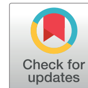

BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 31(2), 161-167 (2023)

Hycanthone Inhibits Inflammasome Activation and Neuroinflammation-Induced Depression-Like Behaviors in Mice

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Abstract

Despite the various medications used in clinics, the efforts to develop more effective treatments for depression continue to increase in the past decades mainly because of the treatment-resistant population, and the testing of several hypotheses- and target-based treatments. Undesirable side effects and unresponsiveness to current medications fuel the drive to solve this top global health problem. In this study, we focused on neuroinflammatory response-mediated depression which represents a cluster of depression etiology both in animal models and humans. Several meta-analyses reported that proinflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were increased in major depressive disorder patients. Inflammatory mediators implicated in depression include type-I interferon and inflammasome pathways. To elucidate the molecular mechanisms of neuroinflammatory cascades underlying the pathophysiology of depression, we introduced hycanthone, an antischistosomal drug, to check whether it can counteract depressive-like behaviors *in vivo* and normalize the inflammation-induced changes *in vitro*. Lipopolysaccharide (LPS) treatment increased proinflammatory cytokine expression in the murine microglial cells as well as the stimulation of type I interferon-related pathways that are directly or indirectly regulated by Janus kinase-signal transducer and activator of transcription (JAK-STAT) activation. Hycanthone treatment attenuated those changes possibly by inhibiting the JAK-STAT pathway and inflammasome activation. Hycanthone also ameliorated depressive-like behaviors by LPS. Taken together, we suggest that the inhibitory action of hycanthone against the interferon pathway leading to attenuation of depressive-like behaviors can be a novel therapeutic mechanism for treating depression.

Key Words: Neuroinflammation, Depression, Hycanthone, Animal model, Interferon signaling

INTRODUCTION

Depression is one of the most common and serious psychiatric disorders affecting people of all ages (Ferrari *et al*., 2013). It occurs in approximately 15% of the general population (Romain Troubat *et al*., 2021) with implications of suicide as the number one behavioral comorbidity and the most common cause of avoidable death in affected individuals (Orsolini *et al*., 2020). Depression is a condition characterized by decreased interest, loss of appetite, lack of concentration, feelings of sadness and deep sorrow, and a varying degree of hopelessness (Marcus *et al*., 2012).

Several known mechanisms describe the pathophysiology of depression. One of which is the monoamine hypothesis, which predicts that the underlying cause of depression is the alteration of norepinephrine, dopamine, and serotonin levels in the brain (Jesulola *et al*., 2018). This hypothesis is supported by the clinical use of monoamine oxidase inhibitors and tricyclic antidepressants (Krishnan and Nestler, 2008). In addition, studies on depression have been conducted concerning various etiological aspects such as genetic factors (Mondimore *et al*., 2006; Dannlowski *et al*., 2007; López-León *et al*., 2008; Xiang *et al*., 2008), neuroendocrine factors including the hypothalamic pituitary adrenal (HPA) axis dysfunction

Open Access https://doi.org/10.4062/biomolther.2022.073

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Received May 30, 2022 **Revised** Aug 19, 2022 **Accepted** Sep 20, 2022
Published Online Oct 7, 2022

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(Brigitta, 2002; Vreeburg *et al*., 2009; Keller *et al*., 2017; Bao and Swaab, 2019) and immunologic factors (Song *et al*, 2009; Jeon *et al*., 2017; Zhao and Liu, 2019).

Neuroinflammation has been widely implicated in the study of depression with reports suggesting the involvement of immune stimulation and challenges (Raison *et al*., 2006). Depression could also more likely develop in people who are immunocompromised such as those who have infectious and autoimmune diseases (Jeon and Kim, 2017). Several inflammatory cytokines such as C-reactive protein (CRP), tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), and interleukin 10 (IL-10) were implicated in the pathogenesis of depression in recent studies (Strawbridge *et al*., 2017). The administration of interferon-alpha (IFN-α) to treat patients with chronic hepatitis C, induced depressive symptoms as one of the adverse effects (Murakami *et al*., 2016). Similarly, increased levels of pro-inflammatory cytokines in animals with stress-induced depression were associated with microglial activation (Yang *et al*., 2015; Chen *et al*., 2016).

