KMS99220 Exerts Anti-Inflammatory Effects, Activates the Nrf2 Signaling and Interferes with IKK, JNK and p38 MAPK via HO-1

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Neuroinflammation is an important contributor to the pathogenesis of neurodegenerative disorders including Parkinson's disease (PD). We previously reported that our novel synthetic compound KMS99220 has a good pharmacokinetic profile, enters the brain, exerts neuroprotective effect, and inhibits NFκB activation. To further assess the utility of KMS99220 as a potential therapeutic agent for PD, we tested whether KMS99220 exerts an anti-inflammatory effect in vivo and examined the molecular mechanism mediating this phenomenon. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, oral administration of KMS99220 attenuated microglial activation and decreased the levels of inducible nitric oxide synthase and interleukin 1 beta (IL-1β) in the nigrostriatal system. In lipopolysaccharide (LPS)-challenged BV-2 microglial cells, KMS99220 suppressed the production and expression of IL-1β. In the activated microglia, KMS99220 reduced the phosphorylation of IκB kinase, c-Jun N-terminal kinase, and p38 MAP kinase; this effect was mediated by heme oxygenase-1 (HO-1), as both gene silencing and pharmacological inhibition of HO-1 abolished the effect of KMS99220. KMS99220 induced nuclear translocation of the transcription factor Nrf2 and expression of the Nrf2 target genes including HO-1. Together with our earlier findings, our current results show that KMS99220 may be a potential therapeutic agent for neuroinflammation-related neurodegenerative diseases such as PD.

Keywords: heme oxygenase-1, IκB kinase, mitogen-activated protein kinases, neuroinflammation, Nrf2

INTRODUCTION

Accumulating evidence shows that neuroinflammation plays a vital role in the pathogenesis of neurodegenerative diseases including Parkinson's disease (PD) (Glass et al., 2010), with microglial activation, accumulation of proinflammatory cytokines, and activation of the proinflammatory transcription factor NFκB observed in the brains of post-mortem PD patients (Gerhard et al., 2006; Hunot et al., 1997) and experimental models of PD (Członkowska et al., 1996; Ghosh et al., 2007; Herrera et al., 2000; Liberatore et al., 1999). The principal cause of neuroinflammation is prolonged and sustained activation of microglia, the resident immune cells of the brain, which results in production of excessive amounts of the proinflammatory cytokines and nitric oxide (NO).

Production of inflammatory mediators are modulated by two different classes of kinases—the IκB kinase (IKK) (Gamble et al., 2012) and the mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular-signal-regulated kinase (ERK) (Caivano and...
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Cohen, 2000: Han and Ulevitch, 2005: Kaminska, 2005). Therefore, dual inhibition of both IKK and MAPK signaling pathways can be an effective approach for suppressing neuroinflammation.

We have reported the synthesis of a novel chalcone compound, KMS99220, and its pharmacokinetic properties (Lee et al., 2018b). KMS99220 has an excellent pharmacokinetic profile and can enter the brain after oral administration, which makes it a good candidate as a drug for the central nervous system. KMS99220 was found to protect the nigral dopaminergic neurons against the effects of dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lee et al., 2018b), and to downregulate the induction of inducible NO synthase (iNOS) and NO generation in activated BV-2 microglial cells (Lee et al., 2018a). However, whether KMS99220 can actually suppress neuroinflammation in the brain had not been tested. In addition, whether the kinases IKK and/or the MAPKs are involved in the action of KMS99220 was not known.

We previously observed that HO-1 mediates the anti-inflammatory action of KMS99220 and that HO-1 expression is induced by AMP-activated protein kinase (AMPK), whose activity is directly activated by KMS99220 (Lee et al., 2018a). On the other hand, by conducting a surface plasma resonance analysis, we found that KMS99220 can directly bind with high affinity to Keap1 (Lee et al., 2018b), the inhibitor protein of the transcription factor Nrf2 whose liberation from Keap1 is known to induce HO-1 gene expression (Kundu and Surh, 2010). Several reports have shown that chalcone compounds activate Nrf2 and thereby suppress inflammatory response (Bukhari et al., 2013; Foresti et al., 2013; Lee et al., 2015b). Therefore, it was possible that KMS99220 might additionally cause HO-1 induction via the Nrf2 signaling pathway, thereby further contributing to its anti-inflammatory activity.

As an extension of our previous studies to assess the utility of KMS99220 as a potential therapeutic agent for PD, we tested whether KMS99220 exerts an anti-inflammatory effect in vivo, and further examined the signaling pathways involved in this phenomenon. We show that oral administration of KMS99220 results in robust anti-inflammatory effect in the brain, and that KMS99220 suppresses the activities of JNK, p38 MAPK, and IKK in the inflammatory signaling via HO-1, and activates the Nrf2 pathway, which is accompanied by induction of HO-1 and other Nrf2 target genes.

