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Short Communication

Anti-inflammatory effects of synthetic compound KT-14480 in lipopolysaccharide-stimulated microglia cells

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Abstract

Objectives Neurodegenerative diseases have a prominent inflammatory component. Several synthetic fluorovinyloxyacetamide derivatives were screened by microglia cell-based assay in order to identify novel compounds that inhibit the inflammatory activation of microglia.

Methods Microglia cell-based nitric oxide assay was employed to screen the compounds. RT-PCR and ELISA were conducted to evaluate the expression of inflammatory gene expression. Molecular mechanisms were determined by western blot analysis, immunocytochemistry, EMSA, and microglia/neuroblastoma cocultures.

Key findings A fluorovinyloxyacetamide compound KT-14480 significantly suppressed nitric oxide production in lipopolysaccharide-stimulated microglia cells. KT-14480 also suppressed the secretion and expression of several inflammatory mediators such as tumour necrosis factor-α, interleukin-1β and inducible nitric oxide synthase. Additional studies showed that these inhibitory effects were accompanied by the suppression of nuclear factor-κB and neuroprotection in the microglia/neuroblastoma coculture.

Conclusions Our results indicate that the anti-inflammatory compound KT-14480 may be a novel therapeutic drug candidate against neuroinflammatory diseases.

Keywords fluorovinyloxyacetamides; microglia; neurodegenerative diseases; neuroinflammation; neuroprotection; nitric oxide

Introduction

Microglia are the primary immune cells of the brain, and are believed to play a major role in host defence and tissue repair in the central nervous system (CNS). Microglia are activated in response to brain injury as in the inflammatory, ischaemic and neurodegenerative pathologies. Activated microglia undergo dramatic morphological change, from their ramified resting state into an amoeboidal morphology. Activated microglia release both neurotrophic factors that enhance neuronal survival and neurotoxic factors.[1] Until now, the conditions defining whether microglia activation is beneficial or detrimental to neuron survival have been poorly understood. It is becoming more widely accepted that inflammation is capable of actively causing neuronal death/damage and microglial activation is a critical component of the inflammatory and immunological insult to neurons.

Activated microglia produce various inflammatory mediators, which include superoxide, nitric oxide (NO) and proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β). These proinflammatory mediators have emerged as important determinants of cytotoxicity-associated neuroinflammation, which has been implicated in multiple neurodegenerative diseases.[1,2] Thus, inhibition of these proinflammatory mediators could be an effective therapeutic approach to preventing the progression of neurodegenerative diseases.

In this study, we evaluated the anti-inflammatory activity of synthetic compounds that have a fluorovinyloxyacetamide skeletal structure. Using microglia cell-based screening, a novel compound, KT-14480, was identified as inhibiting the production of proinflammatory mediators and as activating nuclear factor-kappaB (NF-κB) in lipopolysaccharide (LPS)-stimulated microglial cells. KT-14480 also showed a neuroprotective effect in the microglia–neuroblastoma coculture. These results suggest that KT-14480 may inhibit neuroinflammation and protect the neurons against activated microglia-mediated neurotoxicity.

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Materials and Methods

Reagents and cell culture

Bacterial LPS from *Escherichia coli* 0111:B4 was obtained from Sigma-Aldrich (St Louis, MO, USA). Synthetic chemical compounds were obtained from the Korea Chemical Bank in the Korea Research Institute of Chemical Technology (Daejeon, Korea). These compounds were solubilized in dimethyl sulfoxide (DMSO) and added to the cell culture at the desired concentrations. The immortalized BV-2 murine microglial cell line,[3] which exhibits phenotypic and functional properties comparable to those of primary microglial cells,[4] was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamicin at 37°C. The highly aggressively proliferating immortalized (HAPI) rat microglial cell line[5] and B35 rat neuroblastoma cell line,[6] stably expressing enhanced green fluorescent proteins (EGFP), were maintained in DMEM containing 10% heat-inactivated FBS, 2 mM glutamine, 10 U/ml penicillin, and 10 μg/ml streptomycin (Gibco, Gaithersburg, MD, USA) at 37°C under a humidified atmosphere with 5% CO_2.