Type I interferons are innate immune molecules with strong pro-inflammatory responses and mediate inflammasome activation. Type I interferons are required for the upregulation of caspases, which resulted in the non-canonical nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasome response or expression priming of NLRP3 components (Malireddi and Kanneganti, 2013). NLRP3 is an intracellular multiprotein complex responsible for the innate immune processes associated with infection, inflammation, and psychiatric conditions such as depression. We searched for a compound that can inhibit the type I interferon and inflammasome activity, and consequently, depressive-like behaviors. We found that hycanthone can inhibit both inflammation and inflammasome response in microglial cell lines.

In the present study, we aimed to investigate the antidepressant effects of hycanthone on the LPS-induced depression in mice model. Our results showed that the production of inflammatory cytokines including TNFα, IL-1β, and IL-18 is reduced by hycanthone in the microglial cell line. Moreover, we also demonstrated the antidepressant effects of hycanthone, at least in part, which is mediated via the modulation of type I interferon signaling pathway. These results imply that hycanthone can be used for the treatment of depression through the regulation of neuroinflammatory responses.

MATERIALS AND METHODS

Animal

Male C57BL/6 mice (6 weeks) were obtained from the Orient Bio (Seongnam, Korea). The mice were housed in an optimal environment at a temperature range of 23 ± 2°C and humidity range of 50 ± 10% in a dark/light cycle (lights on at 12:00 AM; lights off at 12:00 PM). All procedures were approved by Konkuk IACUC, Seoul, Korea (KU20195).

Drugs

Hycanthone (Cat No. HY-B1099) was purchased from MedChemExpress (NJ, USA) and LPS (Cat No. L2630) was obtained from Sigma-Aldrich (MO, USA). Hycanthone (25 and 50 mg/kg) was dissolved in 0.1% dimethyl sulfoxide (DMSO) for *in vitro* assays, and in 10% DMSO plus 10% Tween 80 in saline solution for *in vivo* experiments. Mice were treated with hycanthone or vehicle using intraperitoneal injection. Ruxolitinib (tlrl-rux, InvivoGen, CA, USA) and MCC950 (PZ0280, Sigma-Aldrich) were obtained. Ruxolitinib was dissolved at 0.1 μM and MCC950 at 50 μM in culture media.

Forced swim test

The forced swim test (FST) is a widely used behavior test to identify depressive-like behaviors in rodents (Can *et al*., 2012). At the start of each trial, the mice were placed individually in a test cylinder (diameter: 15 cm, height: 25 cm) filled with water regulated at 25 ± 1°C and allowed to swim or float for 6 min while a video camera recorded the whole trial. Subsequently, the subject mice were carefully removed and dried with a towel before placing them back in their home cage. The water was changed after each trial. The immobility time was manually observed and timed by a blind observer during the last 4 min of each trial.

Cell culture

The mouse microglia cell line, BV-2 cells, were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37°C in 5% CO_2. The cells were pretreated with hycanthone (0.1, 1, 10 μM) 30 min before LPS (10 ng/mL) treatment. To observe the type I interferon-induced Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway activation, HEK-blue IFN α/β cells were used, which were obtained from InvivoGen. HEK-blue IFN α/β cells were cultured in DMEM supplemented with 10% FBS, 1% PS, blasticidin (30 μg/mL), and Zeocin™ (100 μg/mL).

NO assay

BV-2 cells ($1{\times}10^5$/well) were seeded in a 24-well plate and stabilized overnight. The cells were treated with various concentrations of hycanthone before LPS (10 ng/mL) stimulation. After 24 h, the cell supernatant was collected. Equal volumes of cell supernatant and Griess reagent (1% sulphanilamide and 0.1% N-1-napthylenediamine dihydrochloride) were mixed for 10 min at room temperature. The optical density was determined at 540 nm, using SpectraMax® ABS plus (Molecular Devices, CA, USA).

QUANTI-blue assay

HEK-blue IFN α/β cells ($3{\times}10^5$/well) were seeded in 96-well plate and stabilized overnight. The cells were pre-treated (1 h) by various concentration of hycanthone before IFN-α2b (0.1 ng/mL). After 17 h of stimulation, 190 μL of QUANTI-blue solution was added in 10 μL of cell supernatant. Subsequently, 200 μL of supernatant mixture is incubated at 37°C for 0.5 h. The optical density was determined at 655 nm using SpectraMax® ABS plus (Molecular Devices).

