

Effects of 1,7-Substituted Methylxanthine Derivatives on LPS-Stimulated Expression of Cytokines and Chemokines in Raw 264.7 and HK-2 Cells

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Received: October 20, 2014
Revised: November 26, 2014
Accepted: November 30, 2014

First published online
December 12, 2014

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pISSN 1017-7825, eISSN 1738-8872

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Chronic kidney diseases are based on uncontrolled immunological and inflammatory responses to pathophysiological renal circumstances such as glomerulonephritis, which is caused by immunological mechanisms of glomerular inflammation with increased production of renal pro-inflammatory cytokines. Pentoxifylline (PTX) exhibits anti-inflammatory properties by inhibiting cytokine and chemokine production through aggregation of erythrocytes and thrombocytes. We synthesized a series of 1,7-substituted methylxanthine derivatives by the Traube purine reaction, and the formation of purine ring was completed through nitrosation, a reduction of the nitroso to the amine by catalytic hydrogenation as derivatives of PTX. Then we studied biological activities such as renal anti-inflammatory effects of the synthesized compounds in the production of cytokines such as nitric oxide (NO), interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α) and of chemokines such as monocyte chemoattractant protein-1 and IL-8 in Raw 264.7 and HK-2 cells. Renal anti-inflammatory activities of this novel series of N-1 and N-7-substituted methylxanthine showed that the N-7 methyl-group-substituted analogs (S7b) showed selective 61% and 77% inhibition of the production of NO and IL-8. The other replacement of the N-1-(CH₂)₄COCH₃ group, as in the case of compound S6c, also showed an effective 50% and 77% inhibition of TNF- α and IL-8 production in LPS-stimulated Raw 264.7 and HK-2 cells.

Keywords: Anti-inflammatory effect, pentoxifylline, pro-inflammatory cytokine

Introduction

Chronic kidney diseases are the slow progressive loss of kidney function and irreversible destruction of the kidney. There are often no noticeable symptoms, making early detection difficult until severe permanent kidney failure. The progression of such diseases becomes a large burden on the social economy [14]. Chronic kidney diseases (CKD) are caused by glomerulonephritis, which is known to be caused by immunological mechanisms and various cytokines.

Cytokines that are involved in the emergence and progression of glomerulonephritis become active in the early stages of acute kidney diseases (AKDs) within epithelial and endothelial cells. High concentrations of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), IL-6, and tumor necrosis factor- α (TNF- α) as well as chemokines such as monocyte chemoattractant protein-1 (MCP-1) and IL-8

have been reported to correlate with the development of renal dysfunction [16, 17].

In particular, TNF- α plays a role in glomerular inflammation and fibrosis. It stimulates the release of IL-1 β and MCP-1, and the blockade of TNF- α with a monoclonal antibody suppresses inflammation and renal experimental glomerulonephritis [12, 13]. Chemokines are expressed in monocyte renal tubule cells, vascular endothelial cells, and activate white blood cells, and induce the infiltration of inflammatory cells and lesions in the kidney [6, 7].

Pentoxifylline (PTX; 1-(5-oxohexyl)-3,7-dimethylxanthine), a methylxanthine derivative, is a hemorheological drug used in the treatment of peripheral vascular disease [5, 15]. PTX, a competitive nonselective phosphodiesterase inhibitor, has anti-inflammatory effects by inhibiting inflammation mediators such as nitric oxide (NO) and pro-inflammatory cytokines [10, 19], and acts primarily by increasing erythrocyte

flexibility, reducing blood viscosity [1], and decreasing the potential for platelet aggregation [15].

In nephrotoxic animal models induced by gentamicin and amikacin, PTX showed antioxidative and renoprotective effects [11, 18]. Wang *et al.* [19] and Zhou *et al.* [20] also reported its kidney protective effects by inhibiting the appearance of TNF- α , IL-1 β , and NO and inhibiting tubulointerstitial fibrosis in animal models. In some clinical research, PTX has been reported to have anti-inflammatory effects by reducing the production of TNF- α and MCP-1 [8, 9].