MATERIALS AND METHODS

Materials
KMS99220 was synthesized according to the previously published method (Lee et al., 2018b). Fetal bovine serum, Dulbecco’s modified Eagle’s medium, trypsin/EDTA, penicillin-streptomycin, and TRIzol reagent were purchased from Thermo Fisher Scientific (USA). Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), and MPTP were purchased from Sigma-Aldrich (USA). Control small interfering RNA (siRNA), HO-1 siRNA, and Lipofectamine RNAiMax reagent were purchased from Invitrogen (USA). Tin protoporphyrin-IX (SnPP) was purchased from Santa Cruz Biotechnology (USA).

Primary antibodies used are as follows: anti-iNOS, anti-HO-1, anti-NRf2, anti-glutamate-cysteine ligase modifier subunit (GCLM), anti-lamin B, and anti-IKK from Santa Cruz Biotechnology; anti-glutamate-cysteine ligase catalytic subunit (GCLC) from Novus Biologicals (USA); anti-ib-1 from Wako Chemicals (Japan); anti-p-IKK, anti-ERK, anti-p-ERK, anti-p38, anti-p-p38, anti-JNK, and anti-p-JNK from Cell Signaling Technology (USA); anti-NAD(P)H: quinone oxidoreductase 1 (NQO1) from Ab Frontier (Korea); and anti-p-actin from Sigma-Aldrich. Anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG were purchased from Sigma-Aldrich. First strand cDNA synthesis kit for reverse transcription polymerase chain reaction (RT-PCR) was purchased from MBI Fermentas (Canada). Bradford protein assay kit was purchased from Bio-Rad (UK). Interleukin 1 beta (IL-1β) enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (USA), and Vectastain ABC kit and biotinylated secondary antibodies were from Vector Laboratories (USA). Enhanced luminal-based chemiluminescence Western blotting detection system was purchased from Pierce Chemical (USA).

Animals
All procedures were pre-approved by the Institutional Animal Care and Use Committee at Asan Medical Center (IACUC No. 2015-13-088). As previously detailed (Lee et al., 2018b), male C57BL/6 mice weighing 23 to 25 g received 10 or 30 mg/kg KMS99220 suspended in the vehicle solution (10% N-methyl-2-pyrrolidone and 90% dimethyl sulfoxide (DMSO), air-diluted with the culture media. The final concentration of DMSO in the cell culture was 0.01% for both KMS99220-treated and control cells. The animals in the control group was similarly injected with saline. The first MPTP injection was made 1 h after the second KMS99220 administration.

Immunohistochemistry
At 7 days after the first MPTP injection, the animals were euthanized and transcardially perfused with paraformaldehyde. The brain tissues were cut into 20 μm sections and the Mouse Brain Atlas (Franklin and Paxinos, 1997) was used to delineate the substantia nigral region. As previously detailed (Lee et al., 2016), a total of 5 sections, each 80 μm apart, were obtained from each animal and subjected to Iba-1 immunostaining followed by quantitation of immunodensity.

Cell cultures
BV-2 mouse microglial cells (Blasi et al., 1990) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in the presence of 100 IU/L penicillin and 10 μg/ml streptomycin. The cells were maintained at 37°C in 95% air and 5% CO₂ in a humidified atmosphere. For treatment, KMS99220 was prepared as a 100 mM stock solution dissolved in DMSO and diluted with the culture media. The final concentration of DMSO in the cell culture was 0.01% for both KMS99220-treated and control cells.

RT-PCR
RT reactions were performed using 5 μg of total RNA and the
First Strand cDNA Synthesis kit following the manufacturer's directions. The following primers were used for PCR: HO-1 (forward, 5'-AGCAGGAACATGCCCTCT-3'; reverse, 5'-TCTGT-CAGCATCACCTGCCAG-3'), NQO1 (forward, 5'-CCATCTA-AACAGCGATCA-3'; reverse, 5'-TAGCTTTGAATGCTGCT-3'), GCLC (forward, 5'-ATGACCTGTCCACTTGATGCA-3'; reverse, 5'-ACACGCCATCCTAAACACCGATCA-3'), GCLM (forward, 5'-AGCTGGACTCTGTGATCATGGCTT-3'; reverse, 5'-CAAAGCCAGTCAAATCTGGTGGCA-3'), IL-1β (forward, 5'-ATGGCAACTGTTCCTGAACTCACCT-3'; reverse, 5'-CAGGACAGGTATAGATTCTTTCCTTT-3'), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (forward, 5'-CACCACCATGGAGAAGGCTGG-3'; reverse, 5'-TTGTGATGAGTCCCACTTGGCCAGG-3').

