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An Endocannabinoid Uptake Inhibitor from Black Pepper Exerts Pronounced Anti-Inflammatory Effects in Mice

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ABSTRACT: Guineensine is a dietary *N*-isobutylamide widely present in black and long pepper (*Piper nigrum* and *Piper longum*) previously shown to inhibit cellular endocannabinoid uptake. Given the role of endocannabinoids in inflammation and pain reduction, here we evaluated guineensine in mouse models of acute and inflammatory pain and endotoxemia. Significant dose-dependent anti-inflammatory effects (95.6 ± 3.1% inhibition of inflammatory pain at 2.5 mg/kg ip and 50.0 ± 15.9% inhibition of edema formation at 5 mg/kg ip) and acute analgesia (66.1 ± 28.1% inhibition at 5.0 mg/kg ip) were observed. Moreover, guineensine inhibited proinflammatory cytokine production in endotoxemia. Intriguingly, guineensine and LPS independently induced catalepsy, but in combination this effect was abolished. Both hypothermia and analgesia were blocked by the CB1 receptor inverse agonist rimonabant, but the pronounced hypolocomotion was CB1 receptor-independent. A subsequent screen of 45 CNS-related receptors, ion channels, and transporters revealed apparent interactions of guineensine with the dopamine transporter DAT, 5HT2A, and sigma receptors, uncovering its prospective polypharmacology. The described potent pharmacological effects of guineensine might relate to the reported anti-inflammatory effects of pepper.

KEYWORDS: *diet, endocannabinoid system, inflammation, pepper, polypharmacology*

INTRODUCTION

Guineensine belongs to a class of unique natural *N*-isobutylamides bearing a benzodioxane moiety terminal to the alkyl chain and was first isolated from West African pepper (*Piper guineense* Schumach).[1] This fatty acid derived natural product is abundant in several species of the plant genus *Piper*, including the dietary pepper species *P. longum* L. and *P. nigrum* L.,[2] where it is present in about 0.5% of the dry weight in the fruits.[3] Black pepper is one of the most popular spices worldwide and extensively employed for its culinary (i.e., spicy) but also medical properties.[4] Pepper is known to contain different bioactive phytochemicals such as the major alkaloids piperin and its isomer chavicin,[4] which exert numerous biological effects, including activation of TRP and K2P channels[5] which mediate the pungency of piperin. However, relatively little is known about the biological effects of the abundant *Piper* *N*-alkylamides. The outstanding bioactivity of guineensine (Figure 1) *in vivo* was only recently reported.[6] Although guineensine has been shown to be insecticidal,[7] significant to moderate noteworthy effects on mammalian targets have been reported too. For instance, guineensine was shown to inhibit the Acyl-CoA cholesterol acyltransferase (IC_{50} = 3.12 μM)[8] and alpha glucosidase-I (IC_{50} = 19.2 μg/mL).[3] The most potent biological activity of guineensine reported relates to its inhibition of the endocannabinoid uptake process, with an IC_{50} = 290 nM for anandamide (AEA) in U937 cells.[6] This mechanism was matched by indirect activation of cannabinoid CB1 receptors and overall cannabimimetic effects in mice.[6] Interestingly, the natural *N*-alkylamide *N*-benzyl-(9*Z*,12*Z*)-octadecadienamide from Peruvian Maca (*Lepidium meyenii* Walp.) was shown to likewise inhibit anandamide uptake (IC_{50} = 670 nM).[9] Different natural and synthetic *N*-alkylamides have been identified as potential modulators of endocannabinoid transport, and Chicca and Nicolussi *et al.* (2017) recently reported the first potent and selective endocannabinoid cellular reuptake inhibitor, WOBE437, which was derived from the natural 2,4-dodecadienamide scaffold from coneflower (*Echinacea* spp.).[10] Altogether, this strongly suggests that structural similarities between endocannabinoids and *N*-alkylamides from plants can cause noteworthy biological activities within the endocannabinoid system (ECS). Only *N*-alkylamides that are not substrates of serine hydrolases like fatty acid amide hydrolase (FAAH) can exert central pharmacological effects *in vivo*. It was previously shown that guineensine is not metabolized by FAAH or any of the known serine hydrolases involved in the ECS, but

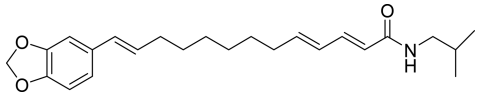

Figure 1. Chemical structure of guineensine ((2*E*,4*E*,12*E*)-13-(1,3-benzodioxol-5-yl)-*N*-(2-methylpropyl)trideca-2,4,12-trienamide).