ELISA

BV-2 cells ($1{\times}10^5$/well) were seeded in a 24-well plate and stabilized overnight. The cells were treated with various concentrations of hycanthone before LPS (10 ng/mL) and adenosine 5'-triphosphate (ATP) (2 mM) treatment. Three h after adding LPS, the cells were co-stimulated with ATP for another hour. The cell supernatants were used to observe the change in the levels of inflammatory cytokines. ELISA kits (TNF-α, DY410-05; IL-1β, DY401; IL-18, DY7625-05) were purchased

Table 1. Sequence of qRT-PCR primers

Gene	Accession number	Forward	Reverse
TNFa	NM_013693	TTC GAG TGA CAA GCC TGT AG	AGA TTG ACC TCA GCG CTG AGT
IL1b	NM_008361	GTC TTT CCC GTG GAC CTT CC	TCG GAG CCT GTA GTG CAG TTG
IL18	NM_008360	TGG CTG CCA TGT CAG AAG ACT	CAG TGA AGT CGG CCA AAG TTG
NLRP3	NM_145827	AAG ATT ACC CGC CCG AGA AAG	TCG CAG CAA AGA TCC ACA CA
iNOS	NM_010927	CAA GAG TTT GAC CAG AGG ACC	TGG AAC CAC TCG TAC TTG GGA
COX2	NM_011198	TGC TGT ACA AGC AGT GGC AA	AGG TGC TCG GCT TCC AGT AT
IDO1	NM_008324	AGT CGG AAG AGC CCT CAA AT	GGT GTT TTC TGT GCC CTG AT
Ifi44	NM_133871	ATG CAC TCT TCT GAG CTG GTG G	TCA GAT CCA GGC TAT CCA CGT G
Gbp7	NM_145545	GGT GTC ATC ACA GCA GAC GAG T	CCG TCT TGG AAA GAA GTG CCT G

from R&D Systems (MN, USA). ELISA was performed according to the manufacturer's instructions. The optical density was determined at 450 nm using SpectraMax® ABS Plus (Molecular Devices).

Quantitative RT-PCR analysis

Quantitative real-time PCR was conducted to confirm the mRNA expression levels in the prefrontal cortex. The total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) and measured by Nanodrop (Nanodrop Technologies, NC, USA). The cDNA was synthesized using 2 μg of total RNA and the RT reaction mixture containing RevertAid Reverse transcriptase, reaction buffer (Thermo Fisher Scientific, MA, USA), and dNTP (Promega, WI, USA). Template cDNA was amplified by QuantStudio3 (Thermo Fisher Scientific) using BlasTaq™ 2X qPCR MasterMix (Applied Biological Materials Inc., BC, Canada). The primer pairs used in qRT-PCR are mentioned in Table 1.

Statistical analysis

All values were described as the mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons in the GraphPad Prism 7 software (GraphPad, CA, USA). The level of statistical significance was considered at a *p* value of <0.05.

RESULTS

Interferon activity was significantly reduced by hycanthone treatment

To evaluate the anti-interferon activity of hycanthone, we performed the QUANTI-blue assay in HEK-blue IFN α/β cells. Secreted alkaline phosphatase (SEAP) reporter system level was significantly increased by the stimulation of interferon-α2b and reduced by treatment of hycanthone at 10 μM (Fig. 1). Ruxolitinib has an inhibitory effect on JAK1/2 and type 1 interferon signaling pathways and was used here as a positive control. The result suggests that hycanthone have modulatory effects on IFN-induced inflammation response.

Production of neuroinflammatory mediators were decreased by hycanthone in microglial cell line

To investigate whether hycanthone affects the neuroinflammatory response, we first stimulated the microglial BV2 cells with LPS. Nitric oxide (NO) production was increased by LPS stimulation and was counteracted by hycanthone in