Nitrite quantification

The production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, using Griess reagents as previously described.[7]

Cell viability

Cell viability was assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[7]

TNF-α ELISA

BV-2 cells were treated with KT-14480 in the absence or presence of LPS. After 24 h of incubation, the levels of TNF-α in culture media were measured by specific ELISA as previously described.[8]

Reverse transcription–polymerase chain reaction (RT-PCR)

BV-2 cells were treated with KT-14480 in the absence or presence of LPS (100 ng/ml) for 6 h. Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA). Reverse transcription and PCR amplification were carried out as previously described.[7] Nucleotide sequences of the primers were based on published cDNA sequences of mouse inducible nitric oxide synthase (iNOS), TNF-α, IL-1β or β-actin: iNOS forward, 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′; iNOS reverse, 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′; TNF-α forward, 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′; TNF-α reverse, 5′-ACT TGG GCA GAT TGA CCT CAG-3′; IL-1β forward, 5′-GCA ACT GTT CCT GAA CTC-3′; IL-1β reverse, 5′-CTC GGA GCC TGT AGT GCA-3′; β-actin forward, 5′-ATC CTG AAA GAC CTC TAT GC-3′; β-actin reverse, 5′-AAC GCA GCT CAG TAA CAG TC-3′.

Western blot analysis

BV-2 cells were pretreated with KT-14480 for 30 min and stimulated with LPS. After LPS stimulation for 1 h, cells were washed with phosphate buffered saline (PBS) and lysed with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% NP-40). Western blot analysis was carried out as previously described.[7]

Immunofluorescence staining

BV-2 cells were cultured on sterile coverslips and pretreated with KT-14480 for 30 min and stimulated with LPS for 1 h. Immunofluorescence staining was carried out as previously described.[7]

Nuclear extract preparation and electrophoretic mobility shift assay

BV-2 cells were pretreated with KT-14480 for 30 min and stimulated with LPS for 1 h. Nuclear extracts were then prepared from the cells as described previously.[9] Electrophoretic mobility shift assay was carried out as previously described.[7]

Microglia–neuroblastoma coculture

The coculture of HAPI rat microglial cells and EGFP-transfected B35 rat neuroblastoma cells (B35-EGFP) was carried out as previously described.[7]

Statistical analysis

All data were presented as mean ± SD from three or more independent experiments, unless stated otherwise. Statistical comparisons between different treatments were performed using the non-parametric Kruskal–Wallis test with Dunn's post-hoc tests and using the SPSS version 14.0K program (SPSS Inc., Chicago, IL, USA). Differences with a value of $P < 0.05$ were considered to be statistically significant.

Results

The identification of KT-14480 as an inhibitor of LPS-induced microglial NO production

NO production represents inflammatory activation of microglia, and has been widely used as a representative measure of inflammatory activation of microglia.[10–12] We therefore initially determined the effects of synthetic compounds on NO production in LPS-stimulated BV-2 microglial cells. In the preliminary study, the BV-2 mouse microglia cells were treated with increasing concentrations of LPS followed by the measurement of NO production. One hundred nanograms per millilitre of LPS was found to be the optimal concentration to induce NO production (data not shown), which was used throughout the experiments. A concentration of LPS higher than 100 ng/ml did not further increase the magnitude of the response (data not shown). The fluorovinyloxyacetamide compounds suppressed the NO production in LPS-stimulated BV-2 microglia cells to varying degrees (summarized in Table 1; the skeletal structure of the compounds is shown in Figure 1). Among the compounds tested, KT-14480 showed the greatest inhibitory effect on LPS-induced NO production in BV-2 microglia cells (Table 1). The inhibitory effect of

Table 1 Fluorovinyloxyacetamide derivatives inhibit LPS-induced NO production in BV-2 microglia cells