**IL-1β measurement**

Lysates of BV-2 cells treated with KMS99220 and 0.2 μg/ml LPS were obtained. The substantia nigra tissue was dissected on ice immediately after sacrifice, homogenized in 10 mM phosphate buffer (pH 7.0) containing 0.1% NP-40, and centrifuged to obtain the resulting supernatant. The level of IL-1β in the cell lysates and tissue homogenates was measured using an IL-1β ELISA kit according to the protocol provided by the manufacturer.

**Western blot analysis**

Total cell lysate and nuclear fraction of BV-2 cells and striatal tissue extracts were obtained according to the previously described method (Lee et al., 2015b; Woo et al., 2014). The blots were incubated with primary antibody against iNOS (1:200), p-IKK (1:1,000), IKK (1:200), p-JNK (1:1,000), JNK (1:1,000), p-p38 (1:1,000), p38 (1:1,000), p-ERK (1:1,000), ERK (1:1,000), HO-1 (1:200), Nrf2 (1:2,000), GCLC (1:3,000), GCLM (1:200), NQO1 (1:1,000), lamin B (1:200),...
or β-actin (1:60,000) at 4°C followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using chemiluminescence substrate and quantitatively analyzed by densitometry.

**siRNA transfection**
Cells were transiently transfected with siRNA (final concentration: 40 nM) for HO-1 or control using Lipofectamine RNAiMax reagent according to the manufacturer’s instructions. After 24 h, the cells were treated with KMS99220 and/or LPS, and Western blot analysis was performed.

**Data analyses**
Statistical tests were carried out using PRISM (GraphPad Software, USA). P values < 0.05 were considered statistically significant. Comparisons of three or more groups were analyzed by one-way ANOVA and post Dunnett’s multiple comparison tests.

**RESULTS**

**KMS99220 has anti-inflammatory effects both in vivo and in vitro**
We first tested whether KMS99220 reduces microglial activation in mice exposed to the dopaminergic neurotoxin MPTP (Kurkowski-Jastrzebska et al., 1999). Immunostaining for the microglial marker Iba-1 in the nigral sections revealed that MPTP administration increased the Iba-1 immunoreactivity, which was effectively nullified by co-treatment with KMS99220 (Fig. 1A). Quantitative densitometric analysis showed that MPTP administration increased Iba-1 immunoreactivity to 155% of vehicle-treated animals, and that KMS99220 cotreatment completely abrogated it (P > 0.05 vs vehicle-treated control; P < 0.01 vs MPTP treated for both 10 and 30 mg/kg KMS99220) (Fig. 1B). The 10 mg/kg regimen of KMS99220 seemed sufficiently effective, as 30 mg/kg was not significantly better in terms of downregulation of Iba-1 immunoreactivity (P > 0.05). The increase in the protein level of iNOS in the MPTP animals was also suppressed by 70% upon KMS99220 cotreatment (Fig. 1C). In the same animals, KMS99220 completely nullified the increase of the proinflammatory cytokine IL-1β (Fig. 1D; P > 0.05 vs vehicle-treated control). We further tested the anti-inflammatory effects of KMS99220 in BV-2 microglial cells and found that the LPS-induced increases in the levels of mRNA (Fig. 1E) and protein (Fig. 1F) of IL-1β were suppressed in a dose-dependent manner by KMS99220 and 10 μM was able to provide complete inhibition (P > 0.05 vs vehicle-treated control for both mRNA and protein). KMS99220 was previously shown to have no cytotoxicity in this concentration range (Lee et al., 2018a).

**KMS99220 inhibits activation of IKK and MAPKs in activated microglia**
We then tested whether KMS99220 affects the LPS-induced phosphorylation of IKK. As shown in Figure 2, LPS increased the phosphorylation of IKK, which was dose-dependently and significantly suppressed by KMS99220. At 10 μM, the phosphorylation was inhibited by 94%. We also examined the effect of KMS99220 on the MAPKs: phosphorylation of JNK, p38 MAPK, and ERK was observed after LPS exposure, and this was effectively suppressed by KMS99220 in a dose-dependent manner. At 10 μM, KMS99220 reduced the phosphorylation levels of JNK and p38 MAPK by 87% and 95%, respectively. The effect of KMS99220 on ERK was smaller, with 10 μM KMS99220 suppressing its phosphorylation level by 40%.

