

Andrographolide modulate some toll-like receptors and cytokines expressions in HL-60 cell line

Abstract

Andrographolide, a labdane diterpenoid, is quantitatively the major bioactive secondary metabolite of *Andrographis paniculata*, which is now considered to be a promising therapeutic lead for prevention and cure of inflammatory disorders. Although involvements of Toll-like receptors (TLR) and diverse cytokines have been implicated in its modes of actions, as yet no reports on its effects on expressions of TLRs have appeared. Observations reported in this communication reveal that a non-cytotoxic concentration of andrographolide (10 μ M) completely suppresses TLR-7 and TLR-8 expressions in HL-60 cells and has no effects on TLR-3 expressions in the cell line. Observed effects of this concentration of andrographolide on the expressions of three quantified cytokines (TNF- α , IL-1 β and IL-10) in HL-60 cells were not as pronounced as its suppressing effects on the two TLR expressions. These observations indicate that modulation of TLR-7 and TLR-8 mediated inflammatory and anti-inflammatory cytokines expression are involved in the modes of actions of andrographolide.

Keywords: andrographolide, toll-like receptor expression, cytokine expression, HL-60 cell

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Abbreviations: CNS, central nervous system; DMSO, dimethyl sulfoxide; HL-60, human leukemia-60; IL, interleukin; RT-PCR, reverse transcription polymerase chain reaction; TLR, toll-like receptor; TNF, tumor necrosis factor

Introduction

Andrographis paniculata (Burm. F.) Wall. Ex Nees. is a medicinal plant of Acanthaceae family widely used in both traditionally known Chinese and Indian systems of medicine as a tonic for prevention and cure of infectious disease, inflammation, fever and common cold. Andrographolide is quantitatively the major bioactive secondary metabolite of the plant, now often considered by modern drug discoverers as a structurally and functionally novel therapeutic lead potentially useful for similar purpose.¹ Recent observations in our laboratories and elsewhere have revealed in addition that it is a modulator of adaptive immune responses with stress response regulating activities.²⁻⁴ Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system regulating the functions of all bodily organs including that of brain. They are expressed on cells of the immune system that allow for the recognition of molecular motifs of pathogenic bacteria, fungi and viruses modulating cytokine homeostasis involved in inflammatory processes.⁵ Cytokines are broad category of small proteins that are important in cell signaling. They are now classified in two broad categories viz. pro-inflammatory and anti-inflammatory ones. Some cytokines like IL-1, IL-1 β and TNF- α are pro-inflammatory, where as others such as IL-4, IL-10 and IL-13 suppressing the activity of pro-inflammatory cytokines, are called anti-inflammatory cytokines. All these cytokines have been implicated in the pathogenesis and progression of inflammatory, neurodegenerative and other disorders caused by, or associated with, dysregulation of stress-responsive neuroendocrine and neurotransmission systems.⁶ Although the

functions of TLR signaling pathways have been implicated in modulating effects of andrographolide and *Andrographis paniculata* extracts against stress triggered inflammatory and other biological responses,^{2,4} as yet no reports on its ability to modulate expressions of TLRs have appeared. Results of some *in vitro* experiments indicating that modulation of TLR-7 and TLR-8 expressions by andrographolide are involved in its anti-inflammatory and other bioactivities are summarized in this article.

Materials and Methods

Drugs and chemicals

Andrographolide (99.0% pure by HPLC) from *A. paniculata* leaf extract (Figure 1) was used. The isolation and analytical characterization details are described elsewhere.³ All other chemicals and reagents used were from commercial sources.

In vitro cell toxicity study

Human Promyelocytic leukemic cell line (HL-60, NCCS Pune, India) was maintained in 10% serum supplemented Iscove's Modified Dulbecco's Medium. A density of 5×10^4 cells/well in a 96-well culture plate was used to assess the effect of andrographolide (10, 30, 50, 100, 300 μ M dissolved in dimethyl sulfoxide; DMSO) on cell viability by MTT assay. In short, the viability of HL-60 cells were quantified in terms of mitochondrial metabolic activity using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate (water-soluble tetrazolium salt-1) based colorimetric cell proliferation assay according to the manufacturers' instructions (MTT Cell Growth Assay Kit, Millipore, USA). After 24h pre-incubations, WST-1 was added to the cells and further incubated for 4h under standard culture conditions. Absorbance was measured at 450nm using iMark™ Microplate Reader (BioRad, USA).

