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Synthesis and biological activity of new phthalimides as potential anti-inflammatory agents

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ABSTRACT

The overproduction of nitric oxide (NO) plays an important role in a variety of pathophysiological processes, including inflammation. Therefore, the suppression of NO production is a promising target in the design of anti-inflammatory agents. In the present study, a series of phthalimide analogs was synthesized, and their anti-inflammatory activities were evaluated using lipopolysaccharide (LPS)-stimulated NO production in cultured murine macrophage RAW264.7 cells. A structure-activity relationship study showed that the free hydroxyl group at C-4 and C-6 and the bulkiness of the *N*-substituted alkyl chain are associated with biological activity. Among the series of phthalimide derivatives, compound **IIh** exhibited potent inhibitory activity, with an IC_{50} value of 8.7 μg/mL. Further study revealed that the inhibitory activity of compound **IIh** was correlated with the down-regulation of the mRNA and protein expression of LPS-stimulated inducible nitric oxide synthase (iNOS). Compound **IIh** also suppressed the induction of the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1β in LPS-stimulated RAW 264.7 cells. The anti-inflammatory activity of compound **IIh** was also found to be associated with the suppression of the Toll-like receptor (TLR)4 signaling pathway by down-regulating the activation of interferon regulatory factor 3 (IRF-3) and interferon-β and signal transducer expression. These findings demonstrate that novel phthalimides might be potential candidates for the development of anti-inflammatory agents.

1. Introduction

Inflammation is a complex biological response of the vascular tissue to harmful stimuli, such as pathogens, damaged cells, or irritants.[1] Consequently, the initiation and progression of many diseases, such as cardiovascular disease, cancer, obesity and insulin resistance, are highly associated with inflammatory responses.[2,3] Macrophages are one of the most dominant and widely distributed inflammatory cells and are involved in the initiation and maintenance of the acute inflammatory response.[4] Lipopolysaccharide (LPS), an endotoxin localized in the outer membrane of Gram-negative bacteria, can elicit significant immune and inflammatory responses in macrophages.[5] In the process of LPS-mediated inflammation, Toll-like receptors (TLRs) are key components. In particular, TLR4 is activated by the interaction with LPS, and activated TLR4 leads to the expression of several pro-inflammatory cytokines and mediators of inflammation, such as nitric oxide (NO).[6,7] LPS/TLR4 signaling has been divided into two major downstream pathways: the myeloid differentiation factor 88 (MyD88)-dependent pathway and the MyD88-independent pathway. The activation of the MyD88-dependent pathway contributes to the early phase of NF-κB activation and pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the late phase of NF-κB activation and the induction of interferon (IFN)-inducible genes.[8]

A variety of classes of compounds from natural or synthetic sources have been shown to be potential inhibitors of LPS-stimulated NO production in macrophages.[1–3] Phthalimides have been reported to have antifungal, anticonvulsant, analgesic, hypolipidemic, antitumor and anti-inflammatory activities.[4,5] In the present study, we synthesized a series of novel phthalimide derivatives and evaluated the biological activity of LPS-induced NO production. We also further explored the underlying molecular mechanism responsible for the anti-inflammatory activity using an active phthalimide analog.

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2. Results and discussion

2.1. Chemistry

The synthesis of a series of novel phthalimide derivatives is outlined in Scheme 1.

Intermediate **2** was prepared using 3,5-dimethoxybenzoic acid and glyoxylic acid. In this procedure, sulfuric acid was diluted with ten times amount of acetic acid to avoid the carbonization of product induced by concentrated sulfuric acid, and **2** was yielded as 64.2%. Decarboxylation was then performed in dimethyl phthalate (DMP) at 180–185 °C to afford **3** with the yield of 95.7%. **3** was oxidated to be substituted phthalic acid (**4**, 93.6%) and then dehydrated to afford anhydride **5** (90.2%). The intermediate **6** was prepared by ammonolysis and condensation of **5** and urea.

Target compounds **Ia–Im** were synthesized using compound **5** and different halohydrocarbons with yields ranging from 62% to 80%. **IIa–IIm** were synthesized by demethylation with **Ia–Im**, respectively, with yields from 60% to 70%.

Compound **5** was reacted with ethanolamine at 185 °C to afford the intermediate **7** (52.2%) and target compounds **IIIa–IIIc** were synthesized using different acyl chloride as acylation reagent to yield from 60% to 73%.

The data of NMR spectroscopy and ESI-MS spectra analysis of the target compounds (Supplementary data) reflected the assigned structures of the compounds.