Compound ID[a]	R1	R2	Nitrite (μM)[b]	Viability (%)[c]	% inhibition[d]
KT-14480	CH_3		2.7 ± 0.3	100.7 ± 1.6	88.5 ± 3.8
KT-14482	C_2H_5		5.8 ± 0.1	99.8 ± 1.6	29.4 ± 1.6
KT-15069	CH_3	CH_3	6.7 ± 1.0	97.0 ± 2.1	12.4 ± 13.3
KT-15070	$C3H_7$	CH_3	5.5 ± 0.2	109.1 ± 0.7	35.6 ± 2.4
KT-15071	$CH(CH_3)_2$	CH_3	6.3 ± 0.5	101.3 ± 1.0	19.3 ± 6.9
KT-16513	CH_3	CH_3O	5.1 ± 0.3	111.2 ± 1.2	42.9 ± 4.2
KT-16547	CH_3	F	5.5 ± 0.6	96.7 ± 1.5	35.4 ± 7.9
KT-16581	$CH(CH_3)_2$	CH_3	6.6 ± 0.9	97.6 ± 7.8	14.2 ± 12.0
KT-16582	$CH(CH_3)_2$	CH_3O	6.7 ± 1.0	96.7 ± 2.9	11.1 ± 13.1

[a]All compounds were tested at 2 μM concentration. See Figure 1 for the positions of R1 to R2 in the chemical structure. [b]Nitrite production (μM): 2.1 ± 0.57 in untreated condition and 7.3 ± 0.34 in LPS-treated condition. [c]Viability was expressed as a percentage of surviving cells in comparison to the untreated control, which was set to 100. [d]Results are mean ± SD. Percentage inhibition was based on following calculation: % inhibition = [(A – B)/(A – C)] × 100. A, B and C indicate NO_2^- concentrations (μM) under following conditions: A, LPS (+), compound (–); B, LPS (+), compound (+); C, LPS (–), compound (–).

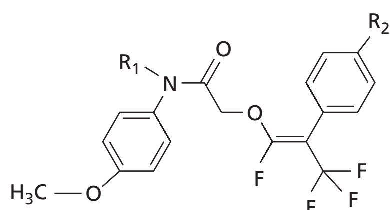

Figure 1 Skeletal structure of fluorovinyloxyacetamide derivatives. See Table 1 for a detailed chemical structure for the positions R1 to R2.

Table 2 KT-14480 inhibits LPS-induced NO production in microglia cells

Concentration of KT-14480 (μM)	BV-2		HAPI	
	% inhibition	Viability (%)	% inhibition	Viability (%)
0.02	17.9 ± 4.3	101.2 ± 5.3	-2.7 ± 4.9	103.7 ± 0.7
0.2	18.7 ± 7.8	100.7 ± 2.9	1.7 ± 3.2	104.4 ± 3.2
2	86.0 ± 9.0	98.0 ± 0.9	42.9 ± 2.3	96.8 ± 1.7

Percent inhibition and viability were calculated as described in Table 1.

KT-14480 was further confirmed at various concentrations using BV-2 mouse microglial cells and HAPI rat microglial cells (Table 2). Higher doses of KT-14480 (above 4 μM) showed cytotoxicity toward BV-2 cells (supplementary Figure 1b). In addition, 2 μM of KT-14480 showed a significant inhibitory activity against LPS-induced NO production, therefore this concentration was chosen for further studies.

Inhibitory effect of KT-14480 on the inflammatory gene expression in microglial cells

To investigate the effect of KT-14480 on the production of TNF-α, BV-2 microglial cells were incubated with LPS in the absence or presence of KT-14480 for 24 h, and the levels of secreted TNF-α were measured by ELISA. Compared to unstimulated microglial cells, the level of secreted TNF-α was increased in LPS-stimulated BV-2 microglia cells, but was markedly reduced by treatment with KT-14480 (Figure 2a). KT-14480 alone did not show any effect on the production of TNF-α (data not shown). The effect of KT-14480 on IL-1β, iNOS and TNF-α gene expression at transcriptional levels was determined by RT-PCR. The LPS-induced expression of these genes was significantly decreased by KT-14480 (Figure 2b).