However, as PTX is reported to have side effects in the gastrointestinal tract and the central nervous system and to interact with other medicines, we aim to develop a new candidate drug that has the anti-inflammatory effects and kidney disease-treating qualities of PTX without its side effects.

In this study, we investigated the renal anti-inflammatory effects of 1,7-substituted methylxanthine derivatives of PTX. We tested the production of cytokines such as NO, IL-1 β , and TNF- α in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells with the synthesized compounds. We also measured the expression of chemokines such as monocyte MCP-1 and IL-8 in HK-2 cells stimulated by LPS to identify the anti-inflammatory effects in the kidney.

Materials and Methods

Compounds

A series of N-1 and N-7-substituted methylxanthine derivatives was synthesized. The formation of the purine ring by the Traube purine reaction method was completed through nitrosation, reduction of the nitroso to the amine by catalytic hydrogenation. Structures of the synthesized compounds were established on the basis of IR, ¹H-NMR, ¹³C-NMR, and GC-mass/mass spectrometry.

Cell Culture

The murine macrophage cell line Raw 264.7 cells and the human epithelial renal proximal tubule cell line HK-2 cells were obtained from the Korea cell line bank. Raw 264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Invitrogen, UK) containing 10% fetal bovine serum (FBS; Gibco Invitrogen, UK), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. HK-2 cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco Invitrogen, UK) containing 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified 5% CO₂ incubator.

Cytotoxicity Assay

The cytotoxicity of 1,7-substituted methylxanthine derivatives to the culture cells was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA) assay. Raw 264.7

cells were seeded on 96-well plates at a density of 1×10^4 cells/well with 1,7-substituted methylxanthine derivatives and incubated for 24 h. The cells with medium alone were used as the untreated control. The incubation medium was removed and 100 μ l of MTT solution was added to each well. After incubation for 4 h at 37°C, MTT solution was removed and 100 μ l of dimethyl-sulfoxide (DMSO) was added to each well. Viable cells were then detected by measuring the absorbance at 580 nm.

Nitric Oxide Assay

The effect of 1,7-substituted methylxanthine derivatives on nitric oxide production was evaluated using the Griess Reagent System. Raw 264.7 cells were seeded on 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C in a humidified 5% CO₂ incubator. After being incubated for 24 h, the media were replaced with fresh media containing 1 μ g/ml of LPS (Sigma, USA) and the cells were treated with 1,7-substituted methylxanthine derivatives for 24 h. The supernatant of each sample was mixed with Griess reagent at room temperature for 10 min. The absorbance was measured at 540 nm. Standard calibration curves were prepared using sodium nitrite as the standard.

Measurement of Cytokines

To measure IL-1 β and TNF- α production, Raw 264.7 cells were treated with LPS (1 μ g/ml) with or without 1,7-substituted methylxanthine derivatives, as described above, and 100 μ l of the supernatant was transferred into 96-well plates to determine the level of IL-1 β and TNF- α . IL-1 β and TNF- α production was determined using commercial enzyme-linked immunosorbent assay kits (ELISA kits; BD Biosciences, USA) according to the manufacturer's instructions.

Measurement of Chemokines

To measure MCP-1 and IL-8 production, HK-2 cells were treated with LPS (10 μ g/ml) with or without 1,7-substituted methylxanthine derivatives, as described above, and 100 μ l of the supernatant was transferred into 96-well plates to determine the level of MCP-1 and IL-8. MCP-1 and IL-8 production was determined using commercial ELISA kits (BD Biosciences, USA) according to the manufacturer's instructions.

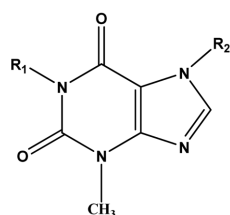
Statistical Analysis

All results are expressed as the mean \pm SD. Statistical significance was assessed at $p < 0.05$, $p < 0.001$ for all comparisons. Statistical comparison was conducted using Student's *t*-test after ANOVA.

