Immunostimulatory Effects of Cycloartane-Type Triterpene Glycosides from *Astragalus* Species

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In the course of our research on the oligoglycosidic constituents of Turkish *Astragalus* species, we have isolated a number of cycloartane-type triterpene glycosides. The current study examines the immunostimulatory effects of nineteen of these cycloartane-type compounds using a transcription-based bioassay for Nuclear Factor kappa B (NF-κB) activation in a human macrophage/monocyte cell line, THP-1. All compounds were inactive at 100 μg/ml except astragaloside I which increased NF-κB directed luciferase expression to levels about 65% as compared with maximal stimulation by *E. coli* lipopolysaccharide (LPS) at 10 μg/ml. None of the compounds were active at low dosage levels (0.1 μg/ml) in combination with 50 ng/ml LPS. Astragaloside I also increased mRNA expression of the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) as measured using reverse transcriptase-polymerase chain reaction (RT)-PCR. Based on these results it is clear that certain structural features are required for immunostimulation of cycloartane-type triterpene glycosides.

Key words *Astragalus*; immunostimulatory; astragaloside I; macrophage; THP-1; nuclear factor kappa B (NF-κB)

Astragalus L., the largest genus in the family Leguminosae, is represented by 380 species in the flora of Turkey.[1] The roots of *Astragalus* species represent a very old and well-known drug in traditional medicine for its usage as an antiperspirant, diuretic and tonic drug. It has also been used in the treatment of diabetes mellitus, nephritis, leukemia and uterine cancer. In the district of Anatolia, located in South Eastern Turkey, an aqueous extract of the roots of *Astragalus* is traditionally used against leukemia and for its wound-healing properties.

Known biologically active constituents of *Astragalus* roots represent two major classes of chemical compounds, polysaccharides and saponins.[2] *Astragalus* polysaccharides are known to have anticancer and immune enhancing properties in both *in vitro* and *in vivo* experiments.[3—5] Chemical studies on *Astragalus* saponins have reported the presence of cycloartane-type triterpenoid glycosides which were found to exert biological activities (*e.g.* anti-inflammatory, analgesic, diuretic, hypotensive and sedative effects).[6]

Our earlier investigations performed on *Astragalus* species resulted in the isolation of a series of cycloartane-type triterpenic saponins.[6—13] Eight of these compounds have been tested in a number of bioassays in order to evaluate the traditional properties that have been ascribed to *Astragalus*. All compounds tested showed either weak activity or no activity in the antimicrobial and cytotoxic bioassays that were used. However, these compounds appear to have immunostimulatory activity when tested at low dosage levels in human lymphocytes.[13]

The aim of the present study was to further investigate the immunostimulant effects of 19 cycloartane-type saponins on macrophage activation and expression of inflammatory cytokines. Macrophage activation was measured using a novel transcription-based bioassay for the activation of NF-κB. The inducible transcription factor NF-κB controls gene expression and regulation of many immune and inflammatory responses mediated by macrophages.[14]

MATERIALS AND METHODS

Materials Cycloartane-type triterpenoid glycosides were isolated from the following species of *Astragalus*: **1**—**3** from *A. oleifolius*[7]; **4** from *A. prusianus*[15,16]; **6**, **12**, **17**—**19** from *A. microcephalus*[8]; **8**, **10**—**11**, **14**—**16** from *A. trojanus*[10—11]; **5** and **7** from *A. cephalotes*[12]; and, **8**—**14** from *A. melanophrurius*.[13] All compounds were identified based on spectral data (IR, ^{1}H- and ^{13}C-NMR, 2D-NMR and MS).

Bacterial lipopolysaccharide, *E. coli*, serotype 026:B6 (LPS) and polymyxin B were obtained from Sigma Chemical Co. THP-1 human monocytes were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). LucLite™ luciferase reporter gene assay kit was purchased from Packard (Downers Grove, IL, U.S.A.). NF-κB plasmid construct was a gift from Riccardo Dalla-Favera that contains two copies of NF-κB motif from HIV/IgK.[17] Reverse Transcriptase (RT)-PCR kits were obtained from Promega (Madison, WI, U.S.A.) and for RNA isolation the TRI Reagent® system was used (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). RT-PCR primers for IL-1β, TNF-α and GAPDH were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.).

Macrophage Assay Macrophage activation was measured using a luciferase reporter gene (transcription factor) assay with THP-1 human monocyte cells. THP-1 cells were grown in 10% FBS, RPMI 1640 medium containing amikacin (60 mg/l) at 37 °C, 5% CO_2. In brief, cells were transiently transfected using DEAE-dextran (10 μg/1×10^6 cells) and a luciferase construct containing two binding sites for NF-κB (1 μg/1×10^6 cells). Transfection solution containing THP-1 cells was incubated for 7 min in 37 °C water bath. Transfected cells were then resuspended in 10% FBS, RPMI medium and transferred to 96-well plates (0.2×10^6 transfected cells/well). Astragalus pure compounds were added 24-h after transfection for 4 h and compared relative to maximal activation by LPS (10 μg/ml). Cells were harvested using Packard filter plates and lysed using 30 μl of luciferase

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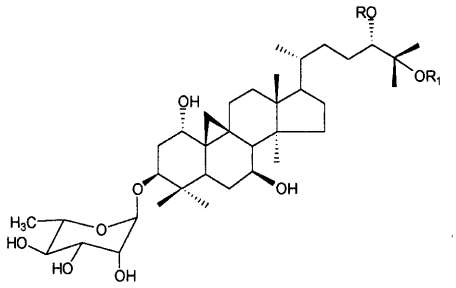
mix (1 : 1, LucLite™ luciferase: 1×PBS, 1 mM Ca and Mg). Luciferase light emission was measured using a Packard microplate scintillation counter.