**HO-1 mediates the inhibitory effects of KMS99220 on IKK, JNK, and p38 MAPK**
We tested if the inhibitory effects of KMS99220 on the phosphorylation of IKK and the MAPKs are mediated by HO-1. We first silenced HO-1 expression in BV-2 cells by siRNA transfection (Fig. 3A); in these HO-1 knockdown cells, KMS99220 could no longer inhibit the LPS-induced phosphorylation of IKK (Fig. 3B). We further tested whether pharmacological inhibition of HO-1 enzyme activity has the same effect. Indeed, co-treatment with the HO-1 inhibitor SnPP abolished the inhibitory effect of KMS99220 on the phosphorylation of IKK (Fig. 3C).

Similar phenomena were observed when the same analyses were performed for JNK and p38 MAPK. In the HO-1 knockdown cells, KMS99220 could no longer inhibit the effects of LPS on the phosphorylation levels of JNK and p38 MAPK, as LPS-treated cells and LPS + KMS99220-treated cells did not show significant differences (Fig. 3B). The HO-1 inhibitor SnPP was also able to dose-dependently reverse the effect of KMS99220 on JNK and p38 MAPK. Therefore, the enzymatic activity of HO-1 appeared to mediate the inhibitory action of KMS99220 on JNK and p38 MAPK as well. On the other hand, inhibition or silencing of HO-1 did not
KMS99220 is an anti-neuroinflammatory chalcone compound that has been shown to activate the Nrf2 pathway and induce expression of microglia in vitro.

**KMS99220 activates the Nrf2 pathway and induces expression of HO-1 in microglia**

HO-1 gene expression is controlled by the transcription factor Nrf2 (Alam et al., 1999), and we have previously shown that KMS99220 binds with high affinity to the Nrf2 inhibitor protein, Keap1 (Lee et al., 2018b). Therefore, we tested whether KMS99220 might cause Nrf2 activation and consequently HO-1 induction in microglia. As shown in Figure 4A, within 3 h of KMS99220 exposure, the level of nuclear Nrf2 was increased in a dose-dependent manner and reached $2.1 \pm 0.1$ fold compared with control at $10 \mu M$; this suggests that upon binding of KMS99220 to Keap1, Nrf2 is released and moves into the nucleus. We studied whether this change is accompanied by subsequent induction of the Nrf2 target gene HO-1, and observed that KMS99220 elevated the mRNA level of HO-1 in a dose-dependent manner, reaching $2.8 \pm 0.1$ fold at $10 \mu M$ (Fig. 4F). This was accompanied by an increase in HO-1 protein level to $1.7 \pm 0.1$ fold at 24 h (Fig. 4B).

In order to further confirm that KMS99220 causes Nrf2 activation, we tested whether the well-known Nrf2 target genes such as NQO1, GCLC, and GCLM (Itoh et al., 1997; Jaiswal, 2004) are induced during the same time frame. We observed that all three genes were indeed induced by KMS99220 in a dose-dependent manner. Upon treatment with KMS99220 ($10 \mu M$), NQO1 mRNA and protein levels were increased by $6.5 \pm 0.3$ and $2.5 \pm 0.1$ folds, respectively (Figs. 4C and 4G); moreover, GCLC and GCLM were increased at the mRNA level by $2.3 \pm 0.1$ and $2.6 \pm 0.1$ folds, respectively (Figs. 4H and 4I), and at the protein level by $2.2 \pm 0.1$ and $3.1 \pm 0.1$ folds, respectively (Figs. 4D and 4E). Therefore, KMS99220 was able to concurrently induce the expression of Nrf2 target genes in microglial cells.

Finally, to confirm that the same phenomenon occurs in microglia in their activated state, we carried out the same experiment in LPS-challenged BV-2 cells. As shown in Figure 4I, LPS alone slightly elevated the mRNA level of HO-1, in agreement with previously reported results (Lee et al., 2015a), and co-treatment with KMS99220 caused further increases in a dose-dependent manner. Under the same condition, the mRNA levels of NQO1, GCLC, and GCLM were also similarly elevated.

**DISCUSSION**

Prolonged activation of microglia causes an exaggerated inflammatory response that leads to neuronal cell death through excessive release of proinflammatory cytokines: the cell death signals from neurons in turn aggravate reactive microgliosis, thereby leading to neurodegeneration (Yang and Zhou, 2019). Therefore, the attenuation of microglia activation is a viable therapeutic strategy for neuroinflammation-mediated neurodegenerative diseases (Hurley and Tizabi, 2013). To this end, we have previously developed several novel synthetic compounds that suppress microglia activation and provide neuroprotection (Lee et al., 2015b; 2016; Son et al., 2012; 2016). In this current study, we demonstrate that the chalcone compound KMS99220, which has a good pharmacokinetic profile as a central nervous system drug as well as neuroprotective effects in an animal model of PD (Lee et al., 2018b), also exerts anti-inflammatory effects both in vitro and in vivo. We report that KMS99220 activates the Nrf2 signaling and induces its target gene expression including HO-1, and inhibits the inflammatory signaling by interfering with both IKK and MAPKs activation via HO-1.