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exhibits central cannabimimetic effects in mice,[6] namely, hypothermia, catalepsy, hypolocomotion, and analgesia (collectively referred to as tetrad), typically observed upon activation of central CB1 receptors.[11] The indirect cannabinoid receptor activation by guineensine is assumed to be related to its inhibition of central reuptake of the major endocannabinoids AEA and 2-arachidonoylglycerol (2-AG), similar to the synthetic endocannabinoid reuptake inhibitor WOBE437, which is more selective and more potent.[10]

CB1 receptors, which mediate retrograde signaling leading to the inhibition of synaptic circuits at both glutamatergic and GABAergic synapses, are abundantly expressed in the CNS and have been associated with analgesia, but also immunomodulation.[12] In our previous study, the analgesic effect of guineensine measured in the hot plate assay could be inhibited by the CB1 antagonist/inverse agonist rimonabant, while the effects on body temperature and locomotion were only partially blocked.[6] Endocannabinoids also interact with other proteins, including ion channels and nuclear receptors involved in immunomodulation.[13] Moreover, the ECS is involved in regulating numerous biochemical processes related to cellular stress and homeostasis, and it modulates also fundamental pathophysiological processes, including inflammation and pain.[13]

Given the emerging link between nutrition and the ECS,[14] here we studied the anti-inflammatory and analgesic effects of guineensine, the most potent natural endocannabinoid uptake inhibitor reported so far, in different mouse models of inflammation. To our knowledge, guineensine has not previously been studied in the context of inflammation despite the reported anti-inflammatory and analgesic effects of black pepper.[4] In our previous study, guineensine induced significant hypolocomotion under basal physiological condition that could only partially be inhibited by CB1 receptor antagonism.[6] Therefore, we profiled guineensine *in vitro* in a screening panel of 45 receptors, ion channels, and transporters related to CNS pharmacology, with the aim to further explore its potential polypharmacology. Our data show that upon intraperitoneal administration, guineensine exerts significant anti-inflammatory effects in different mouse models, including endotoxemia. Moreover, we propose additional CNS targets for this dietary natural product which may explain, at least in part, the CB1 receptor-independent extrapyramidal effects.

MATERIALS AND METHODS

Chemicals. Guineensine (≥95% pure in HPLC) was isolated and purified as previously described.[6] Formalin, indomethacin (Indo), and dimethyl sulfoxide (DMSO) puriss. and sterile filtered were obtained from Sigma-Aldrich (St. Louis, MO). Rimonabant (≥98% pure) was obtained from pharmaserv AG (Stansstad, Switzerland).

Materials. Lipopolysaccharide (LPS; *Escherichia coli*, serotype O55:B5) and carrageenan were obtained from Sigma-Aldrich (St. Louis, MO). Bio-Plex Pro Mouse Chemokine assays were obtained from Bio-Rad (Hercules, CA).

Animals. Male BALB/c mice (8 to 10 weeks old; 20−25 g body weight) were supplied by the Centro de Investigación Biomédica de Occidente—Instituto Mexicano del Seguro Social and kept under standard environmental conditions (24 ± 2 °C; light−dark cycle of 12:12 h) with food and water *ad libitum*. Mice were handled according to Mexican Federal Regulations for the Care and Use of Laboratory Animals NOM-062-ZOO-1999 (Mexican Ministry of Health), which is in accordance with the Code of Ethics of the Directive 2010/63/EU. For all the experiments, guineensine, rimonabant, or vehicle (DMSO) was administered intraperitoneally (ip) in a volume of 20 μL. At the vehicle dose used (1 mL/kg) no significant effects on the disease models were observed.

Formalin Test. Formalin provokes a biphasic painful behavior when it is administered intraplantarly. The first phase lasts 0 to 10 min after injection, and then, after a quiescent period, the second phase lasts 15 to 60 min.[15] The test was carried out individually with a BALB/c male mouse in a Plexiglas cage (20 × 20 cm). After a habituation period of 5 min, formalin was injected in the right hind paw (20 μL at 1% formalin in saline solution). Immediately, mice were gently placed in the cage and paw shaking was recorded during 60 min. Paw shaking was counted for 1 min every 5 min, and data were analyzed as percentage per phase.

Carrageenan-Induced Paw Edema. Paw inflammation was induced in BALB/c male mice by subplantar administration of 1% carrageenan in saline solution (w/v, 50 μL) into the right hind paw. Paw thickness was measured with a digital micrometer (Mitutoyo; Takatsuku, Japan) immediately prior to the injection of carrageenan (T_0) and thereafter at every 30 min for 3 h after carrageenan injection. Inflammation was evaluated as the increase in paw thickness (edema) at every time point (T_X) relative to T_0 and was reported in percentage. In addition, hyperalgesia was evaluated by mechanical sensitivity, with a digital algometer (Bioseb; Pinellas Park, FL) at T_0 and 4 h after carrageenan injection. The pain threshold was reported as percentage relative to T_0.

LPS-Induced Hypothermia. When high doses of LPS (2−5 mg/kg) are injected into mice, this leads to an inflammatory reaction with cytokine release and hypothermia.[16] For this, BALB/c male mice were injected ip, always in the evening (2−5 pm), with 2.5 mg/kg of LPS in saline. Rectal temperature was measured with a thermocouple probe (Physitemp Instruments; Clifton, NJ) right before LPS injection (T_0) and at every 30 min (T_X) for 2 h. Data were expressed as temperature change for each time point ($T_X - T_0$), and the area under the curve was calculated with the GraphPad Prism software version 6.