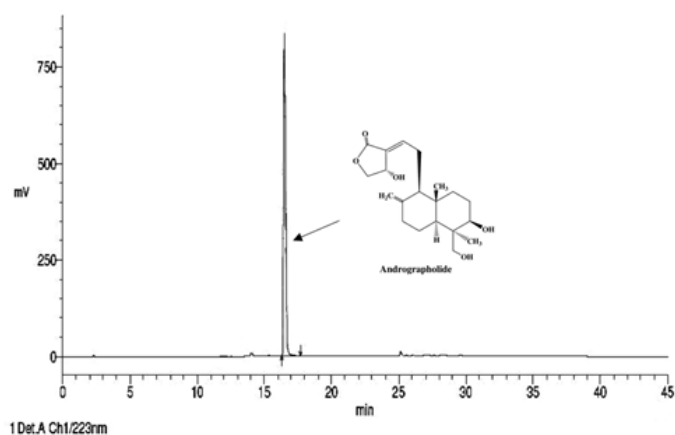


Figure 1 HPLC fingerprint and chemical structure of andrographolide.

TLRs and cytokines expressions

HL-60 cell line was used to assess the effect of andrographolide at 10 μ M concentration on expressions of toll like receptors and cytokines.

Table 1 Primers for RT-PCR

Gene	Primer sequence	Product size (bp)	Annealing temperature ($^{\circ}$ C)
TLR-3	Sense 5'-GCTAGCAGTCATCCAACAGA-3' Antisense 5'-TTGTGGGTAGATCATCGGGT-3'	177	54
TLR-7	Sense 5'-CCTTGTGCGCCGTGTA AAAA-3' Antisense 5'-GGGCACATGCTGAAGAGAGT-3'	114	55
TLR-8	Sense 5'-GCAAATAGCTCCTGCAGCC-3' Antisense 5'-TCTTCGGCGCATAACTCACA-3'	120	55
TNF- α	Sense 5'-TCAGAGGGCCTGTACCTCAT-3' Antisense 5'-GGAGGTTGACCTTGGTCTGG-3'	127	54
IL-10	Sense 5'-CGAGATGCCTTCAGCAGAGT-3' Antisense 5'-CGCCTTGATGTCTGGGTCTT-3'	189	56
IL-1 β	Sense 5'-GCTGCTCTGGGATTCTCTTCA-3' Antisense 5'-CAGGTCCTGGAAGGAGCACT-3'	172	56
β -actin	Sense 5'-CTCACCATGGATGATGATATCGC-3' Antisense 5'-AGGAATCCTTCTGACCCATGC-3'	163	55

The HL-60 cells were cultured (1×10^6 cells/well/ml of RPMI media) in a 24 well culture plates. Expressions of TLRs viz., TLR-3, TLR-7 and TLR-8 and cytokines (TNF- α , IL-1 β and IL-10) were quantified by RT-PCR method as per manufacturers' instructions (High Capacity cDNA Reverse Transcription Kit, Applied BiosystemsTM, USA). The ligand of TLRs viz. polyinosinic-polycytidylic acid (Ploy I: C; 1 μ g/ml), imiquimod (8mM), resiquimod (R848; 1 μ g/ml) were used as specific agonist to trigger the expressions of TLR-3, TLR-7 and TLR-8, respectively. These agonistic ligands were dissolved in DMSO and incubated with HL-60 cells for 24h at 37 $^{\circ}$ C, 5% CO₂ and 95% O₂, alone or along with andrographolide. Hereupon the TLRs agonist and andrographolide were added simultaneously to the cell cultures. All mRNA sequences were retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov/nucleotide>). The gene-specific primers were designed using primer 3 software (<http://fokker.wi.mit.edu/primer3>) and synthesized at Integrated DNA Technologies, Inc, USA (Table 1). The PCR was carried out in a thermal cycler (Applied Biosystems, CA, USA). The density of each band was measured using densitometry software provided with Alphamager[®] gel documentation system (Alpha-Imager, India). Amplifications were carried out in triplicate and the relative expression of cytokine mRNA genes was determined using β -actin housekeeping gene expression as an internal control.

