RESEARCH ARTICLE

Novel Synthesized Coumarin Derivative as a Pro-inflammatory Cytokines Inhibitor

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Abstract

The purpose of this study was to investigate the effect of the novel synthesized coumarin derivative, RKNU026 on pro-inflammatory cytokine inhibitory efficacy. The results demonstrated that RKNU026 could inhibit IL-1β and TNF-α production in a concentration dependent pattern. The half maximal inhibitory concentrations (IC₅₀) of RKNU026 for IL-1β and TNF-α were 0.024 µM and 729 µM, respectively. Compared with dexamethasone at the same final concentration of 1 µM, the efficacy of RKNU026 on IL-1β inhibition was still lower than dexamethasone (70.78% versus 89.53%) while RKNU026 had no efficacy on TNF-α inhibition at this concentration. Our work suggests that RKNU026 has anti-inflammatory efficacy through pro-inflammatory cytokine inhibition. Furthermore, it is considered to be an IL-1β inhibitor rather than a TNF-α inhibitor.

Keywords: inflammation, pro-inflammatory cytokines, novel synthesized coumarin derivative
ฤทธิ์ยับยั้งไซโตไคน์ก่อการอักเสบของสารอนุพันธ์คูมารินส์สังเคราะห์ใหม่

ประยุทธ์ ภูวรัตนาวิวิ, 1 ธิปไตย หิรัณยเอกภาพ, 2 นันทีทิพ ลิ้มเพียรชอบ, 1 เรืองวิทย์ กิจบรรณเดช, 2 ชวัญชัย รัตนมณี 1

1 ภาควิชาเภสัชกรรมปฏิบัติ คณะเภสัชศาสตร์ มหาวิทยาลัยนเรศวร พิษณุโลก
2 ภาควิชาเภสัชเคมีและเภสัชเวท คณะเภสัชศาสตร์ มหาวิทยาลัยนเรศวร พิษณุโลก

บทศึกษา

วัตถุประสงค์ของการศึกษาในครั้งนี้คือ เพื่อทดสอบฤทธิ์ของสารอนุพันธ์คูมารินส์สังเคราะห์ใหม่ในการยับยั้งไซโตไคน์ก่อการอักเสบ นอกจากนี้ยังเปรียบเทียบประสิทธิภาพของสารกับยาเดกซามีดอนที่มีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบ ผลการศึกษาแสดงให้เห็นว่าสารอาร์เคเอ็นยู-26 สามารถยับยั้งการสร้างไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้า และทูเมอร์เนคโครซิสแฟกเตอร์-แอลฟา แบบแปรผันโดยตรงกับความเข้มข้น ค่าความเข้มข้นของสารอาร์เคเอ็นยู-26 ที่ให้ผลยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้า และทูเมอร์เนคโครซิสแฟกเตอร์-แอลฟาเป็นครึ่งหนึ่ง คือ 0.024 ไมโครโมลาร์ และ 729 ไมโครโมลาร์ ตามลำดับ และเมื่อเปรียบเทียบกับยาเดกซามีดอนที่มีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้า ยังคงต่ำกว่ายาเดกซามีดอนที่มีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้า และเมื่อเปรียบเทียบกับยาเดกซามีดอนที่มีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้า พบว่ามีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้ามากกว่ายาเดกซามีดอนที่มีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้ามากกว่ายาเดกซามีดอนที่มีคุณสมบัติในการยับยั้งไซโตไคน์ก่อการอักเสบชนิดอินเตอร์ลิวคิน-1 เบต้า

คำสำคัญ: ภาวะอักเสบ, ไซโตไคน์, การยับยั้ง, สารอนุพันธ์คูมารินส์สังเคราะห์ใหม่
Introduction

Inflammation is one of the most important pathogenesis of human diseases, including Alzheimer’s disease (AD), which is a significant and growing public health problem worldwide. Inflammation relates to AD as a consequence of the damaged neurons. Previous studies demonstrated that the stimuli of inflammation in Alzheimer’s brain were the activating glia cells (astrocytes and microglia) and the over-deposition of highly insoluble amyloid β peptide and neurofibrillary tangle. These stimulators induce the production of inflammatory molecules including pro-inflammatory cytokines such as interleukin 1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) which produce neuroinflammatory signals and affect neurophysiologic mechanisms regarding cognition and memory. Thus far, there are no anti-inflammatory drugs approved for the treatment of AD.

