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Immunopharmacology and inflammation

Ethyl rosmarinate inhibits lipopolysaccharide-induced nitric oxide and prostaglandin E_2 production in alveolar macrophages

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ABSTRACT

In this study, a series of rosmarinic acid and analogs were investigated for their anti-inflammatory potential against LPS-induced alveolar macrophages (MH-S). Our results showed that, among the test compounds, ethyl rosmarinate (**3**) exhibited the most potent inhibitory effect on NO production in LPS-induced MH-S cells, with low cytotoxicity. Compound **3** exhibited remarkable inhibition of the production of PGE_2 in LPS-induced MH-S cells. The inhibitory potency of compound **3** against LPS-induced NO and PGE_2 release was approximately two-fold higher than that of dexamethasone. Compound **3** significantly decreased the mRNA and protein expression of iNOS and COX-2 and suppressed p65 expression in the nucleus in LPS-induced MH-S cells. These results suggested that compound **3** inhibited NO and PGE_2 production, at least in part, through the down-regulation of NF-κB activation. Analysis of structure-activity relationship revealed that the free carboxylic group did not contribute to inhibitory activity and that the alkyl group of the corresponding alkyl ester analogs produced a strong inhibitory effect. We concluded that compound **3**, a structurally modified rosmarinic acid, possessed potent inhibitory activity against lung inflammation, which strongly supported the development of this compound as a novel therapeutic agent for the treatment of macrophage-mediated lung inflammatory diseases, such as COPD.

1. Introduction

Lung inflammation is fundamental to the etiology and persistence of respiratory disease conditions, including asthma and chronic obstructive pulmonary disease (COPD). In particular, COPD is a major health problem that results in morbidity and mortality worldwide (Barnes, 2015). A recent systemic review and meta-analysis suggested a high and growing prevalence of COPD, both globally and regionally (Adeloye et al., 2015). Among the various cell types, alveolar macrophages and neutrophils are thought to be responsible for COPD-related inflammation (Barnes, 2004; Meijer et al., 2013). However, there is increasing evidence to show that alveolar macrophages play a key role in the pathogenesis of COPD (Barnes, 2004). COPD is a disease of the small airways and lung parenchyma, of which inflammation and tissue destruction are the pathological hallmarks (Di Stefano et al., 2004). Alveolar macrophages are the predominant cell type in the lung (Shapiro, 1999; Pons et al., 2005), and a marked increase (5–10 fold) in the numbers of macrophages both in airways and in lung parenchyma was evident in patients with COPD (Keatings et al., 1996; Pesci et al., 1998). Furthermore, the number of parenchymal alveolar macrophages, but not neutrophils, was found to be directly proportional to the severity of the disease (Finkelstein et al., 1995; Di Stefano et al., 1998). Moreover, the pathological role of alveolar macrophages has been demonstrated, as the depletion of lung macrophages conferred protection against the development of emphysema in an experimental model of COPD (Beckett et al., 2013). Upon activation by harmful stimuli (predominantly cigarette smoke and bacterial infections) alveolar macrophages release inflammatory mediators including nitric oxide (NO), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Barnes, 2016). The uncontrolled production or excessive accumulation of these mediators can destroy lung tissue, which leads to respiratory failure and dysfunction. As a vital organ for gaseous exchange, chronic or excessive inflammation of the lung can be life threatening. Currently, bronchodilators and inhaled corticosteroids are prescribed as the first-line therapies for COPD. However, owing to the limited improvements in

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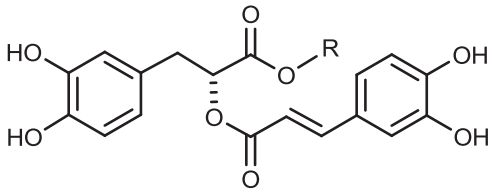
symptom control and survival rate (Callahan et al., 1991; Jiang and Zhu, 2016), this approach has not provided a satisfactory solution. More importantly, a certain proportion of patients with COPD are reported to be resistant to corticosteroids and experience a deterioration of their symptoms (Marwick et al., 2007; Malhotra et al., 2011; Jiang and Zhu, 2016). When used at high doses or for a prolonged time, corticosteroids can lead to problematic side effects, such as osteoporosis, aseptic joint necrosis, adrenal insufficiency, gastrointestinal, hepatic, and ophthalmologic effects, hyperlipidemia, growth suppression, and possible congenital malformations (Buchman, 2001). A significant increase in the risk of pneumonia with the long-term use of inhaled corticosteroids has also been reported in patients with COPD (Sonal and Loke, 2010). Therefore, the discovery and development of new agents for the effective treatment of COPD are an urgent necessity.