Statistical analysis

Means±standard error of mean (SEM) was calculated for the observed values. Statistical analysis for Figure 2A was performed by one way analysis of variance (ANOVA) followed by Student-

Newman-Keuls multiple comparison test and t-test for Figure 2B. GraphPad Prism-6 software (GraphPad Software Inc., CA, USA) was used for statistical analysis. P-values less than 0.05 were always considered as statistically significant.

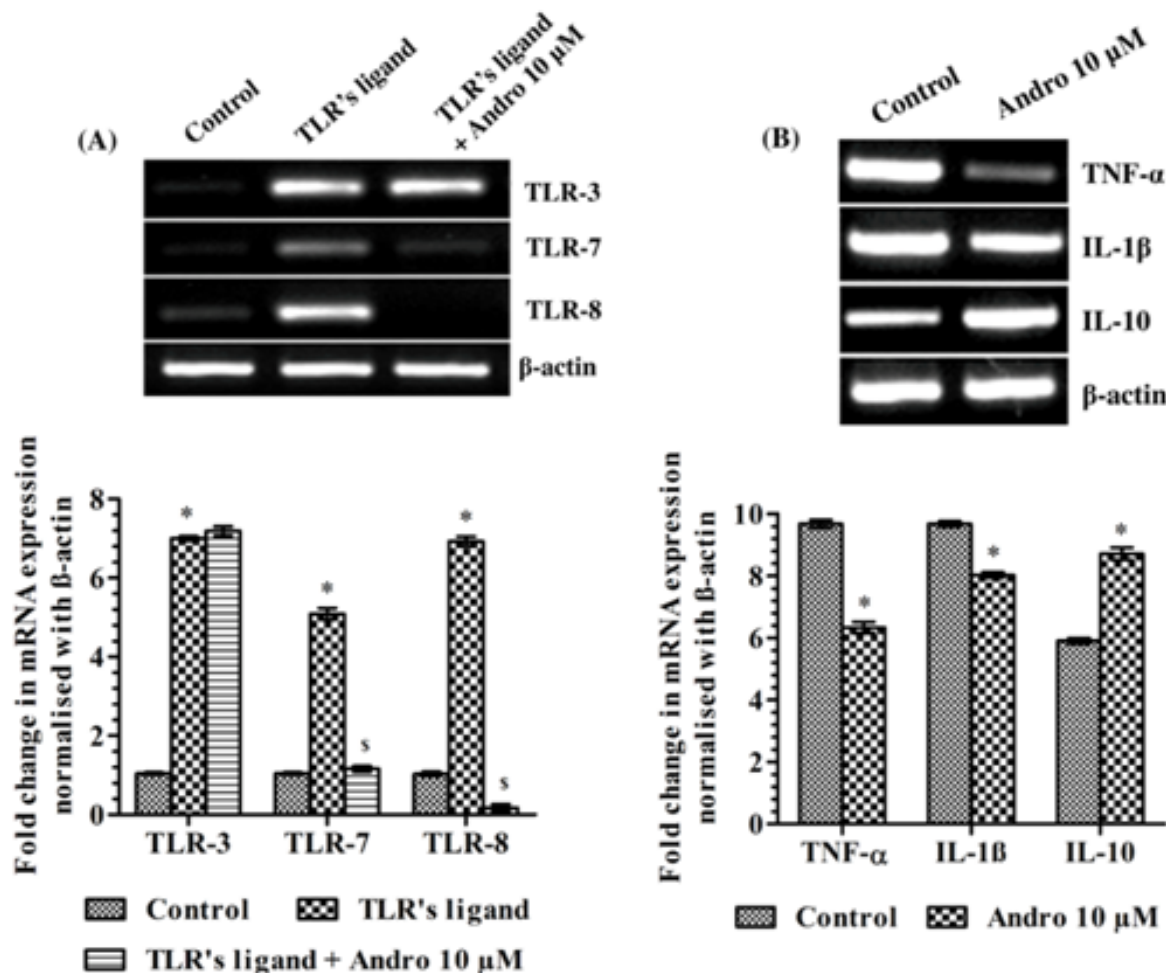


Figure 2A Effect of andrographolide on TLRs expression viz. TLR-3, TLR-7 and TLR-8.

