CKD-712, (S)-1-(α-naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, Inhibits the NF-κB Activation and Augments Akt Activation during TLR4 Signaling

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Since CKD-712 has been developed as an anti-inflammatory agent, we examined the effect of CKD-712 during TLR4 signaling. Using HEK293 cells expressing TLR4, CKD-712 was pre-treated 1 hr before LPS stimulation. Activation of NF-κB was assessed by promoter assay. The activation of ERK, JNK, p38, and Akt was measured by western blotting. CKD-712 inhibited the NF-κB signaling triggered by LPS. The activation of ERK, JNK, p38 or IRF3 was not inhibited by CKD-712. On the contrary the activation of these molecules was augmented slightly. The activation of Akt with stimulation of LPS was also enhanced with CKD-712 pre-treatment at lower concentration, but was inhibited at higher concentration. We suggest that during TLR4 signaling CKD-712 inhibits NF-κB activation. However, CKD-712 augmented the activation of Akt as well as Map kinases. Therefore, we suggest that CKD-712 might have a role as an immunomodulator.

Keywords: CKD-712, TLR4, Akt, immunomodulator
via inhibition of phosphatidylinositol 3 (PI3K)-protein kinase C (PKC) signaling after LTA or LPS stimulation in macrophage cells (9).

In this study we attempted to determine which signaling pathway is affected by CKD-712 after TLR4 stimulation, since TLR4 is the major PRRs responsible in development of sepsis. HEK293-TLR4 cells (kindly provided by Professor Douglas T, Golenbock, Massachusetts University, USA) were cultured in 10% fetal bovine serum-RPMI1640 medium. HEK293-TLR4 cells express MD2 and CD14 as well. CKD-712 was kindly provided by ChongKunDang Pharm, Cheonan. LPS was purchased from Sigma-Aldrich, St, Louis, MI, USA. To assess cell viability after treatment of CKD-712, cells were reacted with 5 mg/ml of tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) (Sigma-Aldrich). After 3 hrs of incubation (37°C, 5% CO2) the medium containing MTT was removed and 200 μl of DMSO was added into the well and the absorbance was measured at 540 nm. For the luciferase assay, 2×10⁵ cells/well were seeded in 12-well plates and cultured for 2 days. NF-κB promoter and pCMV β-gal vector were co-transfected overnight, and CKD-712 was pre-treated before LPS treatment. LPS was treated for 6 hrs. Luciferase activity was measured by a Luciferase Reporter Assay System (Promega, Madison, WI, USA) and β-galactosidase activity was measured with O-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich), as the substrate. Luciferase activity was normalized for transfection efficiency with the β-galactosidase activity. Western blotting was done using anti-TLR4, anti-ERK, anti-p38, anti-JNK, anti-IRF3 or anti-Akt antibodies as well as control antibodies. All of the antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

First, we determined the optimal concentration of CKD-712 in HEK293-TLR4 cells. When treated with CKD-712 for 24 hrs the cell viability was 50% at 100 μM and 40% at 200 μM (Fig. 1A). Next, HEK293-TLR4 cells were treated with LPS and NF-κB activation was measured. CKD-712 was pre-treated 1 hr before stimulation of LPS. We found that 50 μM of CKD-712 inhibited NF-κB activation in TLR4 signaling}.
modulates MAP kinase activation. After stimulation with LPS, CKD-712 did not inhibit activation of ERK, JNK or p38 kinase in HEK293-TLR4 cells (Fig. 2). Rather, pre-treatment with CKD-712 delayed activation of these kinases. As shown in Fig. 3, MAP kinases were phosphorylated 15~30 min following stimulation with LPS, after which activation subsided in 60 min. However, CKD-712 treatment extended the activation of ERK and JNK, which were found to be activated 60 min after TLR4 stimulation (Fig. 2). Phosphorylation of IRF3 was not inhibited and showed a sustained activation like the MAP kinases (Fig. 2). Interestingly, the activation of Akt was augmented slightly with pre-treatment of CKD-712 (50 μM) after LPS stimulation (Fig. 3, left). But higher concentration of CKD-712 (100 μM) inhibited the activation of Akt completely (Fig. 3, right).

With the brief data presented here, we suggest that CKD-712 activated Akt and MAP kinases after TLR4 stimulation at 50 μM. Emerging evidence has shown the existence of a close relation between TLRs and the PI3K/Akt pathway. One of the early evidences demonstrated that PI3-kinase is involved in TLR4-mediated cytokine expression in mouse macrophages (10). Even in non-immune cells, such as, in foam cells, binding of LPS and TLR4 increases Nox1 expression through the phospholipase A2 β-Akt signaling pathway (11). Another paper reporting that HMGB-1 induced phosphorylation of Akt, which led to significant protection in a cerebral injury model, suggesting that the PI3 kinase-Akt pathway is involved with TLR2 signaling as well as TLR4 signaling (14). More recently the association of endogenous TBK1 and Akt was observed in macrophages when stimulated with poly (I:C) and LPS, showing that Akt activation mediated by TBK1 contributes to TLR3 and TLR4-mediated immune responses (15).

The effect of CKD-712 on phosphorylation of Akt depends on the cell types. In immune cells, CKD-712 inhibits Akt activation in macrophages (9) and human endothelial cells (15). But in rat heart muscle cells, CKD-712 enhances Akt activation to prevent myocardial ischemia and further inflammation (16). Our data show that in HEK293 cells, CKD-712 at low concentration augments the activation of Akt, whereas high concentration of CKD-712 has an inhibiting effect. Therefore pharmaceutical agents regulating the PI3-Akt pathway may be used as immunomodulators in the innate immune response. Although the mechanism of the augmented activation of Akt by CKD-712 and the inhibition of NF-κB activation have yet to be clarified, CKD-712 still has a potential as an immunomodulator.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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