We show that KMS99220 suppresses the LPS-induced
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The phosphorylation of both IKK and the MAPKs, the kinases involved in the inflammatory signaling pathway, IKK phosphorylates IκB and causes the release and nuclear translocation of NFκB, the key transcription factor in the development of inflammation (Scheidereit, 2006). The MAPKs phosphorylate the transcription factors AP1 and CREB, which in turn increases the mRNA levels of genes of inflammatory mediators (Caivano and Cohen, 2000; Kang et al., 2008; Kristof et al., 2001). As such, the deficiency of microglial p38α MAPK prevents neuronal damage by suppressing the production of proinflammatory cytokines (Xing et al., 2011). In an MPTP model of PD, inhibition of JNK decreased the expression of inflammatory mediators and protected the dopaminergic neurons (Wang et al., 2009). Many genes of inflammatory mediators are under the control of both the MAPKs and NFκB. For example, the iNOS gene contains NFκB- and AP1-binding sites, and its expression is elevated by both NFκB and phosphorylated Jun (Kleinitert et al., 2004). The IL-1β gene contains NFκB-, CREB-, and AP1-binding sites as well (Gray et al., 1993). Therefore, the concurrent interruption of both MAPK signaling and IKK signaling by KMS99220 should be useful in effectively suppressing inflammation.

We also show that the inhibitory effects of KMS99220 on JNK, p38 MAPK, and IKK are mediated by HO-1, as both the knockdown and pharmacological inhibition of HO-1 abolished the effect of KMS99220. We have previously demonstrated that KMS99220 reduces the phosphorylation of IκB, activation of NFκB, expression of iNOS and generation of NO in activated microglia; these effects were also deemed to be mediated by HO-1, as KMS99220 was not effective in cells in which HO-1 was knocked down or pharmacologically inhibited (Lee et al., 2018a). Taken together, HO-1 appears to mediate the anti-inflammatory effect of KMS99220 by inhibiting the MAPK pathways and the IKK/IκB/NFκB pathway, ultimately reducing the levels of iNOS and IL-1β.

HO-1 gene is under the control of the transcription factor Nrf2, and the existing evidence delineates a prominent role of Nrf2/HO-1 in the regulation of neuroinflammation (Ahmed et al., 2017; Innamorato et al., 2008): in Nrf2-deficient animals, HO-1 expression was reduced, the levels of inflammatory molecules were greatly increased, and microglial activation was exacerbated in response to LPS and MPTP (Innamorato et al., 2008; Rojo et al., 2010). Nrf2 is known to be activated when it is released from its cytosolic inhibitor protein Keap1 (Kundu and Surh, 2010), and development of molecules that interfere with the protein-protein interaction between Keap1 and Nrf2 is an emerging strategy to selectively and effectively activate Nrf2 (Jiang et al., 2016). KMS99220 directly binds...
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with high affinity to Keap1 (Lee et al., 2018b), and induces microglial nuclear translocation of Nrf2 and expression of HO-1 as well as other Nrf2 target genes NQO1, GCLC, and GCLM. Therefore, it is probable that KMS99220 activates the Nrf2/HO-1 system by interfering with the protein-protein interaction between Keap1 and Nrf2.

We have previously demonstrated that KMS99220 directly activates the enzyme AMPK (Lee et al., 2018a). It has been previously reported that AMPK causes accumulation of Nrf2 by attenuating its export from the nucleus (Joo et al., 2016) and induces the Nrf2-dependent HO-1 expression (Liu et al., 2011; Zimmermann et al., 2015) in other cell types, suggesting that such a crosstalk may additionally contribute to the anti-inflammatory effect of KMS99220 in microglia. On the other hand, we found that the AMPK-dependent HO-1 induction can occur in the absence of Nrf2 and that inhibition of AMPK activity does not completely block the suppressive effect of KMS99220 on NO production in BV-2 microglia (Lee et al., 2018a). Therefore, it is most likely that an AMPK-independent mechanism initiated by the release of Keap1 from Nrf2 also plays an important role in the anti-inflammatory effect of KMS99220.