Results

Synthesis

We have synthesized a series of 1,7-substituted methylxanthine derivatives (**S6a**, **S6c**, **S7b**, **S8b**) by Traube purine synthesis as illustrated in Table 1. Structures of the synthesized compounds were established on the basis of IR, ¹H-NMR,

Table 1. Cell viability of 1,7-substituted methylxanthine derivatives.

Compound	R ₁	R ₂	Cell viability (% of control)
S6a	(CH ₂) ₂ COCH ₃	H	93.55 ± 3.52*
S6c	(CH ₂) ₄ COCH ₃	H	95.08 ± 1.35**
S7b	(CH ₂) ₃ COCH ₃	CH ₃	96.22 ± 0.45**
S8b	(CH ₂) ₃ COCH ₃	CH ₂ CH ₃	92.90 ± 1.09**
PTX	(CH ₂) ₄ COCH ₃	CH ₃	-

Values are means of three determinations with standard deviation of $\pm 10\%$ at 1 μ M.

Significant difference, * $p < 0.05$, ** $p < 0.001$.

¹³C-NMR, and GC-mass/mass spectrometry (data not shown).

Cell Viability

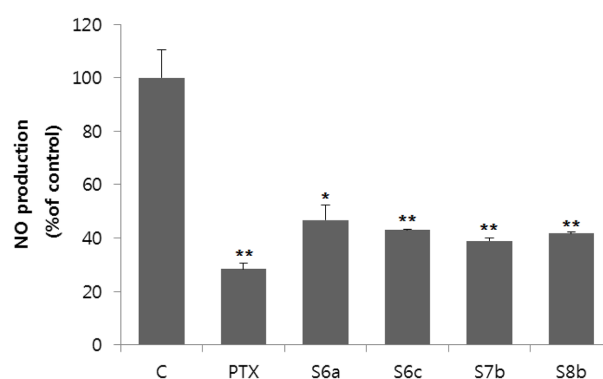
Raw 264.7 cells were treated with 1,7-substituted methylxanthine derivatives for 24 h and the cell viability was tested by MTT assay as described previously. As shown in Table 1, the 1,7-substituted methylxanthine derivative treatments resulted in more than 90% cell viability compared with untreated cells. None of the synthesized compounds exhibited cytotoxicity in Raw 264.7 cells.

Nitric Oxide Assay

Nitric oxide is an important inflammatory mediator released by activated macrophages. We investigated the inhibitory effects of PTX and 1,7-substituted methylxanthine derivatives on NO production in LPS-stimulated Raw 264.7 cells. They were treated with 1 μ g/ml of LPS with or without 1,7-substituted methylxanthine derivatives. The levels of nitric oxide were determined after 24 h. The production of NO was decreased in LPS-stimulated Raw 264.7 cells (Fig. 1). The level of NO was significantly decreased by treatment with 1,7-substituted methylxanthine derivatives. S6a, S6c, and S8b decreased NO production by more than 50% compared with untreated cells. S7b inhibited production of NO by about 61% and PTX resulted in about 71% decrease.

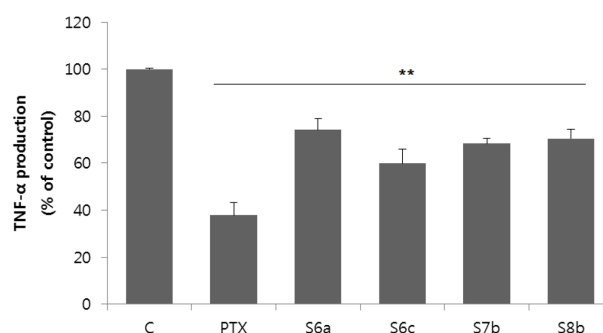
Measurement of Cytokines

To determine the effects of PTX and 1,7-substituted

**Fig. 1.** Effect of 1,7-substituted methylxanthine derivatives on the production of nitric oxide (NO) in LPS-stimulated Raw 264.7 cells.

