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Anti-Inflammatory Effect of 3-Methylcarbazoles on RAW 264.7 Cells Stimulated with LPS, Polyinosinic-Polycytidylic Acid and Pam3CSK

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ABSTRACT

In the present study, 3-methylcarbazole and 1-methoxy-3-methylcarbazole were isolated from the culture of *Streptomyces* sp. LJK109, endophyte of *Alpinia galanga* Swartz. 3-methylcarbazole, a carbazole derivative, has been found to be highly potent as anti-inflammatory agent. The immunomodulatory activity of these agents in toll like receptor (TLR)-activated RAW 264.7 macrophages induced by lipopolysaccharide (LPS), Poly(I:C), and pam3CSK was investigated by assessing nitric oxide (NO) and pro-inflammatory cytokines. The 3-methylcarbazoles dose-dependently suppressed the release of NO, PGE_2, TNF-α, IL-1β, IL-6 and IL-10 in LPS- and pam3CSK-activated macrophages but not in Poly(I:C)-activated macrophages. Our results suggest that 3-methylcarbazoles can be further developed as a promising anti-inflammatory remedy.

Keywords: 3-Methylcarbazoles; Anti-Inflammatory Activity; RAW 264.7 Cells; *Streptomyces* sp.

1. Introduction

Some of actinomycete could be isolated from the tissue of healthy plants which was called endophytic actinomycetes [1,2]. Several reports refer to endophytic actinomycetes produced bioactive compounds [3-7]. Recently, we isolated an endophytic actinomycete from the root tissues of *Alpinia galanga* Swartz (Zingiberaceae) which has antifungal activity and it was identified as *Streptomyces* sp. LJK109. Extraction of the culture medium of this strain afforded 3-methylcarbazoles as a major active ingredient, which displayed very strong antifungal activity. Because of 3-methylcarbazoles were a derivative of 6-chloro-a-methylcarbazole-2-acetic acid (carprofen, Imadyl), which was used as a nonsteroid anti-inflammatory agent [8,9]. Thus, the anti-inflammatory effects of 3-methylcarbazoles on macrophages and its inhibitory mechanisms remain to be elucidated. We investigated the immunomodulatory activity of 3-methylcarbazoles on activated macrophages induced by toll like receptor (TLR) ligands such as LPS (a TLR4 ligand), polyinosinic-polycytidylic acid (Poly(I:C)) (a TLR3 ligand), and *N*-palmitoyl-*S*-[2,3-bis(palmitoloxy)-(2*RS*)-propyl]-Cys-Ser-Lys_4 (pam3CSK) (a TLR2 ligand); in addition to nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-10 assays were utilized.

2. Materials and Methods

2.1. Organisms and Compounds

Streptomyces sp. LJK109 was isolated from the root tissues of *Alpinia galanga* by the surface sterilization technique [10]. Identification of the isolate to genus level was based on morphological, cultural, physiological, and biochemical characterizatics and also 16S rDNA sequencing as described by Taechowisan and Lumyong [11]. Solid medium for sporulation and bioactive compounds production was International *Streptomyces* Project medium 4 (ISP-4) and ISP-2, respectively. The 10-day-old cultures were extracted three times with ethyl acetate. This organic solvent was pooled and then taken to dryness under flash evaporation to give a dark brown solid (340.5 mg). The solid was separated by column chromatography using silica gel 60 (Merck, 0.040 - 0.063 mm) and CH_2Cl_2/MeOH (22:3) as the eluent to give 3-methylcarbazole (**1**) as a colorless prisms (102.6 mg); m.p. 206˚C -207˚C (from acetone); have the molecular formulae $C_{13}H_{11}$ N (M+, *m*/*z* 181.233) and 1-methoxy-3-methylcarbazole (**2**) as a brown needles (85.4 mg); m.p. 188˚C - 190˚C (from benzene); have the molecular for-

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mulae $C_{14}H_{13}$ NO (M+, *m/z* 211.259). Their ^{1}H- and ^{13}C-NMR spectral data were identical with those of 3-methylcarbazole and 1-methoxy-3-methylcarbazole previously reported [12-14].