KMS99220 has an excellent neuroprotective property against MPTP and was shown to alleviate PD-like motor deficits in vivo (Lee et al., 2018b). In the brain parenchyma where the dopaminergic neurons and microglia are in close contact and form a vicious cycle in neurodegeneration, KMS99220 may utilize multiple mechanisms to provide neuroprotection (Fig. 5). First, by inducing the antioxidant enzymes HO-1, NQO1, and GCL via Nrf2 in neurons, KMS99220 can reduce oxidative stress, to which the dopaminergic neurons are particularly vulnerable (Hwang, 2013). Accordingly, NQO1, HO-1, and GCL activities have been shown to provide neuroprotection in dopaminergic neurons (Loboda et al., 2016). Second, in microglia, KMS99220 may suppress the inflammatory signaling pathways triggered by IKK, JNK, and p38 MAPK, and this is mediated by HO-1. Because the IKK signaling pathway and the MAPK signaling pathway both lead to the production of proinflammatory mediators, this dual inhibition of the two pathways may provide robust protection against neuroinflammation. Third, KMS99220 activates the Nrf2 pathway leading to HO-1 induction, in addition to the AMPK pathway, and this can further contribute to the efficient anti-inflammatory action of KMS99220. Fourth, KMS99220 induces the production of enzymes NQO1 and GCL in microglial cells, which can also add to the anti-inflammatory activity as previously suggested (Kimura et al., 2018; Rahman and MacNee, 2000).

In conclusion, our novel compound KMS99220 effectively suppresses LPS-induced microglial activation and the production of proinflammatory mediators in MPTP-treated mice. KMS99220 also suppresses the activation of IKK, p38 MAPK, and JNK in the inflammatory signaling pathway, which is mediated by the induction of HO-1. KMS99220 induces nuclear translocalization of Nrf2 and expression of HO-1, NQO1, and GCL in microglial cells. Taken together with our previous findings, KMS99220 may be a valuable compound toward the development of therapy for neuroinflammation-mediated diseases including PD.

Disclosure
The authors have no potential conflicts of interest to disclose.

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REFERENCES
Ahmed, S.M., Luo, L., Namani, A., Wang, X.J., and Tang, X. (2017). Nrf2 signaling pathway: pivotal roles in inflammation. Biochim. Biophys. Acta Mol. Basis Dis. 1863, 585-597.
Alam, J., Stewart, D., Touchard, C., Boipapally, S., Choi, A.M., and Cook, J.L. (1999). Nrf2, a Cap’n’Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J. Biol. Chem. 274, 26071-26078.
Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., and Bistoni, F. (1990). Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J. Neuroimmunol. 27, 229-237.
Bukhari, S.N., Jantan, I., and Jasamai, M. (2013). Anti-inflammatory trends of 1, 3-diphenyl-2-propen-1-one derivatives. Mini Rev. Med. Chem. 13, 87-94.
Caivano, M. and Cohen, P. (2000). Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. J. Immunol. 164, 3018-3025.
Członkowska, A., Koubutnic, M., Kurkowska-Jastrzębska, I., and Członkowski, A. (1996). Microglial reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson’s disease mice model. Neurodegeneration 5, 137-143.
Foresti, R., Bains, S.K., Pitchumony, T.S., de Castro Brás, L.E., Drago, F., Dubois-Randé, J.L., Bucolo, C., and Motterlini, R. (2013). Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells. Pharmacol. Res. 76, 132-148.

Franklin, K.B.J. and Paxinos, G. (1997). The Mouse Brain in Stereotaxic Coordinates (San Diego: Academic Press).

Gamble, C., Mcintosh, K., Scott, R., Ho, K.H., Plevin, R., and Paul, A. (2012). Inhibitory kappa B kinases as targets for pharmacological regulation. Br. J. Pharmacol. 165, 802-819.

Gerhard, A., Pavese, N., Hotton, G., Turkheimer, F., Es, M., Hammers, A., Eggert, K., Oertel, W., Banati, R.B., and Brooks, D.J. (2006). In vivo imaging of microglial activation with [11C(R)-PK11195 PET in idiopathic Parkinson's disease. Neurobiol. Dis. 21, 404-412.

Ghosh, A., Roy, A., Liu, X., Kordower, J.H., Mufson, E.J., Hartley, D.M., Ghosh, S., Mosley, R.L., Gendelman, H.E., and Pahan, K. (2007). Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A. 104, 18754-18759.

Glass, C.K., Sajio, K., Winner, B., Marchetto, M.C., and Gage, F.H. (2010). Mechanisms underlying inflammation in neurodegeneration. Cell 140, 918-934.