Effect of KT-14480 on LPS-induced NF-κB and MAPK activation in microglial cells

As the transcriptional regulation of some proinflammatory cytokines and iNOS requires NF-κB activation,[13,14] the effects of KT-14480 on this signalling pathway were examined. The NF-κB pathway activation includes IκB degradation and a subsequent nuclear translocation of the free NF-κB (p65) from IκB and activation of genes with NF-κB binding sites, therefore the effect of KT-14480 on the nuclear translocation of the p65 subunit of NF-κB was investigated by immunofluorescence staining (Figure 3a). LPS induced translocation of the NF-κB from the cytoplasm into the nucleus, while the presence of KT-14480 suppressed the LPS-induced NF-κB p65 nuclear translocation. Electrophoretic mobility shift assay revealed that KT-14480 also suppressed the LPS-induced NF-κB DNA-binding activity in microglia cells (Figure 3b). The supershift assay and the unlabelled probe containing the NF-κB binding sequence were conducted in separate experiments in order to confirm the binding specificity of NF-κB (data not shown). The effect of KT-14480 on the p38 mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways, which are considered to regulate NF-κB activation and induce proinflammatory mediators in activated microglia,[15] was examined. Although phosphorylation of ERK seemed to be decreased by the compound, these changes were not statistically significant. Phosphorylation of p38 and JNK was not significantly affected either (Figure 4). The results are based on the assessment of KT-14480 effects on MAPKs at a single time point. Further evaluation at different time points is necessary to draw a firm conclusion. Since the levels of total p38, JNK and ERK MAPKs

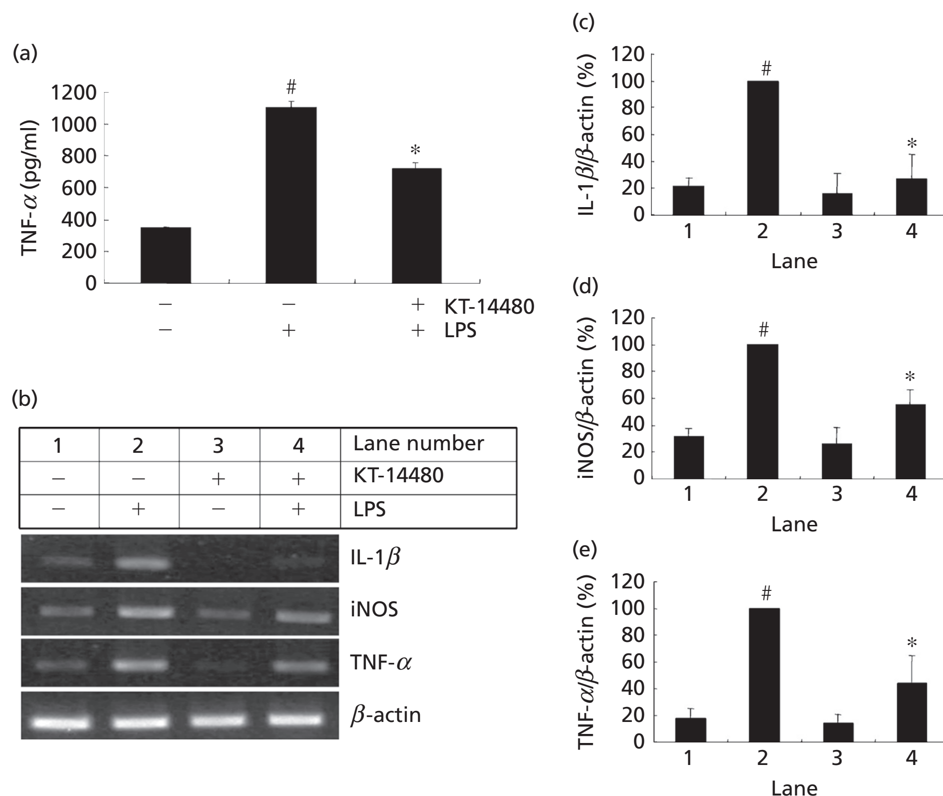

Figure 2 Suppression of the expression of IL-1β, iNOS and TNF-α in LPS-stimulated BV-2 microglial cells by KT-14480. (a) TNF-α ELISA. Data from triplicate determinations are shown (mean ± SD). $^{\#}P < 0.01$ vs untreated control; $^{*}P < 0.01$ vs LPS only. (b) Determination of mRNA levels by RT-PCR analysis. (c, d, e) Densitometric quantification. Data from triplicate determinations are shown (mean ± SD). $^{\#}P < 0.01$ vs untreated control; $^{*}P < 0.01$ vs LPS only.

were not changed by LPS or KT-14480 treatment in the separate experiments (data not shown), α-tubulin was used to normalize the levels of phosphorylated MAPKs.