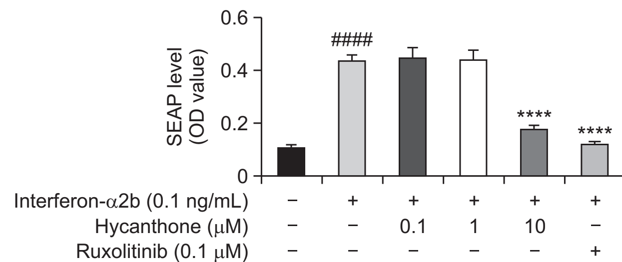

Fig. 1. The anti-interferon activity of hycanthone. HEK-blue IFN α/β cells were stimulated by interferon-α2b (0.1 ng/mL) for 17 h after pre-treatment with hycanthone (μM) (N=6 per group). Data were analyzed by one-way ANOVA. $^{####}p<0.0001$ vs. vehicle group, $^{****}p<0.0001$ vs. Interferon-α2b group.

a dose-dependent manner (Fig. 2A). The elevation of TNF-α expression after LPS stimulation was also reduced by 10 μM hycanthone (Fig. 2B). To activate the NLRP3 inflammasome (Coll *et al*., 2015), LPS and ATP were co-stimulated in BV2 cells (Xie *et al*., 2014). The release of IL-1β and IL-18 was increased by co-stimulation of LPS and ATP, and significantly inhibited by 10 μM hycanthone (Fig. 2C, 2D). MCC950 is a well-established inhibitor of NLRP3 inflammasome activation and was used as a positive control (Coll *et al*., 2015). These findings suggest that hycanthone can inhibit the production of pro-inflammatory factors associated with neuroinflammation and inflammasome activation.

Hycanthone ameliorated depressive-like behaviors in mice

Various studies have demonstrated that neuroinflammation is involved in the pathophysiology of depression by enhancing the expression of inflammatory cytokines (Jeon and Kim, 2018). To determine the antidepressant effect of hycanthone, we treated C57BL/6N mice with hycanthone for 10 days and subsequently induced depressive-like behaviors via LPS stimulation (O'Connor *et al*., 2009) a day before the experiment (Fig. 3A). Any effect on the body weight was not noted in hycanthone-treated groups (Fig. 3B). Consistent with other studies, LPS administration induced depressive-like behaviors in mice (Fig. 3C) (Sulakhiya *et al*., 2016; Li *et al*., 2019). Meanwhile, sub-chronic treatment with hycanthone (25 and 50 mg/kg, i.p.) significantly attenuated the LPS-induced increase in immobility time in FST, showing the antidepressant potential of hycanthone (Fig. 3C).