Open Field Test. Motor changes after LPS-induced hypothermia were evaluated in an open field box (40 × 40 × 30 cm, Plexiglas). The mice were individually placed in the center and allowed to move freely for 5 min. The locomotion activity was recorded with the OpenFieldTest (programed by Mario Coutiño), and the active time and distance were evaluated automatically. After every experiment, the box was cleaned with 70% ethanol to remove odors.

Cytokine Serum Levels. Mice were sacrificed by decapitation 2.5 h after LPS injection, the blood was recovered immediately thereafter, and serum was separated by centrifugation. IL1-β, TNF-α, and IL-6 were measured with Bio-Plex Pro Mouse Chemokine assays (Bio-Rad; Hercules, CA) and quantified with the MAGPIX Multiplex Reader (Luminex; Austin, TX), according to the manufacturer's instruction.

Tetrad Test. The tetrad test battery was conducted to evaluate the central cannabimimetic effect of guineensine upon LPS-induced hypothermia. For this, guineensine or vehicle was administered ip 1 h after LPS (2.5 mg/kg, ip) and the tetrad test battery was carried out 1 h later, as previously described.[10] In short, rectal temperature was measured right before and 1 h post guineensine or vehicle injection, and change in body temperature was expressed as the difference between these temperatures. Catalepsy was measured using the bar test, where the mouse was retained in an imposed position with forelimbs resting on a bar of 4 cm height. Latency in this position was measured until both front limbs were removed or remained over 120 s. Hypolocomotion was determined using the rotarod test where the animals were trained to walk under a rotarod (Erweka; Heusenstamm, Germany) at 4 rpm. For the test, latency to fall was measured with a cutoff time of 120 s. Catalepsy and hypolocomotion were measured independently in three trials. The hot plate tests were performed to evaluate analgesia, using a 54−56 °C hot plate (Thermo Scientific; Waltham, MA) with a Plexiglas cylinder. Animals were gently placed in the plate, and latency to first nociceptive response (paw lick or foot shake) was measured. Pretreatment with rimonabant (SR1) was used to evaluate CB1 antagonism. SR1 was administered ip 30 min before guineensine injection.

***In Vitro* Pharmacological Screening.** Guineensine was profiled in more detail in an extended CNS adverse drug reaction screening panel and tested for the interaction with the below indicated potential off-target proteins (45); receptors, transporters, and ion channels expressed in the CNS. The threshold to exclude a significant interaction was set as

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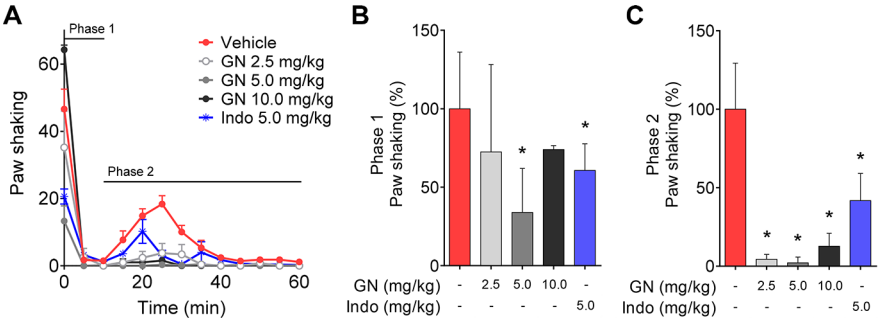

Figure 2. Effects of guineensine in male BALB/c mice on persistent pain in the formalin test. (A) Time course of paw shaking. (B) Percentage of pain inhibition in phase 1. (C) Percentage of pain inhibition in phase 2. Indomethacin (Indo; 5.0 mg/kg) was used as positive control. Vehicle (DMSO), guineensine (GN), or Indo was administered ip 30 min before formalin injection (20 μL, 1%, intraplantar). Data show mean values of at least 2 independent experiments (n = 5−16), *p < 0.05 compared to vehicle in Kruskal−Wallis test followed by Mann−Whitney U *post hoc* test.

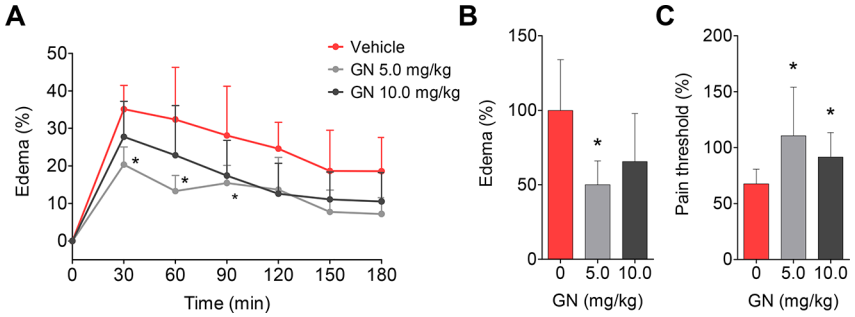

Figure 3. Evaluation of guineensine in carrageenan-induced paw edema in mice. (A) Time course of paw inflammation, evaluated as the differences in paw thickness. *p < 0.05 compared to vehicle using two-way ANOVA. (B) Effect of guineensine on overall inflammation as edema over 180 min (area under the curve). (C) Paw pain threshold, evaluated as mechanical sensitivity before and after 4 h of saline or carrageenan injection. Vehicle (DMSO) or guineensine (GN) was administered ip 30 min before saline or carrageenan injection (50 μL, 1%, intraplantar). Data show mean values of at least 2 independent experiments (n = 5−16), *p < 0.05 compared to vehicle in Kruskal−Wallis test followed by Mann−Whitney U *post hoc* test.