Coumarins are bicyclic aromatic compounds found in many plants (Rutaceae and Umbelliferae families), essential oils, and foods. Previous studies have shown that coumarin compounds have many useful pharmacological properties and therapeutic potential as effective treatment for cancers, coagulation disorders and inflammation. Currently coumarins are one of the chemical groups of interest in the pipeline of drug development, particularly the 7-hydroxycoumarin (7-HC) derivatives. These derivatives are non-genotoxic to human cells. In addition, several studies demonstrated various pharmacological effects of 7-HC derivatives such as immunomodulation, anti-oxidant, anti-tumor, anti-infection, prolonged anti-nociceptive and anti-inflammation (Figure 1).

![Figure 1. Pharmacological effects of 7-hydroxycoumarin derivatives.](image)

7-((7-(piperidin-1-yl)heptyl)oxy)-2H-chromen-2-one (RKNU026, Figure 2), a novel 7-HC derivative with amine spacer arm, previously displayed a potent inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Our preliminary data showed that RKNU026 had no acute cytotoxic effect on SH-SY5Y cells at $10^{-4}$ M. Since inflammation is one of the pathogenesis of AD, in this study we investigated the anti-inflammatory actions of RKNU026 and the mechanisms in which RKNU026 reduced cytokine-provoked inflammation in whole blood.
Materials and Methods

Materials and chemicals

Ready-SET-Go!® enzyme-linked immunosorbent assay (ELISA) kits for quantitative determination of IL-1β and TNF-α were purchased from eBioscience (San Diego, CA, USA). Dexamethasone, lipopolysaccharides from Escherichia coli strain 026:B6 (LPS), RPMI medium, penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were analytical grade.

RKNU026 was synthesized by Dr. Reungwit Kitbunnadaj (Figure 2). RKNU026 was dissolved in 1% dimethyl sulfoxide and was diluted to the final concentrations ranging from $10^{-2}$ to $10^{-9}$ M in the culture medium.

![Chemical Structure of RKNU026](image)

Figure 2. The chemical structure of 7-((7-(piperidin-1-yl)heptyl)oxy)-2H-chromen-2-one (RKNU026)

Whole blood sample collection and preparation

Sample collection

Whole blood samples were obtained from three healthy volunteers aged 20 years or older (20 mL each). The volunteers included in this study were evaluated for their health status and met all of the following inclusion criteria: (1) The volunteers had no infectious disease, cancer, inflammatory or autoimmune diseases; and (2) The volunteers had not taken any medications known to be capable of stimulating or inhibiting cytokine release such as antibiotics, anti-inflammatory agents, or immunosuppressive medications for at least 6 weeks before participating in the study. All subjects provided written informed consent before admission to the study. Whole blood samples were aseptically collected by a registered nurse. The study protocol was approved by the Research Ethics Committee of Naresuan University, Phitsanulok, Thailand.

Preparation of blood samples

Sodium citrate (3.8% w/v) was added to each whole blood sample as an anticoagulant. The samples were preserved in RPMI1640 supplemented with 100U/ml of penicillin, 100 µg/ml of streptomycin and incubated at 37°C for 1 h. Then RKNU026 and positive control (dexamethasone at the final concentration of 1 µM) were added to each whole blood cell culture. The negative control was blood samples without RKNU026. Inflammation was induced by adding 100 ng/mL LPS and further incubated at 37°C for 24 h. The LPS-stimulated whole blood samples were then centrifuged and the supernatants were collected for pro-inflammatory cytokines inhibition assay.

Measurement of pro-inflammatory cytokines

The concentrations of IL-1β and TNF-α were measured by using double-sandwich ELISA technique. In brief, the capture antibody was diluted in coating buffer overnight and blocked with blocking buffer, then incubated overnight.
Samples were added and incubated for 2 h. The detection antibody was added and incubated for 1 h, and then HRP-conjugated streptavidin was added and incubated for 30 min. Unbound and non-specific materials were washed before working on the connecting step. The colorimetric detection reagent was added and led the reaction to develop for 30 min and terminated the reaction with stop solution. The solution was measured by spectrophotometer at 450/650 nm. The concentrations of IL-1β and TNF-α (pg/mL) were determined and calculated as the percentages of pro-inflammatory cytokine inhibition. Dexamethasone was used as the positive control in this study because it is known to be a potent anti-inflammatory agent.

Statistical analysis

Data are expressed as means±standard errors (SEM). The results were taken from at least three independent experiments. All experiments were performed in triplicates. The half maximal inhibitory concentrations (IC50) were determined by using GraphPad Prism (Version 2.01, GraphPad Software, Inc., USA). The differences between the means were analyzed for statistical significance by independent samples two-sided t-test with significance level of 5% (α = 0.05). Microsoft Excel Software, Inc., USA was used for this calculations.