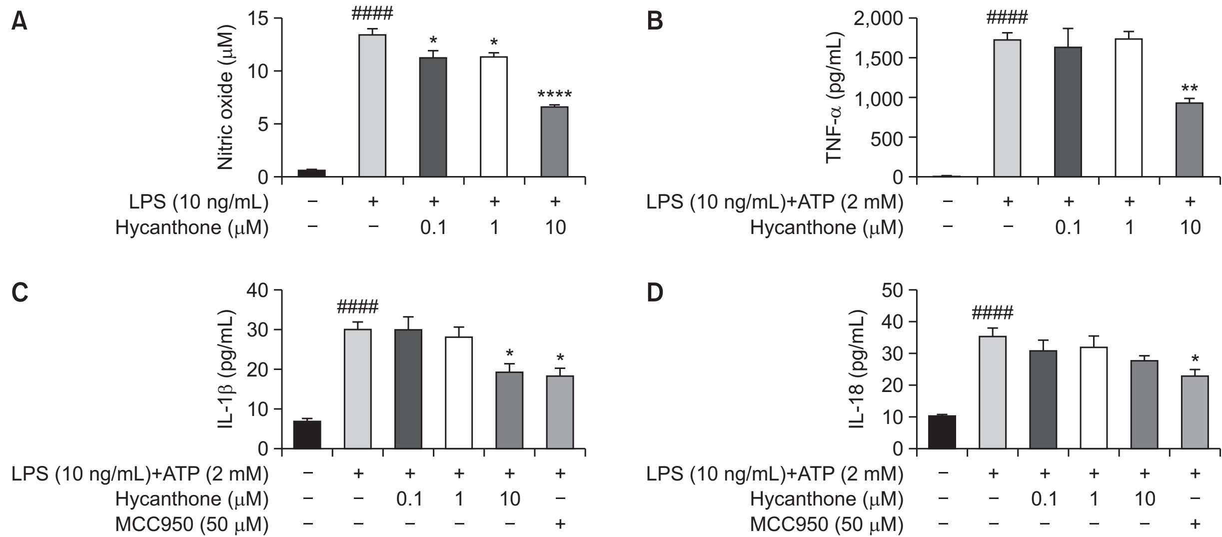

Fig. 2. The anti-inflammatory activity of hycanthone. (A) BV2 cells were stimulated by LPS (10 ng/mL) for 24 h. (B-D) In addition, the cells were treated by LPS (10 ng/mL) for 3 h and subsequently added by ATP (2 mM) for 0.5 h after pre-treatment with hycanthone (μM) (N=3 per group). Data were analyzed by one-way ANOVA. $^{####}p$<0.0001 vs. vehicle group, ****p<0.0001, **p<0.01, *p<0.05 vs. LPS or LPS+ATP group.

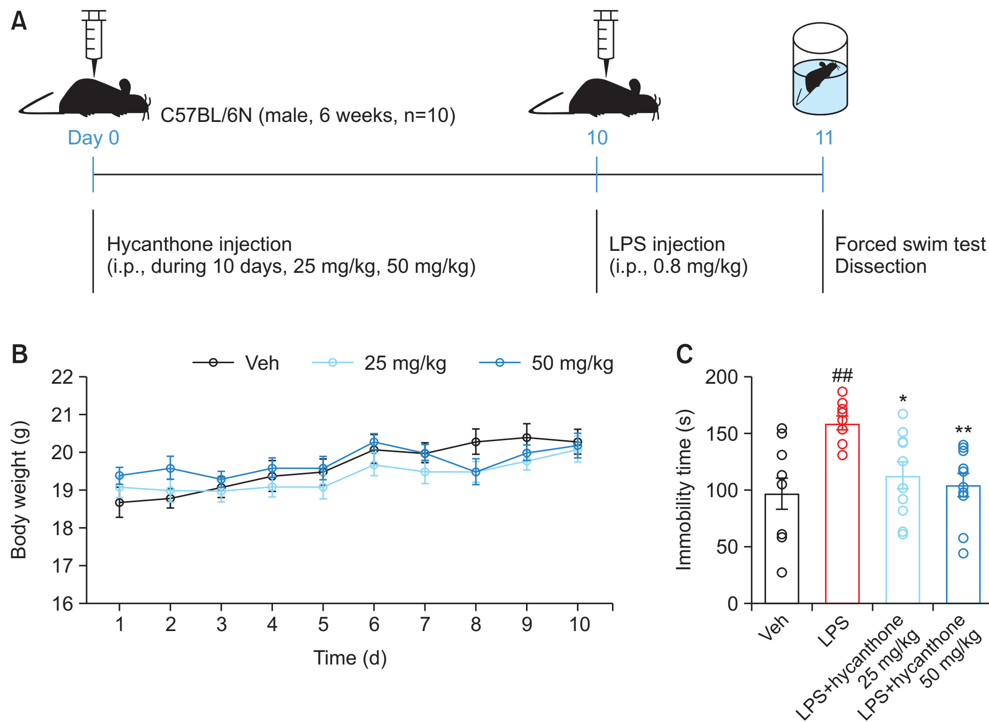

Fig. 3. The anti-depressant effect of hycanthone in an LPS-induced animal model of depression. (A) C57BL/6N mice were treated with hycanthone (25 and 50 mg/kg, i.p.) for 10 days and then LPS (0.8 mg/kg, i.p.) on the 10th day to induce depressive-like behaviors. (B) Body-weight profile during hycanthone treatment. (C) Forced swim test was done to check the antidepressant effect of hycanthone (N=10 per group). Data were analyzed by one-way ANOVA. $^{##}p$<0.01 vs. vehicle group, **p<0.01, *p<0.05 vs. LPS group.