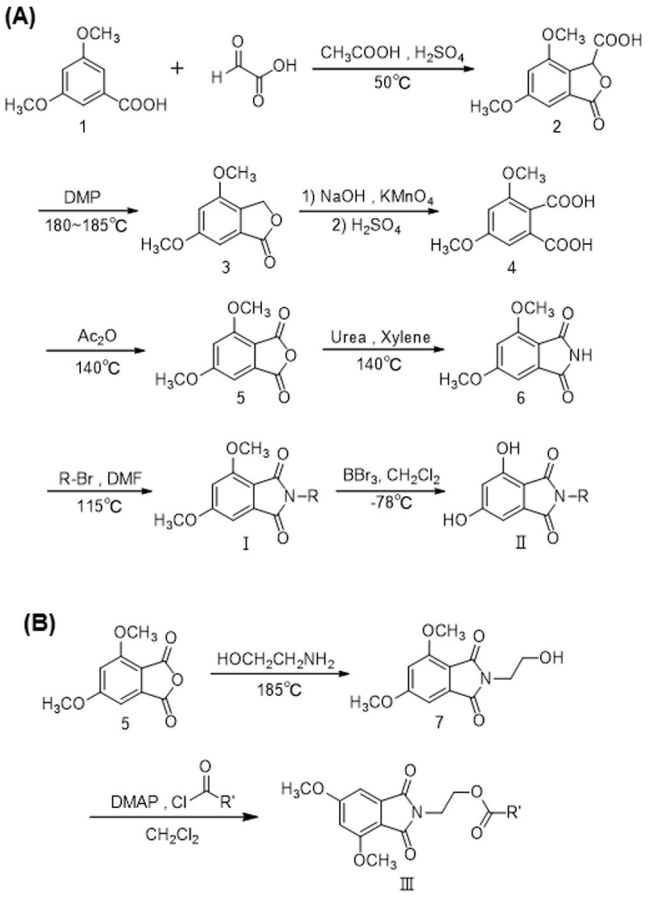
2.2. Inhibitory activity of NO production by phthalimide derivatives and structure-activity relationship

The overproduction of NO by inducible nitric oxide synthase (iNOS) is significant in inflammatory processes. Therefore, high levels of NO are considered to be a biomarker for inflammatory disorders and a useful target for the procurement of anti-inflammatory agents. The synthetic phthalimide analogs were initially evaluated for inhibitory effects on the production of NO in LPS-stimulated RAW264.7 macrophage cells. When the cells were treated with LPS (1 μg/mL) for 22 h, the NO production was markedly stimulated from the basal level of 0.46 ± 0.2 to 25.3 ± 0.3 μM. The positive control, AMT, showed 98.0% inhibition at the test concentration of 50 μM under the same experimental conditions. The inhibitory activity of the synthetic phthalimide analogs (**Ia–Im**, **IIa–IIm**, **IIIa–IIIc**) is summarized in Table 1.

Among the tested compounds, 12 phthalimides inhibited the LPS-stimulated NO production by at least 50% at the test concentration of 20 μg/mL. Regarding the *N*-alkyl chain, the biological activity varied depending on the length of the carbon chain and the substitution patterns. Activity decreased when the number of carbon atoms exceeded 8 in the *N*-alkyl chain. The introduction of halides or ester groups into the alkyl chain also decreased the activity. The substitution of a methoxy group in the R′and R″ positions decreased the activity compared to the free hydroxyl group in the position. The C6- to C7-alkyl chains maintained the biological activity without any substitutions in the alkyl chain and the R′and R″groups. Among the tested compounds, the C7-alkyl chain with a free hydroxyl group in the R′and R″, compound **IIh**, was the most active in the inhibition of NO production. Therefore, the inhibitory activity of NO production by phthalimides is dependent on the size of the carbon chain, the spatial geometry of the *N*-alkyl chain and the substitutions at the aromatic R′ and R″ positions.

2.3. Mechanism of action studies with compound IIh

Based on the SAR study of phthalimide analogs, compound **IIh** (Fig. 1), which showed the highest activity (Table 1), was selected for further detailed mechanism of action studies in RAW264.7 cells.



Scheme 1. The synthesis of *N*-substituted phthalimide analogs. (**A**) Procedure for the synthesis of type I and II *N*-substituted phthalimide analogs. (**B**) Procedure for the synthesis of type III *N*-substituted phthalimide analogs.

Compound **IIh** exhibited concentration-dependent inhibition of LPS-induced NO production in RAW264.7 cells, with an IC_{50} value of 8.7 μg/mL (Fig. 2A).

Employing MTT assay the cytotoxic effect of compound **IIh** was also analyzed at the test concentrations of up to 40 μg/mL. The compound did not show any significant cell viability at the concentrations of up to 20 μg/mL (>95% survival), but the compound **IIh** at the test concentration of 40 μg/mL affected to the cell viability (approximately 17% survival). Therefore, further study for the mechanism of action in the anti-inflammatory activity with the compound **IIh** was observed at the test concentrations of up to 20 μg/mL. Thereby, the inhibition of NO production by compound **IIh** was not attributable to cytotoxic effects at the test concentrations. To further elucidate the possible mechanisms of active phthalimides, the suppression of NO production by compound **IIh** was investigated in relation to the levels of iNOS protein and mRNA expression in the LPS-stimulated RAW 264.7 cells. The RAW264.7 cells were incubated with LPS (1 μg/mL) in the presence or absence of various concentrations of compound **IIh**. After 4 h and 12 h of treatment, total RNA and protein were isolated for real-time PCR and western blot analysis, respectively. The iNOS protein level was markedly increased in LPS-induced RAW264.7 cells, but co-treatment with compound **IIh** significantly suppressed the iNOS protein level in a concentration-dependent manner (Fig. 2B). In addition, treatment with compound **IIh** significantly suppressed iNOS mRNA expression in LPS-stimulated RAW264.7 cells in a concentration-dependent manner (Fig. 2C).