Results

As shown in Table 1 and 2, RKNU026 inhibited IL-1β and TNF-α production in a concentration dependent pattern (Figure 3). RKNU026 at the highest concentration (10⁻² M) has been shown to inhibit IL-1β and TNF-α productions by 79.75 and 74.96%, respectively. However, RKNU026 did not show inhibitory effect on LPS induced TNF-α production at the concentrations lower than 10⁻⁷ M. The IC50 of RKNU026 for inhibition of IL-1β and TNF-α production in LPS-stimulated human whole blood cells are 0.024 µM and 729 µM. Dexamethasone (positive control) at 1 µM inhibited the productions of IL-1β and TNF-α by about 90%. When compared to dexamethasone at the same concentration (1 µM), RKNU026 showed significantly lower inhibitory effect on IL-1β production, (70.78% versus 89.53%), whereas no inhibitory effect on TNF-α production.

Table 1. The percentage inhibitions and IC50 of IL-1β production by RKNU026.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>% IL-1β Inhibition</th>
<th>RKNU026</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻² M</td>
<td></td>
<td>79.75±1.24</td>
<td></td>
</tr>
<tr>
<td>10⁻³ M</td>
<td></td>
<td>77.44±1.14</td>
<td></td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td></td>
<td>75.96±1.26</td>
<td></td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td></td>
<td>74.90±0.60</td>
<td></td>
</tr>
<tr>
<td>10⁻⁶ M</td>
<td></td>
<td>70.78±1.03</td>
<td>89.53±6.68</td>
</tr>
<tr>
<td>10⁻⁷ M</td>
<td></td>
<td>54.82±0.41</td>
<td></td>
</tr>
<tr>
<td>10⁻⁸ M</td>
<td></td>
<td>47.77±0.39</td>
<td></td>
</tr>
<tr>
<td>10⁻⁹ M</td>
<td></td>
<td>30.75±0.44</td>
<td></td>
</tr>
<tr>
<td>IC₅₀ (µM)</td>
<td>0.024</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The percentages of IL-1β inhibition were expressed by mean ± SEM.
Table 2. The percentage inhibitions and IC\textsubscript{50} of TNF-\(\alpha\) production by RKNU026.

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>% TNF-(\alpha) Inhibition</th>
<th>RKNU026</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10^{-2}) M</td>
<td>74.96±0.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10^{-3}) M</td>
<td>56.91±0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10^{-4}) M</td>
<td>28.90±0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10^{-5}) M</td>
<td>Not inhibit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10^{-6}) M</td>
<td>Not inhibit 88.19±1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC\textsubscript{50} (µM)</td>
<td>729</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The percentages of TNF-\(\alpha\) inhibition were expressed by mean ± SEM.

Figure 3. Inhibitory effects on IL-1\(\beta\) and TNF-\(\alpha\) production by RKNU026.
(The grey box indicates the safety zone of RKNU026 in SH-SY5Y cell line)

Discussion

At the beginning of the inflammatory process, pro-inflammatory cytokines are the principal mediators that cause both physiologic and pathologic events at the inflammatory sites.\textsuperscript{1,2} IL-1 and TNF-\(\alpha\) are ones of the most important pro-inflammatory cytokines.\textsuperscript{2,6} The elevations of IL-1 and TNF-\(\alpha\) levels were observed in the brain of patients with AD\textsuperscript{2,6} while the elevation and overexpression of IL-1\(\beta\) levels were observed in the serum, cerebrospinal fluid and brain of the patients.\textsuperscript{23-25} IL-1\(\beta\) is a major activator of astrocytes and microglia which further induce cytokine release and nitric oxide synthase activity to produce nitric oxide leading to neurotoxicity.\textsuperscript{6,26} In addition, IL-1\(\beta\) can enhance the production of amyloid precursor protein and amyloid \(\beta\) from neurons.\textsuperscript{27-29} An \textit{in vivo} study\textsuperscript{30} demonstrated that inhibiting the production of IL-1\(\beta\) helped to improve inflammatory response of the mouse brain. Furthermore, the study in IL-1\(\beta\) receptor antagonist knockout mice\textsuperscript{31} demonstrated increase in neuronal damage induced by amyloid \(\beta\). Increase in TNF-\(\alpha\) was also observed in AD serum and brain after exposure to amyloid \(\beta\).\textsuperscript{32} However, the pathophysiologic actions of TNF-\(\alpha\) are different from those of IL-1\(\beta\). The neurotoxic effect of TNF-\(\alpha\) in human cortical neurons was demonstrated\textsuperscript{33}. 
whereas its neuroprotective effect such as inducing the expression of protective molecules (manganese superoxide dismutase) was reported in cultured neurons. Nevertheless, the study in transgenic mice overexpressing TNF-α showed severe inflammation and neurodegeneration that caused fatal outcome. In addition, the results from a meta-analysis of forty studies showed that the levels of IL-1β and TNF-α in peripheral blood of Alzheimer’s patients were significantly high, suggesting that inflammation was involved in many steps of neurodegenerative cascade which led to AD pathologies. Therefore, inhibiting the production of IL-1β and TNF-α or reducing their levels may be an alternative approach to prevent or reduce the severity of inflammation as well as the disease.