2.2. Structure Elucidation of the Compounds

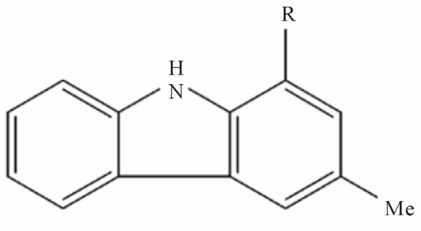
The structures of the active compounds (**Figure 1**) have been identified using NMR and mass spectral data. The melting point of the compounds was determined on a Buchi-540 melting point apparatus. Optical rotation were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, 1H and 13C NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and FAB-MS, respectively, on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

2.3. Cell Culture and Sample Treatment

RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37˚C in DMEM medium supplement with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. For each experiment, cells were detached with a cell scraper. Experiments were performed at a cell density of 2×10^6 cells/ml; at this density, more than 99% of cells were viable according to Trypan blue staining. The stock solution (100 mg/ml) of 3-methylcarbazoles was dissolved in 100% DMSO. Non-cytotoxic concentrations (0 - 20 μg/ml) of 3-methylcarbazoles were prepared by dilution with DMEM medium. After RAW 264.7 cells were incubated for 18 h, cells were pretreated with 3-methylcarbazoles (0 - 20 μg/ml) for 30 min. Next, cells were stimulated with LPS (1 μg/ml), Poly(I:C) (1 μg/ml), and pam3CSK (10 μg/ml), and incubated for 24 h.

2.4. Nitrite Assay

Nitrite accumulation, an indicator of NO synthesis, was measure in the culture medium by Griess reaction, as described previously [7].



1 R = H
2 R = OMe

Figure 1. Chemical structures of 3-methylcarbazole (1), and 1-methoxy-3-methylcarbazole (2).

2.5. PGE_2, TNF-*α*, IL-1*β*, IL-6 and IL-10 Assay

The inhibitory effect of methylcarbazoles on PGE_2, TNF-*α*, IL-1*β*, IL-6 and IL-10 production was deter- mined by analyzing PGE_2, TNF-*α*, IL-1*β*, IL-6 and IL-10 levels with enzyme immunoassay (EIA) kits (Stressgen, USA) according to the manufacturer's instructions.

2.6. MTT Assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [7].

3. Results and Discussion

Since the first report of a carbazole alkaloid, murrayanine, from the stem bark of *Murraya koenigii* [15], a number of carbazole alkaloids have been isolated from this plant [16-20] and other plants [13,21]. Although there are many carbazole alkaloid derivatives that have special ability to scavenge reactive oxygen species-free radicals, such as hydroxyl radicals, superoxide radicals, or hypochlorous acid, and to influence processes involving free-radical injury [22,23], they have also been found to inhibit lipid peroxidation and to possess vasorelaxant [24-26] and anti-inflammatory/antioxidant activity [20]. Moreover they have been reported for their other pharmacological activities such as anticancer [27], antidiarrhoeal [28], antidiabetic [29], antiasthmatic [30], antiplasmodial [31], antibacterial, antifungal and anthelmintic activities [32].

Previous reports indicated that 3-methylcarbazole was produced by numerous species of plants including *Murraya euchrestifolia* [13], *Murraya koenigii* [19], *Clausena dunniana* [33], *Micromelum hirsutum* [34]. This compound has many biological activities for example: cytotoxicity against both mouse melanoma B16 and adriamycin-resistant P388 mouse leukemia cell lines [19], and growth inhibitory activity on human fibrosarcoma HT-1080 cells [33].

In our study, 3-methylcarbazoles was obtained from culture of an endophytic *Streptomyces* sp. LJK109, isolated from the root tissues of *Alpinia galanga*. It was the major anti-inflammatory component, so we selected 3-methylcarbazoles with a potent NO inhibitory action from the crude extract.

It is well-known that macrophages play a crucial role in both nonspecific and acquired immune responses. We investigated the anti-inflammatory potency of 3-methylcarbazoles using TLR-activated macrophages. As depicted in **Figure 2**, 3-methylcarbazoles suppressed macrophage production of the inflammatory mediators NO, PGE_2, TNF-*α*, IL-1*β*, IL-6 and IL-10 in a dose-dependent manner. The 3-methylcarbazoles mediated suppression regard to the TLR ligand used, the LPS (a TLR4 ligand)

