isoforms but not DAGLα or DAGLβ. In sickle mice, KT109 (30 µg/mouse = 1.3 mg/kg) produced a dramatic decrease in 2-AG and its downstream metabolite, PGE2-G, with no effect on AEA. The decrease in PGE2 observed in HbSS mice treated with KT109 may be attributed directly to inhibition of PLA2, as suggested in the ABPP assay, and indirectly to a decreased contribution of the hydrolysis of 2-AG to the pool of AA, its precursor.

The present data are consistent with a report on lipopolysaccharide-stimulated murine macrophages where treatment with KT109 (5 mg/kg), but not KT-195, reduced 2-AG. Similarly, treatment with KT109 reduced arachidonic acid, PGE2 and PGD2 in macrophages. However, there were no changes in 2-AG or AA in brain tissue where DAGLα primarily contributes to the generation of 2-AG.
Lipids were measured in plasma 60 min after systemic injection of KT109; the time of maximum anti-hyperalgesia. The effective anti-hyperalgesic doses we determined in HbSS mice following systemic (~ 1.3 mg/kg, i.p) administration are consistent with effective doses reported in murine models of acute LPS-induced inflammation, chemotherapy-induced neuropathy and nerve injury. It is likely the anti-hyperalgesia observed in vivo was specific to inhibition of DAGLβ and not ABHD6 because the effect was not mimicked by KT195 or KT182 which are each selective for ABHD6. Moreover, the effect is independent of cannabinoid receptors as the anti-nociceptive effect of KT109 in acute LPS-induced inflammation was maintained in CB1 (-/-) and CB2 (-/-) mice. Evidence that KT195 potently bound PLA2 isoforms in an ABPP assay but had no effect on hyperalgesia in vivo supports the conclusion that the effect of KT109 on hyperalgesia in HbSS mice was specific to inhibition DAGLβ and downstream production of PGE2-G and less likely due to a decrease in PGE2. Moreover, the decreased production of PGE2-G following administration of KT109 mitigates the seeming paradox of why a decrease in 2-AG is anti-hyperalgesic when an increase in 2-AG, produced by inhibition of 2-AG hydrolysis, is anti-hyperalgesic in multiple models of peripheral inflammation.

Our behavioral data following i.pl. administration of KT109 are consistent with the contribution of DAGLβ in blood cells to hyperalgesia in SCD. Intraplantar administration of KT109 decreased hyperalgesia in the contralateral paw with a longer latency than in the paw ipsilateral to the injection. The apparent systemic effect of i.pl. KT109 (0.13 mg/kg) exhibited greater potency than intraperitoneal administration of a 10-fold higher dose, suggesting better absorption of the drug in addition to a local action. Although we cannot exclude an effect mediated by the central nervous system, the localization of DAGLβ to immune cells, the absence of binding of KT109 to DAGLα within the central nervous system, and the longer latency for the contralateral effect of KT109 suggest that circulating immune cells contribute to hyperalgesia in HbSS mice.

Evidence that disruption of DAGLβ in macrophages does not result in a general accumulation of triacylglycerides, and DAGLβ is specific for polyunsaturated fatty acids only, supports the therapeutic safety of selective DAGLβ inhibitors. Moreover, the increase in COX-2 in blood cells and increase in pro-nociceptive lipid PGE2-G in plasma in SCD indicates important therapeutic effects of DAGLβ inhibitors for the treatment of pain in SCD. A schematic representation of the biochemical pathway inhibited by KT109 is summarized in Figure 7.
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Table 1. Effect of KT109 on 2-AG, AEA, PGE2 and PGE2-G in plasma.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>HbAA/vehicle (4)</th>
<th>HbSS/vehicle (5)</th>
<th>HbSS/ KT109 (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AG (pmol)</td>
<td>90 ± 2.9</td>
<td>179 ± 4.0</td>
<td>73 ± 5.7</td>
</tr>
<tr>
<td>AEA (pmol)</td>
<td>0.67 ± 0.08</td>
<td>0.24 ± 0.03</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>PGE2 (pmol)</td>
<td>0.61 ± 0.12</td>
<td>3.28 ± 0.34</td>
<td>1.18 ± 0.16</td>
</tr>
<tr>
<td>PGE2-G (fMol)</td>
<td>0.17 ± 0.08</td>
<td>6.48 ± 0.74</td>
<td>2.69 ± 0.75</td>
</tr>
</tbody>
</table>