Protective effect of KT-14480 against microglia-mediated neuroblastoma cell death

As the inflammatory mediators produced by activated microglia are involved in neuronal death,[16,17] the suppression of excessive microglial activation protects neurons. The potential neuroprotective effect of KT-14480 was therefore examined in a coculture system using microglia and neuroblastoma cells. In order to prevent the exposure of B35 rat neuroblastoma cells to the compound, HAPI rat microglia were pretreated for 30 min with KT-14480 prior to the addition of LPS. Then, the culture media were removed and replenished with the fresh media containing the EGFP-expressing B35 (B35-EGFP) neuroblastoma cells and LPS. After an additional incubation for 24 h, viable B35-EGFP neuroblastoma cells were counted. The results showed that LPS-activated microglia decreased the viability of B35-EGFP cells by ~44% compared to control cultures. KT-14480 attenuated the activated microglia-induced cell death of B35-EGFP neuroblastoma cells (Figure 5). Compound pretreatment for 30 min was enough to inhibit LPS-induced activation of HAPI cells (supplementary Figure 1a). LPS or the compound alone did not affect the viability of B35-EGFP neuroblastoma cells without microglia coculture (supplementary Figure 1c). These results indicate that KT-14480 may attenuate microglial neurotoxicity.

Discussion

From the screening of several fluorovinyloxyacetamide derivatives, we have identified KT-14480 as an anti-inflammatory agent that inhibits inflammatory activation of microglia. Since NO is the major proinflammatory mediator and plays an important role in neuroinflammatory diseases, the NO inhibitory effects of several fluorovinyloxyacetamide derivatives were evaluated. KT-14480 showed a strong inhibitory effect against NO production in LPS-stimulated BV-2 microglia cells. Thus, further studies were focused on this compound. In the remaining studies, the inhibitory effect of KT-14480 against IL-1β and TNF-α, which are also known to be major proinflammatory mediators, was investigated. KT-14480 suppressed the secretion of TNF-α protein from LPS-stimulated BV-2 microglia cells. The transcriptional levels of iNOS, IL-1β and TNF-α were also reduced by this compound. These results indicate that KT-14480 effectively inhibits the inflammatory activation of microglia.

NF-κB plays an important role in the transcriptional regulation of proinflammatory mediators.[13,14] The blockade of NF-κB transcriptional activity can suppress iNOS and proinflammatory cytokines such as IL-1β and TNF-α. We therefore investigated the effect of KT-14480 on NF-κB