a binding/response of >50%. CNS-expressed catalogue reference number of each assay is indicated in parentheses referring to the performed CEREP assay (www.cerep.com): a1A, adrenergic receptor (2338); a2B, adrenergic receptor (1344); a2C, adrenergic receptor (0016); BRS3, bombesin-receptor subtype 3 (BB3) (0472); CB1, cannabinoid receptor type 1 (0036); CB2, cannabinoid receptor type 2 (0037); D1, dopamine receptor type 1 (0044); D2, dopamine receptor type 2 (1322); D3, dopamine receptor type 3 (0048); D4.4, dopamine receptor subtype 4.4 (0049); D5, dopamine receptor type 5 (0050); GABAA BZD, GABAA channel at the benzodiazepine binding site (0028); GABAA a1, β2, g2 site (3051); NMDA, NMDA (glutamate) receptor subunit NR1 (0066); TNF-α (0076); H1, histamine receptor type 1 (0870); MT3, melatonin receptor (ML2) (0088); MAO-A, monoamine oxidase A (0443); M5, muscarinic receptor (0097); d2 (DOP) δ-type opioid receptor (0114); μ-type (MOP) opioid receptor (0118); NMDA, PCP (0124); 5-HT1A serotonin receptor (0131); 5-HT2A (0471); 5-HT2B (1333); 5-HT2C (1003); 5-HT3 (0411); 5-HT4e (0501); 5-HT5a (0140); 5-HT6 (0142); 5-HT7 (0144); sigma receptor (3500); AR, androgen receptor (0933); Na^+ channel (site 2) (0169); Cl^- channel (GABA-gated) (0170); NAT, noradrenaline transporter (0355); DAT, dopamine transporter (0052); GABA transporter (0060); 5-HT transporter, serotonin transporter (0439); AChE, acetylcholinesterase (0363); GABA TA, GABA transaminase (0461); Tyr hydroxylase, tyrosine hydroxylase (0214); S1P1, sphingosine receptor (3269); TRPV1, transient receptor potential vanilloid 1 (1640); P-gp, P-glycoprotein (1324). All binding and functional assays were performed as a single screen in duplicate and guineensine was tested at the concentration of 10 μM.

Statistical Analyses. All data are presented as mean ± SD and were analyzed by nonparametrical approached with Kruskal−Wallis test followed by Mann−Whitney U as a *post hoc* test. For time-course analyses of carrageenan-induced paw edema and LPS-induced hypothermia, data were analyzed by two-way ANOVA and Bonferroni post test. A confidence level of P < 0.05 was considered statistically significant. Analyses were carried out using the GraphPad Prism software version v6.0 (La Jolla, CA).

RESULTS

Guineensine Decreased Nociceptive and Inflammatory Pain in the Formalin Test in BALB/c Mice. The pain behavior (paw shaking) observed in the first phase of the formalin test is due to nociceptor activation, but the second phase starts with an acute inflammatory process (Figure 2A). 5.0 mg/kg ip of guineensine was the dose necessary to significantly reduce paw shaking in both phases, with 66.1 ± 28.1 and 97.8 ± 3.7% of pain inhibition per phase, respectively (Figures 2B and 2C). At a dose of 10.0 mg/kg ip, paw shaking was inhibited by 26.0 ± 2.5 and 87.2 ± 8.2% in the first and the second phase, respectively. Thus, the effect of guineensine was biphasic, which may be due to its polypharmacology (see below). At the dose of 2.5 mg/kg ip guineensine selectively but strongly reduced the inflammatory pain by 95.6 ± 3.1% as measured by paw shaking (Figure 2C).

Carrageenan-Induced Edema in BALB/c Mice Was Abolished by Guineensine. Carrageenan is a polysaccharide that induces a strong inflammatory reaction when it is administered via the intraplantar.[17] In Figure 3A, the time course of the edema formation (expressed as percentage of inflammation) relative to basal condition is shown. Guineensine at 5.0 mg/kg ip significantly decreased the degree of edema (inflammation) by approximately 50% between 30 and 90 min after carrageenan injection (Figure 3A). This was also significant (50.0 ± 15.9% inhibition) when the total area under the curve was analyzed as % edema formation (Figure 3B). In addition, the