Gray, JG, Chandra, G., Clay, WC, Stinnett, SW, Haneline, SA, Lorenz, J.J., Patel, IR, Wisely, G.B., Furdon, PJ, Taylor, JD, et al. (1993). A CRE/ATF-like site in the upstream regulatory sequence of the human interleukin 1 beta promoter mediates the induction of phase II detoxifying enzyme genes and gene transcription in lung inflammation: therapeutic approaches. Proc. Natl. Acad. Sci. U. S. A. 90, 7531-7536.

Hurley, L.L. and Tizabi, Y. (2013). Neuroinflammation, neurodegeneration, and inflammation in BV2 microglia cells. Pharmacol. Res. 68, 678-689.

Han, J. and Ulevitch, R.J. (2005). Limiting inflammatory responses during activation of innate immunity. Nat. Immunol. 6, 1198-1205.

Herrera, AJ, Castaño, A., Venero, JL, Cano, J., and Machado, A. (2000). The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. Neurobiol. Dis. 7, 429-447.

Hunot, S., Brugg, B., Richaud, D., Michel, PP, Muriel, MP, Ruberg, M., Faucheux, BA, Agid, Y., and Hirsch, EC. (1997). Nuclear translocation of NF-kB is increased in dopaminergic neurons of patients with Parkinson disease. Proc. Natl. Acad. Sci. U. S. A. 94, 7531-7536.

Hurley, L.L. and Tizabi, Y. (2013). Neuroinflammation, neurodegeneration, and depression. Neurotox. Res. 23, 131-144.

Hwang, O. (2013). Role of oxidative stress in Parkinson's disease. Exp. Neurol. 22, 11-17.

Innamorato, N.G., Rojo, A.I., García-Yague, A.J., Yamamoto, M., de Ceballos, M.L., and Cuadrado, A. (2008). The transcription factor Nrf2 is a therapeutic target against brain inflammation. J. Immunol. 181, 680-689.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., et al. (1997). An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzymes through antioxidant response elements. Biochem. Biophys. Res. Comm. 236, 313-322.

Jaiswal, A.K. (2004). Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic. Biol. Med. 36, 1199-1207.

Jiang, Z.Y., Lu, M.C., and You, Q.D. (2016). Discovery and development of kelch-like ECH-associated protein 1. Nuclear factor erythroid 2-related factor 2 (Kedd1/Nrfr2) protein-protein interaction inhibitors: achievements, challenges, and future directions. J. Med. Chem. 59, 10833-10858.

Joo, M.S., Kim, WD, Lee, KY, Kim, JH, Koo, JH, and Kim, SG. (2016). AMPK facilitates nuclear accumulation of Nrf2 by phosphorylating at serine 550. Mol. Cell. Biol. 36, 1931-1942.

Kaminska, B. (2005). MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. Biochim. Biophys. Acta 1754, 253-262.

Kang, YJ, Chen, J, Otsuka, M, Mols, J, Ren, S, Wang, Y, and Han, J. (2008). Macrophage deletion of p38alpha partially impairs lipopolysaccharide-induced cellular activation. J. Immunol. 180, 5075-5082.

Kimura, A., Kitajima, M., Nishida, K., Serada, S., Fujimoto, M., Naka, T., Fujii-Kuriyama, Y, Sakamoto, S, Ito, T, Handa, H, et al. (2018). NQO1 inhibits the TRD-1-dependent production of selective cytokines by promoting IkB-ζ degradation. J. Exp. Med. 215, 2197-2209.

Kleinit, H., Pautz, A., Linker, K. and Schwarz, PM. (2004). Regulation of the expression of inducible nitric oxide synthase. Eur. J. Pharmacol. 500, 255-266.

Kristof, A.S., Marks-Konczalik, J., and Moss, J. (2001). Mitogen-activated protein kinases mediate activator protein-1-dependent human inducible nitric-oxide synthase promoter activation. J. Biol. Chem. 276, 8445-8452.

Kundu, J.K. and Surh, Y.J. (2010). Nrf2-Keap1 signaling as a potential target for chemoprevention of inflammation-associated carcinogenesis. Pharm. Res. 27, 999-1013.

Kurkowska-Jastrzebska, I., Wrońska, A., Kothutnicka, M., Czlonkowski, A., and Czlonkowski, A. (1999). The inflammatory reaction following 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine intoxication in mouse. Exp. Neurol. 156, 50-61.

Lee, EJ, Ko, HM, Jeong, YH, Park, EM, and Kim, HS. (2015a). β-Lapachone suppresses neuroinflammation by modulating the expression of cytokines and matrix metalloproteinases in activated microglia. J. Neuroinflammation 12, 133.