Samples of blood were collected 60 min after injection of KT109 (30 µg, i.p.) or vehicle (DMSO:Tween 80:Saline, 28:1:71%). The quantification of lipids was based on the area ratio of analytical internal standard/tested lipid. Lipid values were normalized to volume of plasma (ml). All data were expressed as the mean ± SEM. The sample size is indicated in parentheses. One-way ANOVA was run across treatment groups within the same lipid. For simplicity, representation of differences between treatment groups was restricted to two levels of significance: HbSS/vehicle different from HbAA/vehicle at \(^a\) p<0.005; HbSS/vehicle different from HbSS/ KT109 at \(^b\) p<.005.
Figure Legends

Figure 1. Increased 2-AG in plasma is associated with hyperalgesia in HbSS-BERK mice. (A) The level of 2-AG was higher in plasma of HbSS mice compared to HbAA mice and non-hyperalgesic HbSS (nh) mice. *Different from HbAA and HbSS (nh) mice at p=0.004, one-way ANOVA with Bonferroni t-test. Unlike HbAA-BERK and non-hyperalgesic HbSS-BERK mice, hyperalgesic HbSS-BERK mice showed strong mechanical (B) and thermal (C) hyperalgesia. *Different from HbAA and HbSS (nh) mice at p<0.001, one-way ANOVA with Bonferroni t-test. Numbers inside bars indicate group size.

Figure 2. An increase in systemic 2-AG produced hyperalgesia. (A) 2-AG (18 µg/100 µl) was administered by intravenous injection to HbAA and non-hyperalgesic (nh) HbSS mice. Unlike HbAA mice, non-hyperalgesic HbSS mice developed hyperalgesia 60 minutes after injection; hyperalgesia persisted for 24 hours. The vehicle was ethanol in saline (20:80, v:v). *Different from HbAA mice and # different from vehicle at p<0.001 (F[6,54]=5.52, 2-way repeated measures ANOVA with Bonferroni t-test, n=5-6 mice/group). (B) Intraperitoneal injection of JZL184 (0.33 mg/kg, i.p.), an inhibitor of 2-AG hydrolysis did not reduce hyperalgesia in HbSS mice (2-way ANOVA, p=0.913, n=6 mice/group). When injected into non-hyperalgesic HbAS mice, JZL184 (0.33 mg/kg, i.p.) generated mechanical hyperalgesia in comparison to the vehicle (DMSO:Tween-80:saline (12:1:87, v:v:v)). *Different from BL and # different from vehicle at p=0.005 (F[5,50]=3.88, 2-way repeated measures ANOVA with Bonferroni t-test, n=6-5 mice/group). (C) JZL184 (0.33 mg/kg, i.p.) evoked thermal hyperalgesia in non-hyperalgesic HbSS mice. *Different from BL and # different from vehicle at p<0.05 (F[3,24]=2.06, 2-way repeated measures ANOVA with Bonferroni t-test, n=5 mice/group).