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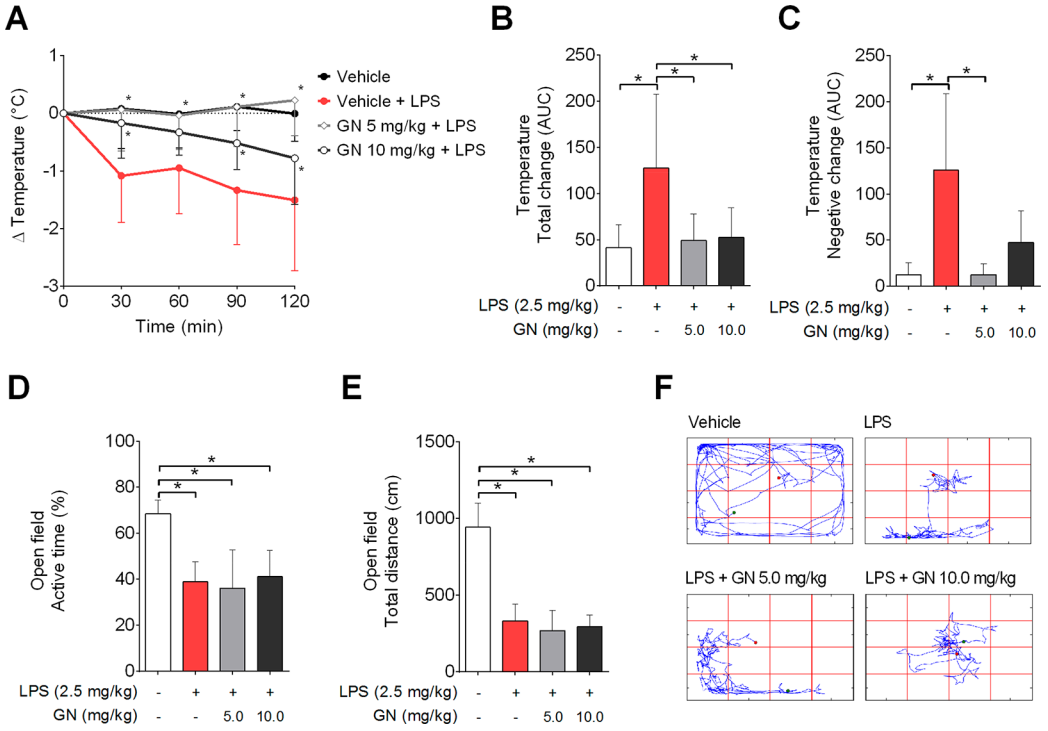

Figure 4. Evaluation of guineensine in LPS-induced hypothermia in mice. (A) Time course of body temperature changes after lipopolysaccharide (LPS) injection. *$p < 0.05$ compared to vehicle using two-way ANOVA. (B) Area under the curve of time-course body temperature change for total area. (C) Area under the curve of time-course body temperature change for negative area. (D) Active time and distance traveled in the open field test. (D) Distance traveled in the open field test. (F) Representative example of travel pathway in the open field test. Vehicle (DMSO) or guineensine (GN) was administered ip 30 min before saline or LPS injection (50 μL, 2.5 mg/kg, ip). *$p < 0.05$ compared with vehicle or LPS, as indicated. Data show mean values of 3 independent experiments ($n = 5–16$), *$p < 0.05$ compared to vehicle in Kruskal–Wallis test followed by Mann–Whitney U *post hoc* test.

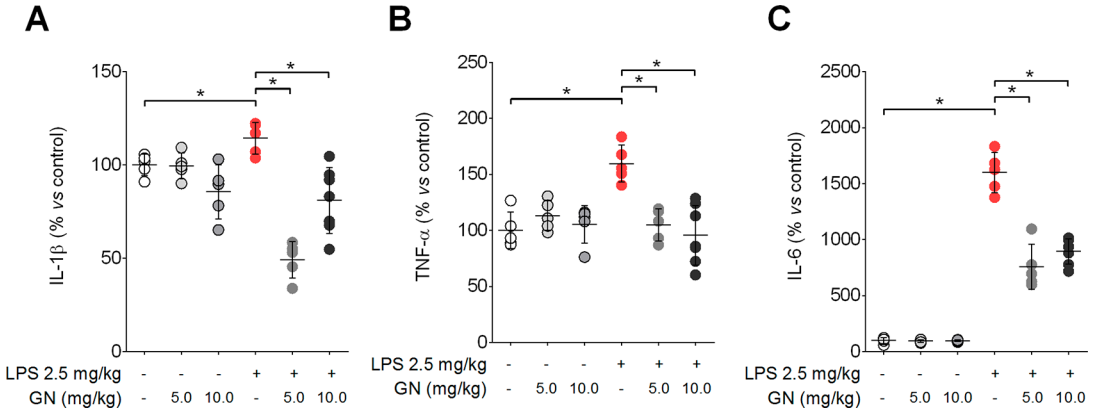

Figure 5. Evaluation of proinflammatory cytokines in response to guineensine and LPS-induced hypothermia in mice. (A) Interleukin 1β (IL-1β), (B) tumor necrosis factor α (TNF-α), and (C) interleukin 6 (IL-6) were quantified in serum re-collected 2.5 h after saline or LPS injection. Values are reported as percentage relative to group control saline + vehicle. Vehicle (DMSO) or guineensine (GN) was administered ip 30 min before saline or LPS injection (50 μL, 2.5 mg/kg, ip). *$p < 0.05$ compared with vehicle or LPS, as indicated. Data show mean values ($n = 5–7$), *$p < 0.05$ compared to vehicle in Kruskal–Wallis test followed by Mann–Whitney U *post hoc* test.

pain response was evaluated after 3 h of 5.0 mg/kg ip of guineensine where it significantly improved the pain threshold compared to vehicle (Figure 3C). Again, there was a biphasic inhibitory effect of guineensine in the carrageenan induced edema with 5.0 mg/kg ip being more efficient than 10.0 mg/kg ip, which may be related to multiple targets at higher doses (see below).