Hycanthone inhibited gene expression of interferon-related factors in LPS-induced depression model

To investigate whether hycanthone affects the expression of interferon-regulated genes in the prefrontal cortex (PFC) from the mice of depression models, qRT-PCR was performed on interferon induced protein 44 (Ifi44) and guanylate binding protein 7 (Gbp7) genes. LPS increased the expression of Ifi44 (Fig. 4A) and Gbp7 (Fig. 4B), and both were normalized

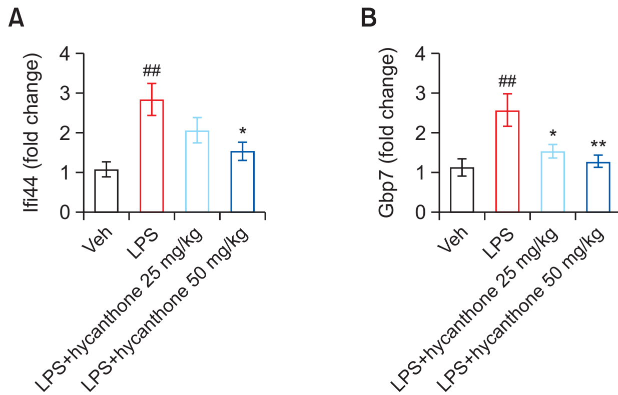

Fig. 4. The gene expression of IFN-related factors in the PFC by hycanthone treatment. The mRNA expression of (A) Ifi44 and (B) Gbp7 was measured by qRT-PCR (N=10). Data were analyzed by one-way ANOVA. ##*p*<0.01 vs. vehicle group, ***p*<0.01, **p*<0.05 vs. LPS group.

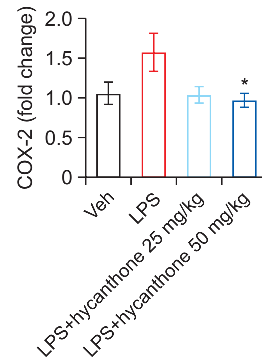

Fig. 5. The gene expression of neuroinflammatory factors in the PFC by hycanthone treatment. The mRNA expression of COX-2 was measured by qRT-PCR (N=10 per group). Data were analyzed by one-way ANOVA. **p*<0.05 vs. LPS group.

by hycanthone treatment, especially at 50 mg/kg. Both genes are classified into the interferon regulated genes, which are critical in controlling inflammation response. The expression of inflammatory factors and cytokines such as TNF-α and IL-1β was increased in LPS model and hycanthone reduced the expression of both cytokines, although statistical significance was absent (Supplementary Fig. 1). In this study, LPS-induced increased expression of COX-2 was significantly inhibited by hycanthone treatment (Fig. 5), reflecting that hycanthone would inhibit the interferon activity in depressive animal models.

Since indoleamine 2,3-dioxygenase 1 (IDO1) is one of the critical mediators in inflammation-mediated depressive-like behaviors (Jeon *et al*., 2017) that is activated by toll-like receptor 4 (TLR4)-related ligands and the IFN family (Hoyo-Becerra *et al*., 2014; Suento *et al*., 2021); we conducted the qRT-PCR of IDO1 as well, which showed similar inhibitory effects by hycanthone (Supplementary Fig. 1).

DISCUSSION

The etiology of depression has been focused mostly on the monoamine deficiency hypothesis (Delgado, 2000) which has progressed to include genetic factors (Shyn and Hamilton, 2010), neuroplasticity and neurogenesis abnormalities (Boku *et al*., 2018). A new insight into the connection between neuroinflammation and depression has been demonstrated in several studies (Jeon and Kim, 2018; Troubat *et al*., 2021). The major pro-inflammatory cytokine, interferon-α (IFN-α), is commonly used in the treatment of viral infection such as hepatitis C . However, IFN-α may induce psychiatric adverse effects such as depressive symptoms and cognitive dysfunctions (Wichers and Maes, 2004). In this study, we showed that hycanthone significantly inhibits the activity of IFN (Fig. 1). Furthermore, the production of pro-inflammatory mediators is also decreased by hycanthone treatment in the microglia cell line (Fig. 2). These results suggest the potential antidepressant activity of hycanthone against neuroinflammation-related depression.