Rosmarinic acid (**1**), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is commonly found in several medicinal plant species, including *Rosmarinus officialis* (Kim et al., 2015) and *Hyptis suaveolens* (Prawatsri et al., 2013). Interest in this compound has increased considerably in the last decade owing to its diverse pharmacological activities, including anti-oxidant (Kelm et al., 2000; Al-Musayeib et al., 2011), anti-cancer (Moore et al., 2016), anti-angiogenic (Cao et al., 2016), anti-viral (Arabzadeh et al., 2013), and anti-microbial properties (Abedini et al., 2013). Rosmarinic acid also exerts anti-inflammatory effects, as demonstrated by its ability to inhibit NO and PGE_2 release in macrophage-mediated inflammation by using RAW 264.7 murine macrophages of peritoneal origin as a model (Huang et al., 2009). In addition, the anti-allergic activity of rosmarinic acid was also been shown in an experimental model of allergic asthma (Costa et al., 2012; Liang et al., 2016). These results indicated the immunomodulatory properties of rosmarinic acid and suggested the possible use of this compound for the treatment of airway inflammation. Nevertheless, marked differences between the inflammatory cells involved in the pathology of asthma and COPD have been described (Ichinose, 2009; Moldoveanu et al., 2009). Moreover, there are functional and phenotypic differences among macrophages from different tissue sites (Guth et al., 2009; Karagianni et al., 2013; Hussell and Bell, 2014). As such, an appropriate therapeutic target for certain airway inflammatory diseases would be of significant concern. As alveolar macrophages and the release of inflammatory mediators play a pivotal role in the pathogenesis of COPD, and owing to the absence of scientific evidence addressing the effects of rosmarinic acid on alveolar macrophages, this study therefore investigated the anti-inflammatory potential of rosmarinic acid in LPS-induced mouse alveolar macrophage MH-S cell lines. We also designed and prepared analogs of this compound and examined their inhibitory activities. The underlying mechanism responsible for the inhibitory effect was also explored.

2. Materials and methods

2.1. Rosmarinic acid (1) and analogs 2–11

Rosmarinic acid (**1**) and methyl rosmarinate (**2**) (Fig. 1) are natural compounds isolated from the weeds *Hyptis sauveolens* by our group (Prawatsri et al., 2013). Structural modifications of rosmarinic acid to the corresponding ester analogs, compounds **3–11,** (Fig. 1) were achieved by conventional esterification of rosmarinic acid to the corresponding esters using appropriate alcohols, with concentrated sulfuric acid as a catalyst at room temperature, except for the benzyl ester **10** that was synthesized by coupling reaction between rosmarinic acid (**1**) and benzyl alcohol using EDCI [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide] and DMAP (4-dimethylaminopyridine) in triethylamine and dichloromethane. The products were purified by column chromatography (Merck silica gel 60, particle size < 0.063 mm) and the structures of the synthesized analogs were confirmed by NMR spectroscopic data (recorded on a Bruker AVANCE 400 FT NMR spectrometer, operating at 400 MHz), and ESI mass spectral data (measured



1, R = H (Rosmarinic acid)
2, R = CH_3 (Methyl rosmarinate)
3, R = CH_2CH_3 (Ethyl rosmarinate)
4, R = $CH_2CH_2CH_3$
5, R = $CH(CH_3)_2$
6, R = $CH_2CH_2CH_2CH_3$
7, R = $CH_2CH(CH_3)_2$
8, R = $CH_2CH_2CH(CH_3)_2$
9, R = $CH(CH_2CH_3)_2$
10, R = $CH_2C_6H_5$
11, R = CH_2CH_2OH

Fig. 1. Chemical structures of the test rosmarinic acid and analogs.

with a Finnigan LC-Q mass spectrometer) (see Supplementary data). The presence of the alkoxyl groups from the starting alcohols was evident from the NMR spectra of the esters. The purity of the compounds was approximately 95%. These compounds were dissolved with dimethyl sulfoxide (DMSO; ≥ 99.5%, Sigma, France) and further diluted in culture medium before bioassay.

2.2. Cell culture and conditions

Murine alveolar macrophage cell line MH-S was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute medium-1640 (RPMI-1640; HyClone, Utah, USA) supplemented with 10% fetal bovine serum (Gibco, South America), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; HyClone, Utah, USA), 2 mM L-glutamine (Gibco, Brazil) and 100 U/ml and penicillin and 100 µg/ml streptomycin (Gibco, USA) in a 5% CO_2 atmosphere at 37 °C.

2.3. Cell viability assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). MH-S cells (1×10^5 cells/well) were plated in a 96-well plate (Nunc™, Roskilde, Denmark) and incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were then exposed to test compounds (final concentration 25 µM) for 24 h. Cells without the test compounds and cells treated with dimethyl sulfoxide (DMSO) served as untreated and vehicle controls, respectively. After the incubation, culture supernatant was discarded and 20 µl MTT solution (5 mg/ml; Sigma, St. Louis, MO, USA) was added. Cells were incubated for another 3 h and the supernatant was removed, subsequently 100 µl of DMSO was added to solubilize the formazan. Absorbance of solubilized formazan solution was measured at 540 nm using a microplate reader (Rayto RT-2100C microplate reader, China). Percentage of viable viability was calculated according to the equation: (absorbance of treated cells/ absorbance of untreated cells) × 100.

2.4. Nitrite and PGE_2 measurements

MH-S cells (1×10^5 cells/well) were plated in a 96-well plate (Nunc™) and incubated for 1 h at 37 °C in an 5% CO_2 atmosphere.