Previously, several compounds have been discovered for the treatment of AD; however, the drugs acting by specific inhibition of inflammation in AD have not been approved. Our group has developed a new chemical, RKNU026, based on knowledge of medicinal chemistry. This compound was designed to possess anti-inflammatory effect by using 7-HC as a core structure linked with amine spacer arm. It has been documented in the previous literatures that 7-HC was safe when tested in human cells and had many useful pharmacological effects. Previous in vitro studies focusing on anti-inflammatory efficacy showed that 7-HC modulated the oxidative stress metabolism, degranulation and microbial killing of human neutrophils. It also interacted with secretory phospholipase A2s and caused some structural modifications that led to a sharp decrease or inhibition of phospholipase A2s activities. Furthermore, in vivo studies showed the potential of 7-HC on the prolonged anti-nociceptive and anti-inflammatory effects at least by reducing the prostaglandin E2 production. Although these studies demonstrated the mechanisms of 7-HC as anti-inflammatory and immunomodulating agents, no study examined 7-HC inhibition activities on human pro-inflammatory cytokine production, especially IL-1β and TNF-α.

In this study, a concentration dependent inhibitory effect of RKNU026 on IL-1β and TNF-α production in LPS-stimulated human whole blood cells has been reported for the first time, with the IC50s of 0.024 and 729 µM, respectively. Another coumarin derivative, isofraxidin (7-hydroxy-6,8-dimethoxycoumarin) has been demonstrated to suppress TNF-α expression in response to LPS-stimulation of peritoneal macrophages. Likewise, RKNU026 inhibited TNF-α expression only at the dose ranges higher than 10 µg/mL. Another study on inhibitory effects of 7-HC derivatives on IL-6, IL-12 and interferon-γ productions showed a poor inhibitory efficacy of IL-6 production (IC50 >10 µg/mL) (Table 3). Other studies in human whole blood demonstrated that the inhibition potencies of RKNU026 on IL-1β and TNF-α production were substantially lower than those of dexamethasone in nanomolar levels.

Our results imply that RKNU026 can inhibit the pro-inflammatory cytokine production of IL-1β and TNF-α, indicating the therapeutic potential of this compound in the diseases where IL-1β and TNF-α are associated with pro-inflammatory actions such as AD, arthritis, atherosclerosis, asthma, cancer, and sepsis.
Table 3. Pro-inflammatory cytokine inhibitory effects of 7-hydroxycoumarin derivatives.

<table>
<thead>
<tr>
<th>7-hydroxycoumarin derivatives</th>
<th>Pro-inflammatory cytokine</th>
<th>Source of inflammation</th>
<th>Methodology</th>
<th>IC₅₀ or effective dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKNU026</td>
<td>IL-1β, TNF-α</td>
<td>human peripheral whole blood</td>
<td>Double sandwich ELISA</td>
<td>IL-1β 0.024 µM TNF-α 729 µM</td>
</tr>
<tr>
<td>7-hydroxycoumarin</td>
<td>IL-1α, IL-6 and TNF-α</td>
<td>P388D1 cells, originating from DBA/2 mice</td>
<td>ELISA</td>
<td>IL-1α 40.8 µg/mL IL-6 77.9 µg/mL TNF-α 39.6 µg/mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>murine influenza virus infection model</td>
<td></td>
<td>30 mg/kg for 2 days (TNF-α was 42.8% of control, IL-6 was 35.3% of control)</td>
</tr>
<tr>
<td>Isofraxidin</td>
<td>TNF-α</td>
<td>peritoneal macrophages</td>
<td>ELISA</td>
<td>&gt; 10 µg/mL</td>
</tr>
</tbody>
</table>

Although the molecular mechanisms of reducing pro-inflammatory cytokines, IL-1β and TNF-α by RKNU026 were not deeply explored here, a previous study suggested that the inhibitory effects of 7-HC on the release of TNF-α and IL-1β was correlated with the reduction of neutrophil migration and the inhibition of prostaglandin E2 production in vivo which is rather different to the whole blood. RKNU026 is needed to be further investigated both in vitro and in vivo regarding the efficacy, safety, and other anti-inflammatory mechanisms such as inhibition of inflammatory enzymes, inhibition of nitric oxide production, and NF-κB expression.

Conclusion

RKNU026 demonstrated an anti-inflammatory effect via inhibiting IL-1β production rather than TNF-α production. Nevertheless, it has a poorer anti-inflammatory efficacy than dexamethasone.

Acknowledgement

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