Raw 264.7 cells were treated with 1 μ M 1,7-substituted methylxanthine derivatives. The values represent the mean \pm SD. Significant difference, * $p < 0.05$, ** $p < 0.001$.

methylxanthine derivatives on the production of the pro-inflammatory cytokines TNF- α and IL-1 β , we performed ELISAs with LPS-stimulated Raw 264.7 cells. As shown in Figs. 2 and 3, TNF- α and IL-1 β were significantly reduced in the cells treated with PTX or 1,7-substituted methylxanthine derivatives. S6a, S7b, and S8b decreased TNF- α release by about 30% compared with controls. The TNF- α production with S6c was 59.89 \pm 6.17% of untreated cells. The maximum inhibitory effect (62.23 \pm 5.36%) on TNF- α release was observed in PTX-treated cells. LPS-induced IL-1 β production was also inhibited by 21% (S7b) and 23% (S6a, S6c, and S8b, equally) compared with untreated cells. PTX decreased IL-1 β release by about 23% compared with results in untreated cells. All of the 1,7-substituted methylxanthine derivatives gave similar results to PTX in terms of IL-1 β production.

**Fig. 2.** Effect of 1,7-substituted methylxanthine derivatives on the production of TNF- α in LPS-stimulated Raw 264.7 cells.

Raw 264.7 cells were treated with 1 μ M 1,7-substituted methylxanthine derivatives. The values represent the mean \pm SD. Significant difference, ** $p < 0.001$.

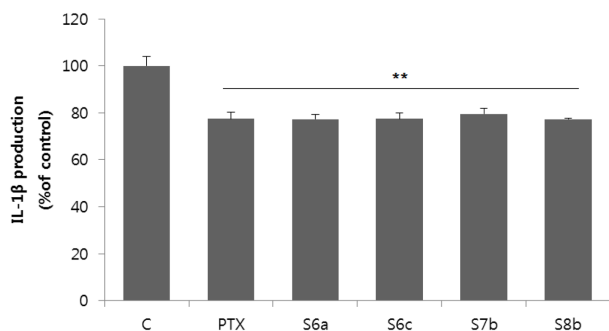


Fig. 3. Effect of 1,7-substituted methylxanthine derivatives on the production of IL-1 β in LPS-stimulated Raw 264.7 cells.

Raw 264.7 cells were treated with 1 μ M 1,7-substituted methylxanthine derivatives. The values represent the mean \pm SD. Significant difference, ** $p < 0.001$.

Measurement of Chemokines

PTX is known as an inhibitor of IL-8 and MCP-1. LPS induces inflammation by activating cytokine and chemokine responses in HK-2 cells. We measured the levels of MCP-1 and IL-8 in HK-2 cells treated with 10 μ g/ml of LPS. 1,7-Substituted methylxanthine derivatives significantly decreased the production of MCP-1 and IL-8 compared with untreated LPS (10 μ g/ml)-stimulated HK-2 cells (Figs. 4 and 5). MCP-1 production was inhibited by 55% to 69% compared with untreated cells. The most potent inhibitor ($68.07 \pm 2.73\%$) of MCP-1 production was S6a among the synthesized compounds. S6c inhibited production of MCP-1 by about 60%. S7b and S8b decreased MCP-1 by 57% and 55% compared with untreated cells. The MCP-1 production of PTX was $3.45 \pm 0.79\%$ of untreated cells. The levels of IL-8 were also reduced by PTX and 1,7-substituted methylxanthine