Various approaches have been used in rodents such as restraint stress, unpredicted chronic mild stress, corticosterone injection, and LPS injection to mimic a human phenotype of depression (O'Connor *et al*., 2009; Frisbee *et al*., 2015; Yi *et al*., 2020; Kim *et al*., 2021). The LPS-induced depressive-like behavior in mice was used in this study to focus on neuroinflammation-mediated depression. Indeed, the immobility time of mice in the forced swim test was significantly increased by LPS injection (0.8 mg/kg). Remarkably, hycanthone treatment attenuated this depressive-like behavior in a dose-dependent manner, suggesting its potential to correct depressive symptoms. Interestingly, hycanthone can reduce interferon induction and synthesis (Hahon and Ong, 1980). These findings support that hycanthone would ameliorate depressive-like behaviors by modulating interferon and releated inflammatory cytokine actions. Several recent studies highlighted the interactions between interferon and inflammasome activation via TLR4 activation. LPS-mediated caspase activations were linked to nuclear factor kappa B (NF-κB) and JAK-STAT signaling, which were important for non-canonical NLRP3 inflammasome activation. NLRP3 inflammasome comprises NLRP3, ASC, and caspase-1. Its activation is classified into two stages: priming and activation stage. In the priming stage, the TLR4 signaling pathway is activated by LPS. In the activation stage, the NLRP3 inflammasome is activated by several external toxins and ATP, forming a single NLRP inflammasome complex (Swanson *et al*., 2019). TLR4 activation increases the levels of inflammatory cytokines, such as IL-1β and IL-18 as inactive and cytosolic pro-forms. Subsequently, caspase-1 in the NLRP3 inflammasome activates IL-1β and IL-18 into active forms and is released (Choi and Ryter, 2014). Therefore, IL-1β and IL-18 can be an indicator of NLRP3 inflammasome activation, and the release of IL-1β and IL-18 was measured in our study.

Among various gene family members involved in interferons, Ifi44 is over-expressed after stimulation of LPS (Ovstebø *et al*., 2008) and is involved in abnormal emotional behaviors (listed in IMPC gene data). Gbp7 is up-regulated by toll-like receptors and interferon signaling pathways (Kim *et al*., 2011; Pilla-Moffett *et al*., 2016). Taken together, the regulation of Ifi44 or Gbp7 may mark and modulate the inflammatory response and might contribute to regulating depressive behaviors. Our finding on the regulatory action of hycanthone on Ifi44 and Gbp7, suggests that its antidepressant effects could

be due to the inhibition of the interferon signaling pathway. Examination of the mechanism and relationship of IFNs and inflammasomes, especially Ifi44 and Gbp7, to depression will be required in future investigations.

The cytokine-activated JAK-STAT pathway plays a crucial role in the regulation of the immune system (Shuai and Liu, 2003) and is activated by inflammatory cytokines such as IFN-α, IFN-γ, and TNF-α (Schindler *et al*., 2007). The regulation of interferon activity to attenuate depressive symptoms could be via the inhibition of the JAK-STAT pathway. LPS stimulation-induced inflammatory reactions were down-regulated by the JNK-STAT1 pathway in microglial cells (Akaishi *et al*., 2022) and loss of stimulator of interferon genes protein (STING)-STAT1 activity reversed the IFN-stimulated genes and interferon induction in human intestinal epithelial cells (Karlowitz *et al*., 2022). Similarly, the type 1 interferon signaling pathway is induced by STAT1-dependent TRIM14 expression (Tan *et al*., 2018). Interferon also binds to interferon type 1 receptors, which activates STAT1 phosphorylation. This phosphorylated STAT1 subsequently activates the transcription of IFN-stimulated genes (Liu *et al*., 2020). We also indirectly showed that the type 1 interferon expression was significantly decreased by hycanthone treatment similar to ruxolitinib using reporter cells. Therefore, in this study, STAT1 activity might regulate interferon expression and response in which hycanthone could modulate this signaling in part. We demonstrated that hycanthone reduced the phosphorylation of STAT1, which could have driven the inhibition of interferon activity, suggesting this pathway could be important because of its antidepressant effect (Supplementary Fig. 2).

In conclusion, hycanthone may be a potential candidate drug for neuroinflammatory disorders, especially depression, potentially via modulating type-1 interferone pathways as well as cytokine production. Potential downstream targets could be Ifi44, Gbp7 and IDO-1, which deserve further attention. Understanding the mechanisms of hycanthone activity related to the interferon signaling pathway would provide new insights into targeting CNS diseases.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2016R1A5A2012284 and NRF-2020M3E5D9080165 to Dr. Shin) and by the Korea Drug Development Fund, funded by Ministry of Science and ICT, Ministry of Trade, Industry, and Energy, and Ministry of Health and Welfare (HN21C1076 to Dr. Jeon).

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