Figure 3. COX-2 was increased in blood cells of HbSS mice compared to HbAA mice. (A) There was no difference in the level of COX-2 between hyperalgesic and non-hyperalgesic (nh) HbSS mice. In both groups the level of COX-2 was higher than that of HbAA mice. COX-2 was detected with rabbit anti-COX-2 (1:500, ABclonal). The secondary antibody was IRDye 800CW goat anti-rabbit (1:15,000; LI-COR). Numbers inside bars indicate group size. *Different from HbSS and HbSS (nh) at p=0.008 (one-way ANOVA with Student-Newman-Keuls test). (B) Representative images of western blot immunoreactive bands corresponding to COX-2 protein isolated from blood cells (top) and the total protein stain for loading control (bottom). A prominent band corresponding to the ~72 kDa protein was identified as COX-2. (C) The specificity of the COX-2 antibody was tested by pre-incubation of the antibody with nickel resin coated with a 10-fold molar excess of COX-2 His-tag protein (b). Negative control (a) included incubation of COX-2 antibody with nickel resin without protein coating (Supplement 1).
**Figure 4.** An increase in the amount of DAGLβ in blood cells contributes to the accumulation of 2-AG in plasma. (A) The relative level of DAGLβ protein was defined as the amount of HbSS immunoreactivity in the sample/average amount of HbAA immunoreactivity of x samples 5 100%. DAGLβ was detected with rabbit anti-DAGLβ (1:500, Abcam). The secondary antibody was IRDye 800CW goat anti-rabbit (1:15,000; LI-COR). The amount of DAGLβ protein in hyperalgesic HbSS mice was greater than that of non-hyperalgesic (nh) HbSS and HbAA mice. Numbers inside bars indicate group size. *Different from non-hyperalgesic HbSS and HbAA mice at p=0.002, one-way ANOVA with Bonferroni t-test. (B) Representative images of immunoreactive bands corresponding to DAGLβ isolated from blood cells [top, HbSS mice (a) and HbAA mice (b)] and the total protein stain for loading control (bottom). (C) The specificity of the rabbit anti-DAGLβ antibody was tested by knocking down the DAGLβ gene with siRNA in cultured fibrosarcoma cells. Western blot analysis was performed on 45 µg of protein (top) and verify by Revert™ 700 Total Protein Stain in each well (bottom). The digits represent positive controls (1, 2), negative controls of scrambled siRNA sequence (3) and GAPDH siRNA (4), and DAGLβ siRNAs s107015 (5) and s107016 (6), respectively. A prominent band corresponding to the ~68 kDa protein was identified as DAGLβ. This band was missing in DAGLβ-/- cells (Supplement 1).

**Figure 5.** Systemic and intraplantar administration of KT109 inhibited mechanical hyperalgesia in HbSS mice. (A) HbSS mice exhibited significant mechanical hyperalgesia prior to drug injections (BL, baseline). KT109 (30 µg) was administered by intraperitoneal injection. Vehicle was DMSO:Tween 80:Saline (30:1:69). A reduction in hyperalgesia occurred at 60 min and persisted through the 3 h testing period (F[12,90]=3.72, p<0.001 for treatment, n=4-7 mice/group, 2-way repeated measures ANOVA). KT109 had no effect in HbAA mice, and the vehicle was without effect in either strain (P=1.0 in HbAA mice, P=0.57 in HbSS mice, 2-way repeated measures ANOVA). *Different from vehicle in HbAA mice at p<0.001, † different from vehicle in HbSS mice at p<0.05, †† different from vehicle in HbSS at p<0.05 (2-way repeated measures ANOVA with Bonferroni t-test). (B) A dose-dependent effect was observed for 3-100 µg KT109 (F[5, 33]=5.341, P<0.001 for treatment, n=4-8 mice/dose, one-way ANOVA). Data for doses were converted to a percent of the maximum possible effect (%MPE). Percent MPE was defined as the average response in the vehicle-treated HbSS mice (V HbSS) minus the post-drug (PD) response in the KT109-treated HbSS mice divided by the average response in the vehicle-treated HbSS mice (V HbSS) minus the average response in vehicle-treated HbAA (V HbAA) mice and multiplied by 100%. %MPE = (V HbSS – PD HbSS)/(V HbSS – V HbAA) x 100%. A dose response analysis confirmed that the dose of 30 µg (i.p.) was the minimally effective dose. The EC50 was 13.1 µg (0.61-283 µg, 95% confidence interval) (GraphPad Prism). Doses were plotted on a log scale. (C) Vehicle was DMSO:Tween 80:Saline, 13:0.5:86.5. HbSS mice injected with vehicle remained different from HbAA mice injected with vehicle throughout the testing period. KT109 (3 µg, i.pl.) blocked mechanical hyperalgesia ipsilateral to the injection through 24 h (F[3,168]=30.4, P<0.001 for treatment effect, n=5-8 mice/group, 2-way repeated measures ANOVA). *Different from HbSS mice injected with KT109 at p<0.05, **different at p<0.001, †† different from HbAA mice at p<0.001 (2-way repeated measures ANOVA).
with Bonferroni t-test). (D) Mechanical hyperalgesia was also blocked in the paw contralateral to the injection (F[1,88]=83.2, P<0.001 for treatment effect, 2-way repeated measures ANOVA), but the effect was not observed until 90 min after intraplantar injection of the drug. Limited data from A are included for perspective. (E) Testing doses of 1, 3 and 10 µg (i.pl.) confirmed that 3 µg was the minimum effective dose to reduce mechanical hyperalgesia ipsilateral to the injection in HbSS mice. *Different from vehicle at p<0.001, one-way ANOVA with Bonferroni t-test; n=5-8 mice/dose. Doses were plotted on a log scale. (F) The analog KT195 did not reduce mechanical sensitivity ipsilateral to the injection in HbSS mice when administered at the effective dose of KT109 (3 µg, i.pl.). (F[12,100]=4.2, P<0.001 for treatment effect, n=6-8 mice/group, 2-way repeated measures ANOVA). *Different from KT195 and vehicle at p<0.001, two-way ANOVA repeated measures with Bonferroni t-test.