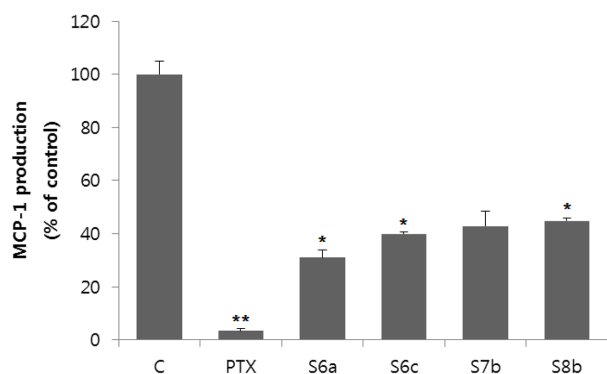


Fig. 4. Effect of 1,7-substituted methylxanthine derivatives on the production of MCP-1 in LPS-stimulated HK-2 cells.

HK-2 cells were treated with 1 μ M 1,7-substituted methylxanthine derivatives. The values represent the mean \pm SD. Significant difference, * $p < 0.05$, ** $p < 0.001$.

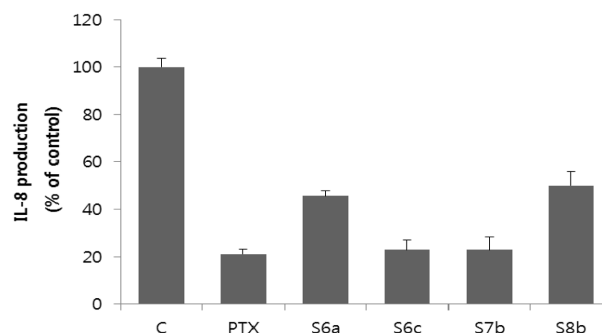


Fig. 5. Effect of 1,7-substituted methylxanthine derivatives on the production of IL-8 in LPS-stimulated HK-2 cells.

HK-2 cells were treated with 1 μ M 1,7-substituted methylxanthine derivatives. The values represent the mean \pm SD.

derivatives.

S6c and S7b decreased IL-8 by about 77% compared with untreated cells. S6a and S8b inhibited the production of IL-8 by about 54% and 50%, respectively, in LPS (10 μ g/ml)-stimulated HK-2 cells. PTX inhibition was $21.05 \pm 2.12\%$ of untreated cells. S6c and S7b were similar to PTX in terms of IL-8 production in LPS (10 μ g/ml)-stimulated HK-2 cells.

Discussion

In the present paper, we report the synthesis and renal anti-inflammatory activity of a novel series of N-1 and N-7-substituted methylxanthine derivatives prepared by the purine ring formation process with subsequent nitrosation, reduction, and ring closure. Cell viability, production of the cytokines NO, IL-1 β , and TNF- α , and of the chemokines IL-8 and MCP-1 were evaluated in LPS-stimulated Raw 264.7 cells and immortalized human proximal tubular cells treated with the compounds. Under pathophysiological circumstances such as kidney disease with acute inflammation, the production of renal pro-inflammatory cytokines is significantly increased [6]. Pro-inflammatory cytokines such as IL-1 β and TNF- α are major mediators responsible for the expression of chemokines such as MCP-1.

We have synthesized a series of 1,7-substituted methylxanthine derivatives (S6a-c, S7a-c, S8a-c, S9a) by Traube purine synthesis, as illustrated in Table 1. Structures of the synthesized compounds were established on the basis of IR, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and LC-mass/mass spectrometry (data not shown). It is noted that the purine compounds having from two to seven carbons of alkyl chain lengths at the N-1 position was prepared. Moreover, it is conceivable that the esters provide a more cell-permeable form of the active compound, whereas the free acids, being ionized at

physiological pH, may cross cell membranes with greater difficulty [2].

The cell viability with 1,7-substituted methylxanthine derivatives was more than 90% compared with untreated cells. None of the synthesized compounds exhibited cytotoxicity in Raw 264.7 cells. The level of NO was significantly decreased by treatment with 1,7-substituted methylxanthine derivatives. S6a, S6c, and S8b decreased NO by more than 50% compared with untreated cells. S7b inhibited the production of NO by about 61%. PTX decreased NO by about 71%.