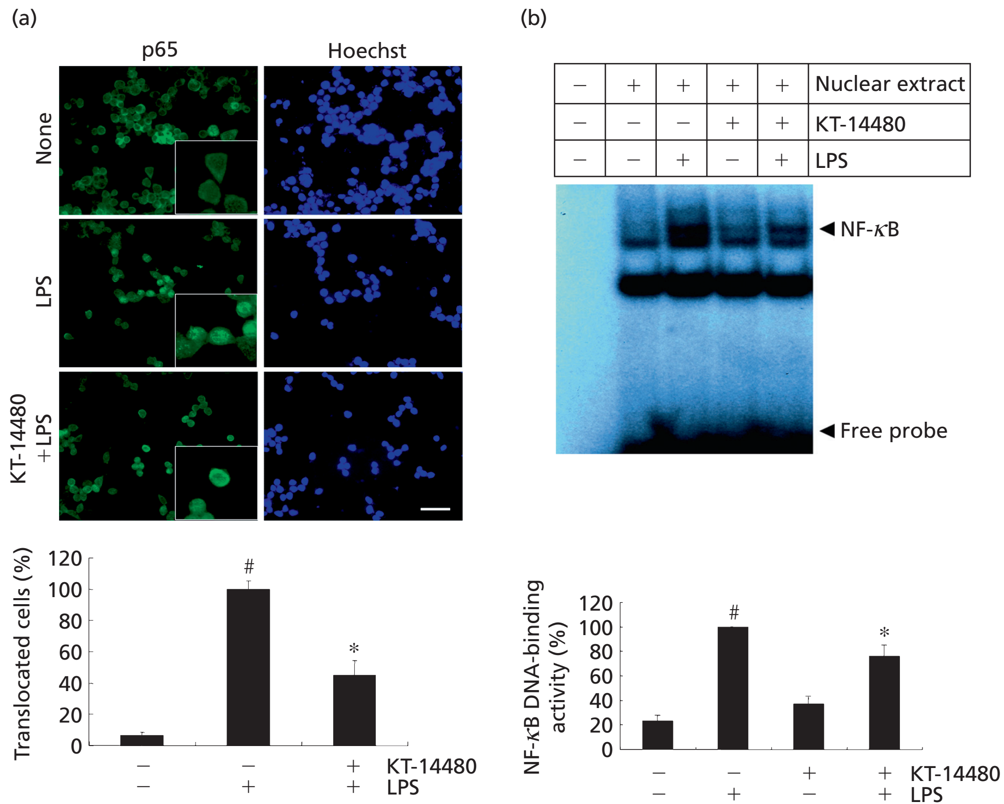

Figure 3 KT-14480 suppressed LPS-induced NF-κB activation in BV-2 microglial cells. BV-2 cells were stimulated with 100 ng/ml LPS in the absence or presence of KT-14480 (2 μM) that had been added 30 min before the stimulation. After 1 h, subcellular location of NF-κB p65 subunit was examined using immunofluorescence staining. LPS-induced NF-κB p65 nuclear translocation, which was colocalized with Hoechst staining, was inhibited by KT-14480 (a). Inset: magnified image of p65 immunofluorescence staining. Scale bar, 50 μm. After LPS stimulation for 1 h, nuclear extracts were isolated for gel shift assay. LPS-induced NF-κB DNA binding activity was also inhibited by KT-14480 (b). Data from triplicate determinations are shown (mean ± SD). $^{\#}P < 0.01$ vs untreated control; $^{*}P < 0.01$ vs LPS only.

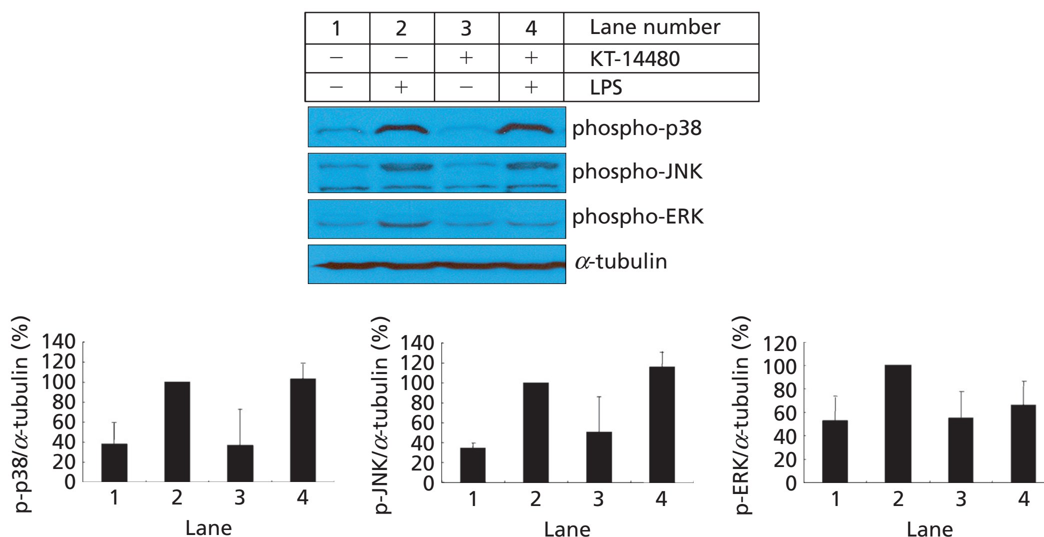

Figure 4 KT-14480 did not affect MAPK pathways in BV-2 microglial cells. BV-2 cells were pretreated with KT-14480 (2 μM) for 30 min and then stimulated with LPS (100 ng/ml) for 1 h. Western blot analysis was then performed to evaluate the activation of MAP kinases. Quantification of phospho-MAP kinases was performed by normalization to α-tubulin and expressed as a relative change in comparison to the LPS treatment, which was set to 100% (lane 2). Data from triplicate determinations are shown (mean ± SD).