**Figure 6.** Intraplantar administration of KT109 reduced heat hyperalgesia in HbSS mice. (A) HbSS mice had a shorter latency to withdraw from the heat stimulus prior to drug injection. *different from the same treatment group in HbAA mice at p<0.001 (F[3,60]=20.7, n=4-6 mice/group, 2-way repeated measures ANOVA with Bonferroni t-test). HbSS mice injected with KT109 maintained this difference from HbAA mice injected with KT109 60 min after drug administration in the paw ipsilateral to the injection, but heat hyperalgesia in HbSS mice was blocked at 2 and 3 hours post drug administration (†different from HbSS mice treated with vehicle at p<0.05). (B) Heat hyperalgesia was also blocked in the contralateral hind paw following intraplantar injection of KT109 into the opposite hind paw in a parallel time course (†different from HbSS mice treated with vehicle at p<0.05). Limited data from A are included for perspective. (C) Testing doses of 1, 3 and 10 µg (i.pl.) confirmed that 3 µg was the minimum effective dose to reduce thermal hyperalgesia ipsilateral to the injection in HbSS mice. *Different from vehicle at p<0.05, one-way ANOVA with Bonferroni t-test; n=6-8 mice/dose. Doses were plotted on a log scale.

**Figure 7.** Biochemical pathways involved in the modulation of pain in SCD by KT109. In contrast to HbAA mice, hyperalgesic HbSS mice demonstrated an increase in DAGLβ in blood cells and 2-AG in plasma. High levels of COX-2 oxidize 2-AG to generate the pro-nociceptive lipid mediator PGE2-G, which causes pain by sensitizing nociceptors. By inhibiting the enzyme activity of DAGLβ, KT109 reduces the accumulation of 2-AG, a target for COX-2, and thus blocks hyperalgesia in HbSS mice.
Methods

Mice

HbSS mice exclusively express human sickle hemoglobin S (HbS) whereas HbAA mice (experimental controls) express normal human hemoglobin A (HbA). Homozygous HbSS or HbAA mice do not express murine alpha- and beta-globins. Hemizygous HbAS mice contain one copy of normal human β-globin and one copy of sickle human β-globin. Hemizygous BERK sickle mice show vascular pathobiology and inflammation and increased mechanical, thermal and deep tissue hyperalgesia, although to a lesser extent than in homozygous HbSS mice.9,50

Western blot analysis

The amount of DAGLβ and COX-2 in blood cell lysates (45 μg of protein per sample) were determined from western blot. Samples were sonicated in RIPA buffer, and protein was quantified using a BCA kit (Sigma-Aldrich). DAGLβ and COX-2 were resolved on a 4-20% gradient SDS-PAGE gel and transferred to a PVDF membrane. Immunoreactivity was visualized using an Odyssey Imaging System (LI-COR). The density of each immunoreactive band was determined using ImageJ software (National Institutes of Health). The amount of DAGLβ and COX-2 proteins was defined as the density of the immunoreactivity of the protein of interest/total protein within each sample (Revert™ 700 Total Protein Stain, LI-COR) and expressed as a percent of the average amount of HbAA.