Effects of Guineensine in LPS-Induced Hypothermia in BALB/c Mice. We employed an endotoxemia model measuring acute LPS-mediated hypothermia.[16] Systemic administration of LPS (2.5 mg/kg) induced a body temperature decrease after 2 h of injection. As shown in Figure 4A, guineensine at 5.0 and 10.0 mg/kg ip significantly prevented this LPS-mediated hypothermia. Analysis of area under the curve revealed that both doses reduced the overall body temperature change (Figure 4B), but when only the negative values were considered, only 5.0 mg/kg ip had a significant effect (Figure 4C). Thus, again the lower dose was more effective than the higher dose of guineensine. We also assessed the spontaneous motor activity in the open field where LPS induced a significant reduction of active time and distance traveled (about 40%), which was however not modulated by guineensine treatment (Figures 4D to 4F).

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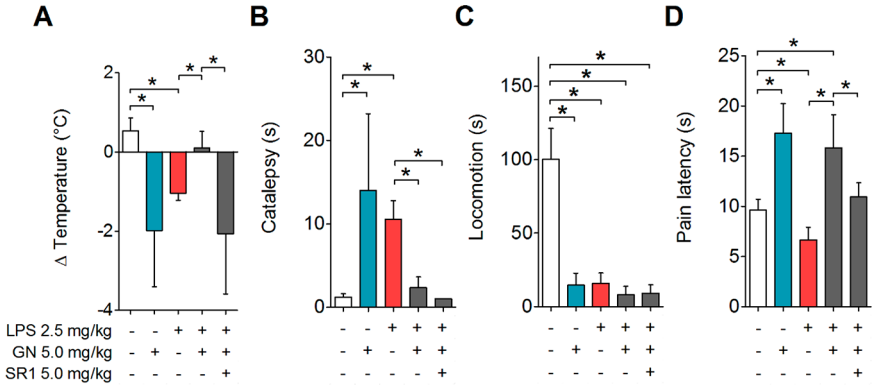

Figure 6. Evaluation of tetrad test in mice with LPS-induced hypothermia and guineensine treatment. (A) Change in rectal temperature. (B) Catalepsy latency in the bar test. (C) Latency to fall in the rotarod test at 4 rpm. (D) Pain latency in the hot plate test at 55 °C. Vehicle (DMSO) or guineensine (GN) was administered ip 60 min after lipopolysaccharide (LPS) injection (50 μL, 2.5 mg/kg, ip). Rimonabant (SR1) was administered ip 30 min before guineensine. Data show mean values of at least 2 independent experiments (n = 5−11), *p < 0.05 compared with vehicle, LPS or SR1 + GN, as indicated in Kruskal−Wallis test followed by Mann−Whitney U *post hoc* test.

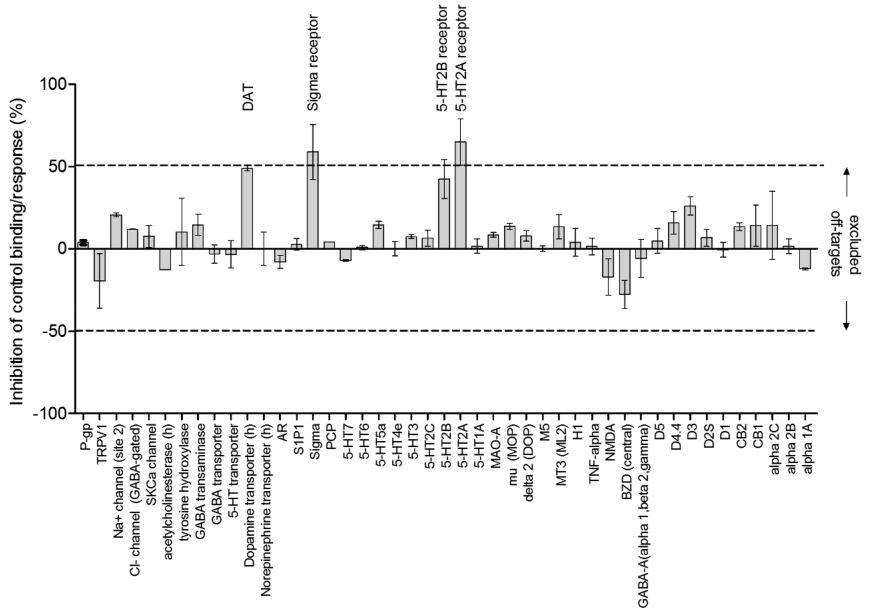

Figure 7. *In vitro* pharmacological screening of guineensine at 10 μM. The CEREP screen was carried out over an extended CNS adverse drug-reaction panel, which included 45 receptors, transporters, and ion channels. The threshold to excluded significant interaction was >50% of binding/response. The assays were performed in duplicate (N = 4). Data show mean values ± SD.

