Anti-Inflammatory Effects of N'-Benzyl-4-Methylbenzene-1,2-Diamine (JSH-21) Analogs on Nitric Oxide Production and Nuclear Factor-kappa B Transcriptional Activity in Lipopolysaccharide-Stimulated Macrophages RAW 264.7

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N'-Benzyl-4-methylbenzene-1,2-diamine (JSH-21) and its analogs were chemically synthesized and their anti-inflammatory potentials investigated. JSH-21 inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophages RAW 264.7 in a dose-dependent manner, with an IC₅₀ value of 9.2 μM, where pyrrolidine dithiocarbamate and parthenolide as positive controls exhibited IC₅₀ values of 29.3 and 3.6 μM, respectively. The inhibitory effect of JSH-21 on the NO production was attributable to its down-regulatory action on LPS-inducible NO synthase (iNOS), which was documented by iNOS promoter activity. In the mechanism of the anti-inflammatory action, JSH-21 exhibited inhibitory effects on LPS-induced DNA binding activity and transcriptional activity of nuclear factor-kappa B (NF-κB). Structural analogs of JSH-21 also inhibited both the LPS-induced NO production and NF-κB transcriptional activity, where diamine substitution at positions 1 and 2 of JSH-21 seems to play an important role in the anti-inflammatory activity.

Key words: N'-Benzyl-4-methylbenzene-1,2-diamine, Chemical preparation, Anti-inflammatory effect, Nitric oxide, Nuclear factor-kappa B, Lipopolysaccharide

INTRODUCTION

Nitric oxide (NO) is a short-lived free radical produced from L-arginine by a NO synthase (NOS) catalyzed reaction in living systems (Palmer et al., 1988). NO plays an important role in the regulation of many physiological functions, such as vasodilation, neurotransmission, neurotoxicity, wound repair, and inflammation (Liaudet et al., 2000; Prast and Philippu, 2001; Ignarro, 2002). Endothelial and neuronal NOS produce moderate amounts of NO, which primarily mediate physiological responses. Meanwhile, NO is also synthesized in the immune system by inducible NOS (iNOS), where it facilitates the killing of invading microorganisms (MacMicking et al., 1997). However, high-output NO by iNOS can provoke septic shock, autoimmune disorders and inflammatory diseases (Bogdan, 2001; Blantz and Munger, 2002). Indeed, NO production in macrophages is related to the level of iNOS protein. The induction of iNOS by lipopolysaccharide (LPS) occurs through the Toll-like receptor (TLR) 4-dependent signaling cascade, resulting in nuclear factor-kappa B (NF-κB) activation (Jones et al., 2001).

NF-κB complex exist in the cytoplasm as homodimer or heterodimer in quiescent forms bound to inhibitory IκB proteins (Beg et al., 1992; Verma et al., 1995; Baueule and Baltimore, 1996). However, LPS treatment of macrophages could turn on the TLR4 signaling pathway to activate the IκB kinase (IKK) complex, resulting in phosphorylation of IκB proteins. The phosphorylated IκB proteins are then subjected to ubiquitination followed by 26S proteasome-mediated degradation. The NF-κB complex, now free of IκB, are moved to the nucleus for transcriptional regulation of pro-inflammatory cytokines, iNOS, cyclooxygenase-2 (COX-2), adhesion molecules, apoptotic and anti-apoptotic proteins (Collins et al., 1995; Tian and Brasier, 2003).
Several inhibitors of NF-κB activation have been reported to control the expression of inflammatory and immune genes, which are linked to inflammatory disorders, such as rheumatoid arthritis, asthma, septic shock, atherosclerosis and cancer (Blantz and Munger, 2002; Cavaillon et al., 2003; Moreland et al., 2004). Lactacystin, PS341 and MG132 are reported as inhibitors of proteasome-mediated IκB degradation (Dick et al., 1997), and PS341 was recently approved, as Bortezomib (Velcade; Millenium Pharmaceuticals), by the FDA for treatment of multiple myelomas (Paramore and Frantz, 2003). As an antioxidant, N-acetylcysteine is reported to inhibit NF-κB activation mainly by interfering with the upstream signals that lead to IκB phosphorylation (Zafarullah et al., 2003). The natural product, calagualine, inhibits the binding of NF-κB-inducing kinase (NIK) to TRAF2 sites and subsequently inhibits the NF-κB activation induced by TNF-α (Manna et al., 2003). Sesquiterpene lactones, such as parthenolide and ergolide, inhibit the IKK complex that phosphorylates IκB proteins (Hehner et al., 1999). Glucocorticoids, like dexamethasone, inhibit NF-κB activation by either interference of the glucocorticoid receptor-mediated DNA binding activity of NF-κB or enhanced synthesis of IκB proteins (Auphan et al., 1995).

In our ongoing study to discover anti-inflammatory agents, \( N'\)-benzyl-4-methylbenzene-1,2-diamine (JSH-21) and its analogs (Fig. 1) were found to inhibit NO production and NF-κB transcriptional activity in LPS-stimulated macrophages RAW 264.7. Chemical preparations of JSH-21 analogs, and their anti-inflammatory potentials, have been documented in this study.

### MATERIALS AND METHODS

#### Chemical preparation of \( N'\)-alkylated benzene-1,2-diamine analogs

\( N'\)-Benzyl-4-methylbenzene-1,2-diamine and its analogs have been chemically prepared, according to the reported principles (Perrin et al., 1982; Yuste et al., 1982; Brown and Rizzo, 1996).

![Chemical structures of JSH-21 and its analogs](image)

\( N'\)-Benzyl-4-methylbenzene-1,2-diamine (4a, JSH-21)

Hydrochloric acid (36%, 0.5 mL) was slowly added to a mixture of \( N'\)-benzyl-4-methyl-2-nitroaniline (3a, 2.0 g, 8.3 mmol) and Fe powder (4.6 g) in 20% ethanol (20 mL) at room temperature. The mixture was refluxed for 1 h and then filtered without cooling. The filtrate was evaporated under vacuum, and subjected to flash column chromatography to isolate the product (4a, 0.9 g), with a yield of 51.4%; Violet solid; Rf=0.3 (hexane:ethyl acetate=3:1); m.p. 111.9-112.4°C; IR (KBr) 3450, 3360, 3050, 2940, 1620 cm\(^{-1}\); \( ^1\)H-NMR (CDCl\(_3\), 400 MHz) \( \delta \) 7.41-7.13 (m, 5H), 6.38 (m, 2H), 5.87 (d, \( J=7.9 \) Hz, 1H), 4.21 (s, 2H), 3.67 (br, NH), 2.17 (s, 3H).

\( 4\)-Methyl-\( N'\)-phenethylbenzene-1,2-diamine (4b, JSH-22)

4-Methyl-\( N'\)-phenethylbenzene-1,2-diamine (JSH-22) was prepared from 4-methyl-2-nitroaniline (5.0 g, 32.9 mmol) in dry toluene (100 mL) with a solution of NaH (2.6 g, 65.8 mmol, 60% in oil) for 5 min at room temperature. 2-(Bromoethyl) benzene (9.1 g, 49.35 mmol) was slowly added, and the resulting mixture heated at 70°C for 30 h. After cooling, the mixture...