Lee, JA, Kim, HR, Kim, J, Park, KD, Kim, DJ, and Hwang, O. (2018a). The novel neuroprotective compound KMS99220 has an early anti-neuroinflammatory effect via AMPK and HO-1, independent of Nrf2. Exp. Neurobiol. 27, 408-418.

Lee, JA, Kim, JH, Woo, SY, Son, HJ, Han, SH, Jang, BK, Choi, JW, Kim, DJ, Park, KD, and Hwang, O. (2015b). A novel compound VSC2 has anti-inflammatory and antioxidant properties in microglia and in Parkinson's disease animal model. Br. J. Pharmacol. 172, 1087-1110.

Lee, JA, Son, HJ, Choi, JW, Kim, J, Han, SH, Shin, N, Kim, JH, Kim, SJ, Heo, JY, Kim, DJ, et al. (2018b). Activation of the Nrf2 signaling pathway and neuroprotection of nigral dopaminergic neurons by a novel synthetic compound KMS99220. Neurochem. Int. 112, 96-107.

Lee, JA, Son, HJ, Kim, JH, Park, KD, Shin, N, Kim, HR, Kim, EM, Kim, DJ, and Hwang, O. (2016). A novel synthetic isothiocyanate ITC-57 displays antioxidant, anti-inflammatory, and neuroprotective properties in a mouse Parkinson's disease model. Free Radic. Res. 50, 1188-1199.

Liberatore, GT, Jackson-Lewis, V, Vukosavic, S, Mandir, AS, Vila, M, McAuliffe, W.G., Dawson, VL, Dawson, TM, and Przedborski, S. (1999). Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat. Med. 5, 1403-1409.

Liu, XM, Peyton, KJ, Shebib, AR, Wang, H, Korthuis, RJ, and Durante, W. (2011). Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. Am. J. Physiol. Heart Circ. Physiol. 300, H84-H93.

Loboda, A, Damulewicz, M, Pyza, E, Jozkowicz, A, and Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell. Mol. Life Sci. 73, 3221-3247.

Rahman, I. and MacNee, W. (2000). Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. Free Radic. Biol. Med. 28, 1405-1420.

Rojo, A.I., Innamorato, N.G., Martín-Moreno, A.M., De Ceballos, M.L., Yamamoto, M., and Cuadrado, A. (2010). Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. Glia 58, 588-598.

Scheiderit, C. (2006). IκB kinase complexes: gateways to NF-κB activation and transcription. Oncogene 25, 6685-6705.
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Son, H.J., Han, S.H., Lee, J.A., Lee, C.S., Seo, J.W., Chi, D.Y., and Hwang, O. (2016). 2-Acetyl-7-hydroxy-6-methoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline exhibits anti-inflammatory properties and protects the nigral dopaminergic neurons. Eur. J. Pharmacol. 771, 152-161.

Son, H.J., Lee, J.A., Shin, N., Choi, J.H., Seo, J.W., Chi, D.Y., Lee, C.S., Kim, E.M., Choe, H., and Hwang, O. (2012). A novel compound PTIQ protects the nigral dopaminergic neurones in an animal model of Parkinson’s disease induced by MPTP. Br. J. Pharmacol. 165, 2213-2227.

Wang, Y., Zhang, Y., Wei, Z., Li, H., Zhou, H., Zhang, Z., and Zhang, Z. (2009). JNK inhibitor protects dopaminergic neurons by reducing COX-2 expression in the MPTP mouse model of subacute Parkinson’s disease. J. Neurol. Sci. 285, 172-177.

Woo, S.Y., Kim, J.H., Moon, M.K., Han, S.H., Yeon, S.K., Choi, J.W., Jang, B.K., Song, H.J., Kang, Y.G., Kim, J.W., et al. (2014). Discovery of vinyl sulfones as a novel class of neuroprotective agents toward Parkinson’s disease therapy. J. Med. Chem. 57, 1473-1487.

Xing, B., Bachstetter, A.D., and Van Eldik, L.J. (2011). Microglial p38α MAPK is critical for LPS-induced neuron degeneration, through a mechanism involving TNFa. Mol. Neurodegener. 6, 84.

Yang, Q.Q. and Zhou, J.W. (2019). Neuroinflammation in the central nervous system: symphony of glial cells. Glia 67, 1017-1035.

Zimmermann, K., Baldinger, J., Mayerhofer, B., Atanasov, A.G., Dirsch, V.M., and Heiss, E.H. (2015). Activated AMPK boosts the Nrf2/HO-1 signaling axis—a role for the unfolded protein response. Free Rad. Biol. Med. 88, 417-426.