It has previously been shown that LPS-induced hypothermia correlates with the release of proinflammatory cytokines, in particular IL-1β, TNF-α, and IL-6.[18] Here, we measured these cytokines 2.5 h after LPS injection (ideal time point to see all three cytokines). As shown, the LPS-induced protein production of IL-1β (Figure 5A), TNF-α (Figure 5B), and IL-6 (Figure 5C) in vehicle treated animals was significantly inhibited by guineensine when preinjected 30 min prior to LPS challenge at 5.0 and 10.0 mg/kg ip. Interestingly, for IL-1β 5.0 mg/kg ip of guineensine was again more efficient than 10.0 mg/kg ip and even lowered the unexpected high basal IL-1β levels in these mice (796 ± 45 pg/mL) (Figure 5A). On the other hand the basal IL-6 levels (169 ± 45 pg/mL) were relatively low but dramatically increased upon LPS challenge (2873 ± 320 pg/mL) (Figure 5C). The high IL-1β levels could be due to the BALB/c strain used where similar levels have been reported.[19] Both doses fully inhibited the LPS-induced TNF-α release but only partially inhibited the expression of IL-6 (approximately 50%). Overall, these data indicate that guineensine attenuates proinflammatory cytokine production.

Differential Effects of Guineensine on Extrapyramidal Behaviors in Endotoxemia. Endotoxin challenge with LPS produced significant hypothermia, catalepsy, hypolocomotion, and hyperalgesia (Figure 6). Thus, LPS triggered three out of four behavioral phenomena that were also induced by guineensine.[6] Here, we assessed the combined effects of LPS and guineensine in these tests. As is shown in Figure 6A, 5.0 mg/kg ip guineensine prevented the hypothermia induced by LPS. Intriguingly, despite 5.0 mg/kg ip guineensine giving a catalepsy in noninflamed mice,[6] it fully abolished the catalepsy induced by LPS (Figure 6B). However, in the locomotion experiment (rotarod), guineensine did not antagonize the effects of LPS (Figure 6C). As expected, guineensine was analgesic and efficiently inhibited the hyperalgesic effects of LPS (Figure 6D). In order to get more insights into the mode of action, we used the CB1 receptor inverse agonist/antagonist rimonabant

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prior to guineensine treatment. As shown in Figure 6, the hypothermia and analgesic effects of guineensine were mediated through CB1 receptors as they could be fully blocked by rimonabant. However, the hypolocomotion and catalepsy presented with LPS/guineensine was apparently independent of CB1 receptors (Figures 6B and 6C).

Pharmacological Profiling of Guineensine on CNS Proteins. Since guineensine by itself induced apparent extrapyramidal effects in mice (Figure 6C) and we previously found that the guineensine-induced hypolocomotion was only partially mediated by CB1 receptors,[6] we carried out a pharmacological profiling on 45 CNS-related receptors, transporters, and ion channels. Employing the CEREP platform, at 10 μM of guineensine, only few potential additional CNS targets could be identified. Considering the commonly used threshold binding/response of >50% at 10 μM, it was found that guineensine was able to interact with the dopamine transporter (DAT), sigma receptor, and 5-HT2A and 5-HT2B receptors (Figure 7). Although no concentration-dependent assessment of these likely molecular interactions has been carried out, the binding interaction with 5HT2A and sigma receptors was clearly outstanding, and these might be relevant biological targets of this dietary *N*-isobutylamide.

DISCUSSION

The dried fruits of different pepper species, including *P. nigrum* L., *P. longum* L., and *P. guineense* Schumach, are widely used spices. They are primarily consumed because of their pungent taste, which is thought to be mediated by TRP and K2P channels,[5] but adaptive biological reasons like counteracting metabolic stress induced by high-calorie diets cannot be excluded.[14] Additionally, the fruits and other plant parts of these peppers are used ethnomedically in different cultures for their medicinal properties, such as antirheumatic, anti-inflammatory, antibacterial, and antifungal.[20] In Asian traditional medicine pepper species have been used for millennia and are known to exert analgesic and anti-inflammatory effects.[21−23] Moreover, *Piper nigrum* L. and *Piper longum* L. are used traditionally to treat psychiatric disorders like affective disorders.[24,25]