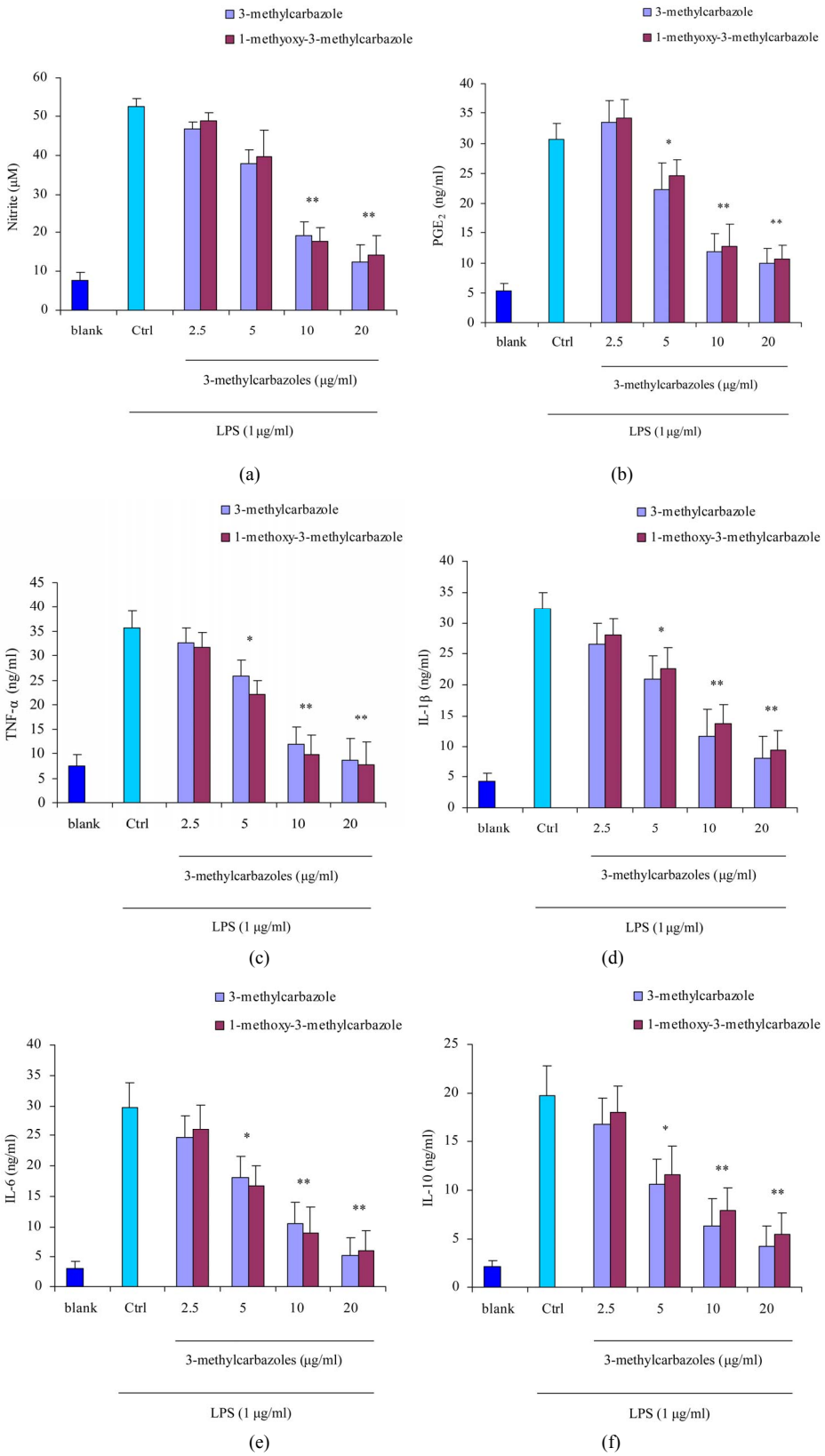


Figure 2. Effect of 3-methylcarbazoles on inflammatory responses *in vitro*. (a)-(f) NO, PGE_2, TNF-*α*, IL-1*β*, IL-6 and IL-10 levels in culture supernatants prepared from LPS-activated RAW264.7 cells pretreated with 3-methylcarbazoles, were determined by Griess reagent and EIA. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the control group.

and pam3CSK (a TLR2 ligand) had more potential effect than poly(I:C) (a TLR3 ligand) (**Figure 3**). The reasons may be, TLR3 localized to intracellular vesicles, its activation required to the cell membrance and lipidraft-mediated endocytosis [35] where as TLR4 and TLR2 were located on the cell surface [36]. No cytotoxic activity of 3-methylcarbazoles was observed under the same conditions (**Figure 4**), indicating that the immunopharmacological effect of 3-methylcarbazoles was not related to cytotoxicity. As found with other carbazoles such as 9-(2-chlorobenyl)-9H-carbazole-3-carbaldehyde suppressed the NO production in LPS/interferon-γ-stimulated murine microglial cells [37] and LPS-stimulated murine RAW264.7 cells [38]. These results imply that the inhibitory activity of 3-methylcarbazoles is derived from its ability to block TLR-mediated inflammatory responses. Further investigations are therefore necessary to understand the molecular mechanisms at a transcriptional level and transcription factors that regulate inflammatory gene expression.