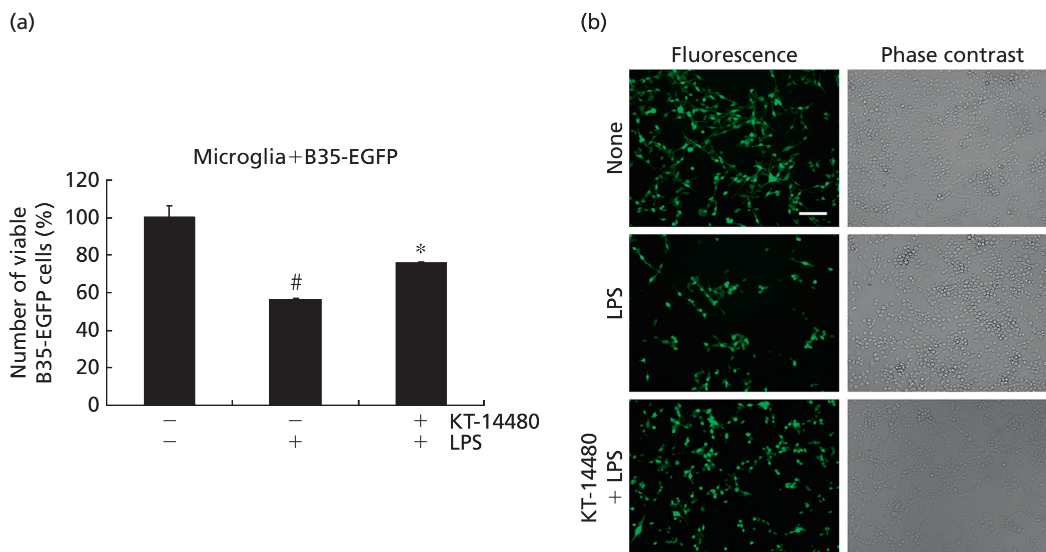

Figure 5 KT-14480 attenuated microglia-mediated neuroblastoma cell death. HAPI microglia cells were pretreated with 2 μM of KT-14480 for 30 min and washed with PBS. Then, LPS (100 ng/ml) and B35-EGFP neuroblastoma cells were added to microglia cells for coculture. Neuroblastoma cell death was assessed after microglia–neuroblastoma coculture for 24 h. At the end of incubation, the number of viable B35-EGFP neuroblastoma cells in the five randomly chosen fields per well was counted under a fluorescence microscope (a). Representative fluorescence or phase contrast images of cells are shown (b). The data represent results obtained from three independent experiments. Results were expressed as a percentage of control (unstimulated HAPI microglia + B35-EGFP neuroblastoma cells) (mean ± SD). Scale bar, 100 μm. $^{\#}P < 0.01$ vs control; $^{*}P < 0.01$ vs LPS only.

activation. KT-14480 decreased NF-κB activity through the suppression of p65 nuclear translocation and DNA-binding activity. The results suggest that KT-14480 inhibits the gene expression of iNOS and proinflammatory cytokines in microglia with molecular mechanisms that might involve the inhibition of NF-κB activation. Several studies have shown that MAPKs mediate the activation of NF-κB.[18,19] We previously reported that another fluorovinyloxyacetamide compound, KT-15087, inhibited microglial activation via suppression of NF-κB, p38 and JNK. Although the two compounds have the same skeletal structure, there was no significant structure–activity relationship between them. The slight difference in the structure between the two compounds may explain the distinct molecular mechanisms of action.

Neuroinflammation is a process that results primarily from abnormally high activation of microglia. This overactive state of the microglia results in increased levels of proinflammatory and oxidative/nitrosative stress molecules, which can lead to neuronal damage/death and contribute to the progression of neurodegenerative diseases. The inhibition of microglia activation could therefore be an effective therapeutic approach for alleviating the progression of neuroinflammatory or neurodegenerative diseases. In fact, some anti-inflammatory agents that inhibit glia activation or production of proinflammatory molecules attenuate neuronal damage or death.[20] In the present study, KT-14480 shows anti-inflammatory activity in activated microglia and also protects neuroblastoma cells against LPS-induced microglial neurotoxicity in microglia–neuroblastoma cocultures. Although the coculture of LPS-stimulated microglia and neuroblastoma cells is not the same as in-vivo conditions, it may mimic pathological conditions in neuroinflammatory diseases where activated microglia contribute to neural damage. Moreover, the coculture system has been successfully used in other studies to test the neuroprotective activity of the chemical compounds.[8,21] Taken collectively, this study suggests that KT-14480 is anti-inflammatory as well as neuroprotective, and the compound can be used for the development of novel therapeutic drugs against neuroinflammatory/neurodegenerative diseases.