The *N*-isobutylamide guineensine was previously identified as a potent dietary uptake inhibitor for both endocannabinoids *in vitro*, increasing the extracellular levels of AEA and 2-AG in U937 and HMC-1 cells. This increase was pharmacologically associated with cannabimimetic effects *in vivo* in the tetrad test.[6] In agreement, increased endocannabinoid levels, for example after inhibition of AEA or 2-AG degradation, show pronounced anti-inflammatory and analgesic effects in several experimental models of inflammatory[26−28] and neuropathic pain.[29,30] Also, some moderately selective AEA uptake/FAAH inhibitors like AM404, OMDM-2, and UCM707[31] have been tested in inflammatory models of acute pain and in models of neuropathic pain, showing anti-inflammatory and analgesic effects mediated by cannabinoid receptors but also by peroxisome proliferator-activated receptors and transient receptor potential vanilloid 1 (TRPV1).[28,32−36] More recently, the highly potent and selective endocannabinoid reuptake inhibitor WOBE437 was shown to exert anti-inflammatory and analgesic effects.[10]

Here, pretreatment with guineensine efficiently prevented the endotoxemia induced by LPS, resulting in a reduction in acute hypothermia and expression of the proinflammatory cytokines IL-1β, TNF-α, and IL-6. The endocannabinoid degradation inhibitors JZL184 (monoacylglycerol lipase inhibitor) and URB597 (FAAH inhibitor) were shown to decrease IL-1β in frontal cortex, hypothalamus, and plasma after an immunological challenge with LPS in rats,[37,38] and JZL184 also decreased IL-6 and TNF-α through cannabinoid receptors.[38] Furthermore, 2-AG was shown to protect against inflammation induced by LPS in a primary culture of caudate nucleus neurons, by decreasing cyclooxygenase-2 expression through a CB1 mechanism.[39] However, contrasting results have been found in rodent models of LPS-induced hypothermia, where CB1 knockout mice or CB1 antagonism prevented the hypothermia and rather decreased TNF-α[40] and IL-6 levels.[41] In other experiments, AEA or JZL184 enhanced LPS-induced hypothermia through a CB1 receptor-dependent mechanism, but did not decrease body temperature by themselves.[40,42] These divergent results could be due to LPS dose, administration route, mouse strain, or time point of circadian rhythm. The general anti-inflammatory effect of guineensine reported here was biphasic, being more potent at the lower concentrations, which might reflect differential polypharmacology.

In our previous study, guineensine potently induced hypothermia, catalepsy, hypolocomotion, and analgesia (tetrad test) under normal physiological conditions.[6] While in normal animals the analgesia and catalepsy could be inhibited fully by rimonabant, the hypothermia and locomotion effects were only partially inhibited by CB1 receptor antagonism. In this study we evaluated whether guineensine treatment in endotoxemic mice showed protective effects that were CB1 receptor-dependent. The endotoxin challenge with LPS induced significant hypothermia, catalepsy, hypolocomotion, and hyperalgesia in mice, in agreement with the literature.[16,43,44] Interestingly, guineensine potently inhibited all LPS mediated effects but not the hypolocomotion induced by LPS. In fact, the locomotion depression may have been additive rather than antagonistic. Moreover, guineensine reduced hypothermia and hyperalgesia by a CB1 receptor-dependent mechanism, in agreement with its previously reported effect on endocannabinoid reuptake.[6] The hypolocomotion after LPS injection has been previously reported being associated with sickness behavior.[45,46] Because guineensine exhibited extrapyramidal effects that appeared to be independent of CB1 receptor activation, at least partially, we profiled this natural product on 45 CNS related receptors, ion channels, and transporters. As a result, guineensine was found to interact significantly with the sigma and 5-HT2A receptors, as well as DAT and the 5-HT2B receptor. Although further biochemical analyses will be necessary to assess the potential role of these targets for the pharmacology of guineensine, the CNS targets identified here might explain some of the extrapyramidal effects in mice. Dopamine is known to modulate motor coordination through direct and indirect pathways in basal ganglia,[47,48] and also 5-HT2A receptors have been reported to increase or improve locomotor activity, particularly due to its expression in cerebellar fastigial nucleus neurons.[49,50] Sigma receptors, mostly sigma 2, are also implicated in locomotion because they modulate dopamine signaling.[51−53] The effects of guineensine on the function of these targets should be tested in future experiments and put into context with the overall bioavailability of guineensine.

In summary, guineensine is a dietary endocannabinoid uptake inhibitor that shows significant anti-inflammatory properties *in vivo* with associated analgesic effects. The acute decrease in locomotion, in particular under inflammatory conditions, might be due to extrapyramidal effects related to its affinity to 5-HT2A,

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sigma receptors, and maybe DAT. In light of the abundance of guineensine in pepper and its explicit CNS polypharmacology, further experiments will have to address the metabolic stability and oral bioavailability of this dietary phytochemical and its likely role as a pharmacologically active natural product. Overall, the here reported bioactivity of guineensine might be associated with the reported anti-inflammatory effects and general health claims of dietary pepper.

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ABBREVIATIONS USED

2-AG, 2-arachidonoylglycerol; 5-HT, 5-hydroxytryptamine or serotonin; AEA, anandamide; CNS, central nervous system; DAT, dopamine transporter; DMSO, dimethyl sulfoxide; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; GN, guineensine; Indo, indomethacin; ip, intraperitoneally; K2P, potassium channel subfamily K member 2; LPS, lipopolysaccharide; SR1, rimonabant; TRP, transient receptor potential

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