The specificity of the DAGLβ antibody was tested by knocking down DAGLβ in mouse fibrosarcoma cells clone NCTC 2472 with small interfering RNA (siRNA) specific
for the DAGLβ gene. siRNA transfection was performed in accordance with the Polyplus INTERFERin protocol. Commercially available siRNAs were used to silence DAGLβ: siRNA #1 (ID s107015, Catalog #4390771, Ambion) and siRNA #2 (ID s107016, Catalog #4390771, Ambion). A scrambled siRNA sequence (Catalog #AM4611) was used to demonstrate the specificity of DAGLβ transfection. The housekeeping gene GAPDH, (Catalog #AM4624) was selected as a positive control to show that silencing of the target gene did not affect cell viability. Cells were homogenized in RIPA buffer and stored at -80°C until analyzed. Western blot analysis was performed on 45 µg of protein and the amount of loading protein was verified by Revert™ 700 Total Protein Stain.

The specificity of the COX-2 antibody was tested by pre-incubation of the antibody with nickel resin (GE Healthcare) coated with a 10-fold molar excess of COX-2 His-tag protein (R&D systems). The antibody-COX-2 protein-nickel resin complex was collected by centrifugation, and the supernatant was applied to the PVDF membrane for determination of immunoreactivity. Incubation of COX-2 antibody with nickel resin alone was used as a negative control.

Behavioral measures of hyperalgesia

Mechanical hyperalgesia. Mice were placed on an elevated mesh platform under glass enclosures and allowed to habituate for 30 minutes prior to testing. Paw withdrawal thresholds were determined using the up-down method32. A series of eight von Frey monofilaments (0.07, 0.16, 0.4, 0.6, 1, 1.2, 2, and 4 g) was used. Testing was initiated with a monofilament that delivered 0.6 g. In the absence of a withdrawal response, a stronger monofilament was applied. If a withdrawal occurred, a weaker
stimulus was presented. Six responses, starting with the negative response immediately before the first paw withdrawal, were recorded. The resulting pattern was tabulated and the 50% paw withdrawal threshold (mechanical threshold) was calculated. The interstimulus interval was 5 s. To determine withdrawal response frequency, a von Frey monofilament with a bending force of 3.6 mN was applied to the plantar surface of each hind paw 10 times, with an interval of approximately 5-10 seconds between applications. *Heat hyperalgesia.* Mice were placed on a glass platform, covered with glass containers, and habituated for ~30 minutes. Radiant heat was applied to the plantar surface of each hind paw from below and paw withdrawal latency was determined. Mean withdrawal latency was calculated from 3 trials. The intensity of the heat source was adjusted so that mice withdrew their hind paws at ~9 seconds during baseline testing. A cutoff time of 16 seconds was imposed to prevent tissue damage.

**Measurement of 2-AG, PGE₂, PGE₂-G and AEA**

Lipids were extracted from each sample in the presence of AEA-d₈, 2-AG-d₅; PGE₂-G-d₅ and PGE₂-d₄ as internal standards.¹⁷ Levels of 2-AG, AEA, PGE₂-G and PGE₂ were analyzed by LC-NSI-MS/MS on a Quantiva™ triple quadrupole (Thermo Scientific™) interfaced to a Dionex Ultimate™ 3000 Rapid separation LC HPLC system (Thermo Scientific™) using a home-packed Luna C18 column (5 μm, 120 Å, 200 mm x 75 μm ID, Phenomenex) at room temperature.