Conclusions

A fluorovinyloxyacetamide compound KT-14480 inhibited LPS-induced microglial activation. It also protected neuroblastoma cells from activated microglia-mediated neurotox city. These effects of KT-14480 were due to the inhibition of the NF-κB pathway in BV-2 microglial cells.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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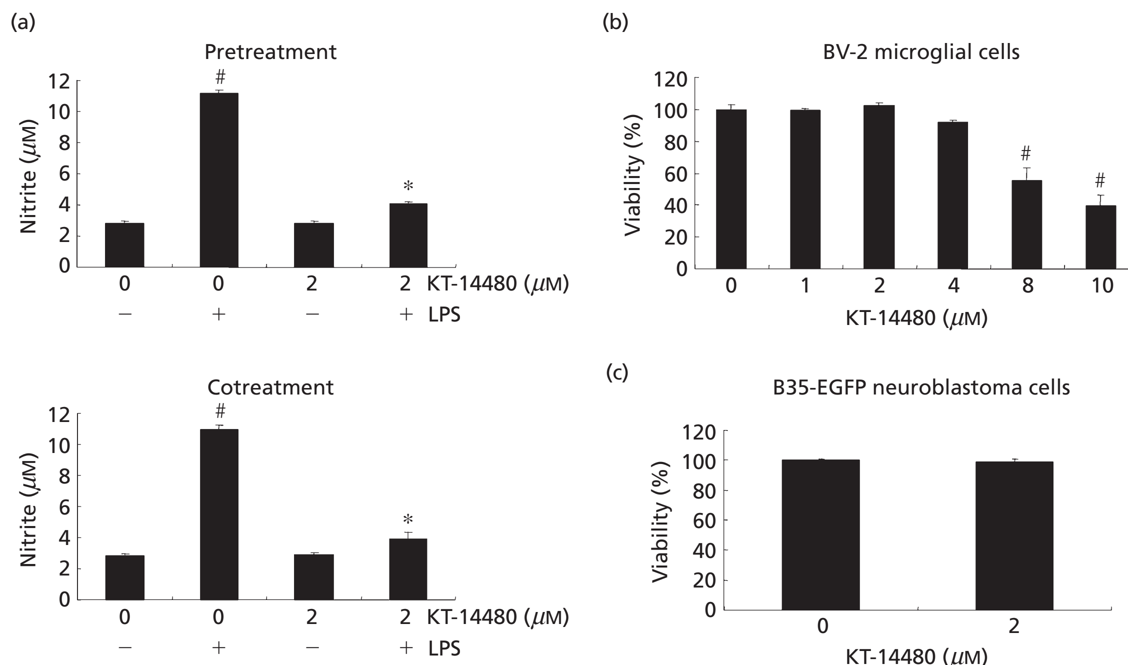

Supplementary Figure 1 The effect of KT-14480 on NO production and cell viability. HAPI microglia cells were pretreated with KT-14480 for 30 min prior to LPS (100 ng/ml) challenge (Pretreatment) or treated with KT-14480 and LPS at the same time (Cotreatment). After additional incubation for 24 h, nitrite content in the culture media was measured using the Griess reaction (a). BV-2 cells or B35-EGFP cells were treated with KT-14480 (1~10 μM for BV-2; 2 μM for B35-EGFP) for 24 h, cytotoxicity of the compound was assessed by MTT assays and the results were expressed as a percentage of surviving cells over control cells (b and c). The data were expressed as the mean ± SD (n = 3), and are representative results obtained from three independent experiments. $^{\#}P < 0.01$ vs. control; $^{*}P < 0.01$ vs. LPS only.

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