Figure 2B Effect of andrographolide on cytokines expression viz. TNF-α, IL-1β and IL-10 in HL-60 cell-line. The gel picture showing expression of TLRs and cytokines and respective histograms showed fold change in mRNA expression normalized with β-actin housekeeping gene. *= $p < 0.05$ vs. control, \$= $p < 0.05$ vs. TLR's ligand. Andro= Andrographolide.

Results and discussion

In cell cytotoxicity experiment, 10 μM andrographolide had no effects on viability HL-60 cells and its calculated IC_{50} for 50% reduction of HL-60 cell viability was 38.62 μM. Reported IC_{50} value of andrographolide for its anti-inflammatory and other activities observed in this and other cell lines vary between 7 and 35 μM.⁷ Therefore, 10 μM andrographolide was used in further experiments conducted to estimate its effects on cytokines and TLRs expressions. An experimental study examining TLRs in the central nervous system (CNS) indicates that these receptors not only play a role in innate immunity in response to infectious diseases but may also participate in CNS autoimmunity, neuro-degeneration and tissue injury.⁸ TLR-3 recognizes double-stranded RNA (dsRNA) released during viral infections, triggering the production of type-1 interferon and inflammatory cytokines/chemokines via the Toll/IL-1R domain-

containing adaptor-inducing interferon-β (TRIF).⁹ TLR-3 activation exacerbates chronic neurodegeneration¹⁰ and triggers nigrostriatal dopaminergic degeneration.¹¹ In addition, activation of the viral innate immune receptor TLR-3 sensitizes the neonatal brain to subsequent hypoxic-ischemic damage.⁹ However, contradictory reports suggest that stimulation of the TRIF pathway may be neuroprotective by reprogramming the cerebral response to stroke.¹² TLR-7 and TLR-8 are highly homologous to each other and are important for immune responses elicited by GU-rich ssRNA as well as synthetic chemicals, including the imidazoquinoline compounds (i.e., imiquimod and resiquimod) and guanosine analogs.¹³⁻¹⁵ The latter compounds (guanosine analogs) were initially described for their ability to activate TLR-7 and TLR-8 and are potent immune response modifiers leading to the production of cytokines (i.e., IFNs) that exert important antiviral and antitumor activities.¹³ There are also evidences that TLR-7 and TLR-8 expressed in microglia^{16,17} and astrocytes.¹⁸

Representative gel pictures showing effects of this andrographolide concentration in incubation medium on mRNA expression of TLRs and cytokines quantified in HL-60 cells (normalized with β -actin as housekeeping gene) are shown in Figure 2A and Figure 2B respectively. It is apparent from the results summarized in Figure 2A that although andrographolide has no effects on TLR-3 expression in these cells, it completely suppresses the expressions of both TLR-7 and TLR-8 in them. As shown in Figure 2B, incubation of the cells with 10 μ M andrographolide alone significantly suppressed the expressions of the anti-inflammatory cytokines TNF- α and IL-1 β , whereas that of the anti-inflammatory cytokine IL-10 was significantly elevated. These results reveal that andrographolide is more effective in suppressing TLR-7 and TLR-8 expressions than suppressing the expressions of the two inflammatory cytokines studied, or for stimulating expression of the anti-inflammatory one IL-10. They could suggest that modulatory effects of andrographolide on cytokine homeostasis might as well be due to its ability to inhibit the expressions of TLR-7 and TLR-8. However, more detailed dose response studies and using different cell lines and *in vivo* or *ex vivo* experiments will be necessary to reaffirm this assertion.

It is now becoming increasingly apparent that TLR-7 and TLR-8 play important roles in the immune response to viral infection and other disorders of auto-immune system by elevating IL-6, TNF- α and IFN- β secretions^{19,20} and that their antagonistic ligands are potential antiviral agents with diverse therapeutic potentials.²¹ Observations reported in this communication are the very first ones revealing its suppressing effects on TLR-7 and TLR-8 expressions and that after its tested concentration it has no effects TLR-3 expression.

Conclusion

Since andrographolide possesses antibacterial and antiviral properties with broad spectrums of therapeutically interesting pharmacological activity profile and safety margin and beneficial effects against adverse stress responses, it seems to be another secondary plant metabolite with pleiotropic protective and curative potentially against broad spectrums of comorbidities commonly encountered in almost all chronically ill patients.

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Conflict of interest

The author declares no conflict of interest.

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