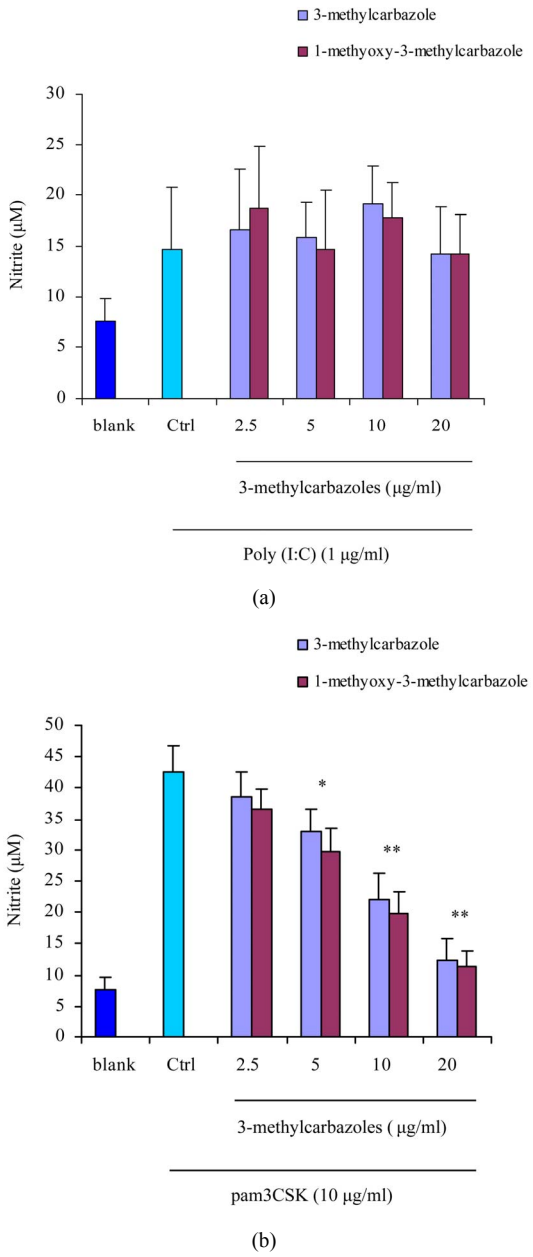


Figure 3. NO production in culture supernatants prepared from Poly(I:C)-, and pam3CSK-activated RAW264.7 cells pretreated with 3-methylcarbazoles was determined by Griess reagent. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the control group.

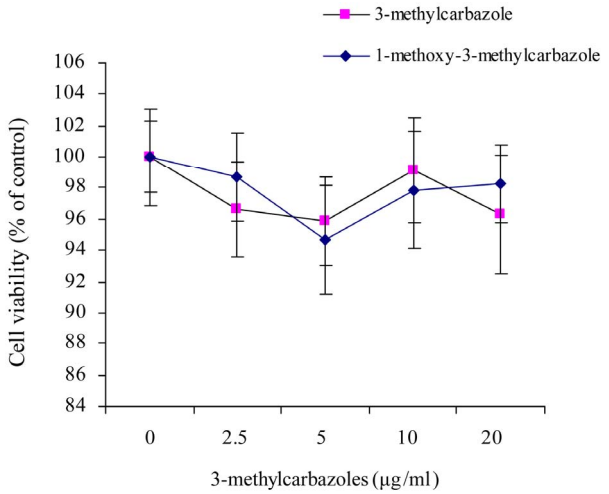


Figure 4. The viability of RAW264.7 cells pretreated with 3-methylcarbazoles was determined by MTT assay.

In conclusion, we found that 3-methylcarbazoles isolated from *Streptomyces* sp. LJK109 was able to suppress NO, PGE_2, TNF-α, IL-1β, IL-6 and IL-10 production in LPS-, and pam3CSK-treated RAW264.7 macrophages. Verification of therapeutic efficacy of 3-methylcarbazoles as a potent anti-inflammatory remedy will be further studied.

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