PTX exhibits anti-inflammatory properties by inhibiting cytokine and chemokine production through aggregation of erythrocytes and thrombocytes. We assessed the production of the pro-inflammatory cytokine to determine whether 1,7-substituted methylxanthine derivatives have anti-inflammatory activity similar to PTX. Reflecting their modest IL-1 β production, the anti-inflammatory activities of replaced functional groups such as N-1-(CH₂)₂COCH₃ or N-7-H were moderately decreased by about 23% compared with PTX. LPS-induced IL-1 β production was also inhibited by 21% (S7b) and 23% (S6a, S6c, and S8b, equally) compared with untreated cells. Bohm *et al.* [3] reported that the activity of methylxanthines in cell regulation may indeed reside in the N-7 substituent. Moreover, Bhat and Madyastha [4] reported the activity of the N-7 position of methylxanthine derivatives, which would affect the anti-inflammatory and antioxidant properties. Among 1,7-substituted methylxanthine derivatives, compound S6c, which differed from PTX in the N-7 substituent, was most effective (40%) on production of TNF- α . S7b, which altered the N-1 position of methylxanthine derivatives, inhibited the release of TNF- α by about 31%. However, replacement of the N-1 and N-7-substituted methylxanthine derivatives (S6a and S8b) inhibited TNF- α production by 25% and 23%, respectively. The inhibitory effect of compound S6c substantiated that the N-7 position of methylxanthine derivatives is critical for TNF- α inhibition.

Recently, several researchers reported that PTX exhibited antioxidative and renoprotective effects in nephrotoxic animal models [11, 18–20]. Our studies confirmed that MCP-1 and IL-8 production was decreased by 1,7-substituted methylxanthine derivatives compared with untreated LPS (10 μ g/ml)-stimulated HK-2 cells. Furthermore, the expression of MCP-1 was also inhibited with compound S7b (55%), S8b (57%), and S6c (60%), containing different functional groups such as N-1-(CH₂)₃COCH₃ or N-7-H. The most potent inhibitor (68%) of MCP-1 production was compound S6a among the synthesized compounds. However, PTX inhibited the MCP-1 production to 3.5% compared with

control cells. The active IL-8 production inhibitors S6c and S7b (77%) exhibited comparable anti-inflammatory potency to PTX (79%) in LPS (10 μ g/ml)-stimulated HK-2 cells, and S6a and S8b showed similar effectiveness (50–54%) compared with control cells. The results of MCP-1 and IL-8 production were similar to those of TNF- α . The replacement of the N-7-substituted methylxanthine derivatives was most effective on the production of chemokines.

The *in vitro* anti-inflammatory activities of this novel series of N-1 and N-7-substituted methylxanthine derivatives have shown that the N-7-substituted analog (S6c) is more selective on TNF- α and IL-8 production in LPS-stimulated Raw 264.7 and HK-2 cells. Furthermore, replacement of the N-1-(CH₂)₃COCH₃ group, as in compound S7b, also showed moderately more selectivity for NO, TNF- α , MCP-1, and IL-8 production than the N-1-(CH₂)₃COCH₃-substituted analog (S8b). This suggests that the N-7 position of methylxanthine derivatives would affect the anti-inflammatory response in LPS-stimulated Raw 264.7 and HK-2 cells. In conclusion, we have identified a novel series of N-1 and N-7-substituted methylxanthine derivatives, which may be developed into potent nonsteroidal anti-inflammatory drugs. In addition, we discussed the rationale for the modification of the derivative structures together with the translation of an *in vivo* animal model with the anti-inflammatory biological evaluation of 1,7-substituted methylxanthine; that is, derivatives of pentoxifylline.

Acknowledgments

This study was supported by the Duksung Women's University research grants 2012.

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