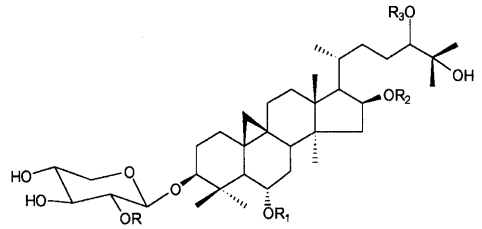
Sequence of PCR Primers Sequences for the primers were described in Su *et al.*[18] IL-1β forward (5′-ATGGCAGAAGTACCTAAGCTCGC-3′); IL-1β reverse (5′-ACACAAATTGCATGGTGAAGTCAGTT-3′); TNF-α forward (5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGC-3′); TNF-α reverse (5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′); GAPDH forward (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′); GAPDH reverse (5′-CATGTGGGCCATGAGGTCCACCAC-3′).

RT-PCR for IL-1β, TNF-α and GAPDH THP-1 cells (3 ml, 1×10^6 cells/ml) were incubated for 2 h with 10% FBS, RPMI 1640 medium at 37 °C, 5% CO_2, under the following conditions: control, LPS (10 μg/ml), astragaloside I (200 μg/ml) and astragaloside II (200 μg/ml). Total RNA was isolated using the TRI Reagent® kit in which cells are lysed using a combination of phenol and guanidine thiocyanate. After the addition of bromochloropropane, RNA is separated into the aqueous phase and subsequently precipitated with isopropanol. Total RNA recovered using this method is about 30 μg.

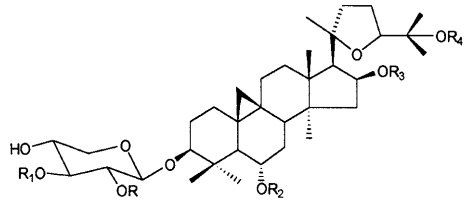
RT-PCR reactions were run using kit reagents from Promega. Each reaction used the following components (total volume of 50 μl): 10 μl AMV/Tfl 5× reaction buffer, 1 μl dNTP mix (10 mM), 2 μl $MgSO_4$ (25 mM), 1 μl AMV reverse transcriptase (5 units/μl), 1 μl Tfl DNA polymerase (5 units/μl), 2 μl of each primer (15 pmol/μl), and 2 ng total RNA (IL-1β and TNF-β) or 10 ng total RNA (GAPDH). The RT-PCR protocol used a Progene automatic thermal cycler. The first cycle consisted of 45 min at 48 °C, followed by 2 min at 94 °C. Amplification was achieved using 35 cycles: denature at 94 °C for 45 s, anneal at 57 °C (IL-1β and TNF-β) or 60 °C (GAPDH) for 1 min, and extend at 68 °C for 2 min. The final cycle held samples at 68 °C for 7 min. Electrophoresis of RT-PCR products (mRNA IL-1β, TNF-α and GAPDH) was accomplished using 5% polyacrylamide gels and ethidium bromide as the staining agent.



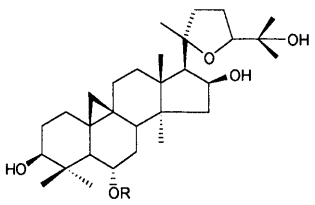
	R	R_1
1.Macrophyllosaponin B	β-D-Xyl	H
2. Macrophyllosaponin C	H	β-D-Glu
3. Macrophyllosaponin D	(2–O–β-D-Xyl)-β-D-Xyl	H



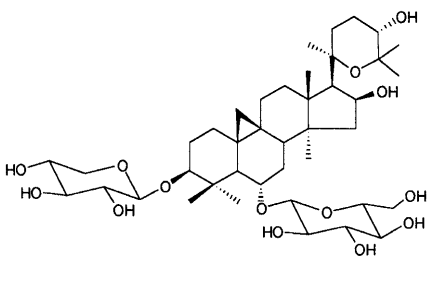
	R	R_1	R_2	R_3
4. Askendoside G	α-L-ara	H	β-D-Glu	H
5. Cyclocanthoside D	H	H	β-D-Glu	H
6. Cyclocanthoside E	H	β-D-Glu	H	H
7. Cephalatoside A	H	H	β-D-Glu	β-D-Xyl



	R	R_1	R_2	R_3	R_4
8. Astrasieversianin II	Ac	Ac	β-D-Xyl	H	H
9. Astrasieversianin X	H	H	β-D-Xyl	H	H
10. Astragaloside I	Ac	Ac	β-D-Glu	H	H
11. Astragaloside II	Ac	H	β-D-Glu	H	H
12. Astragaloside IV	H	H	β-D-Glu	H	H
13. Astragaloside VI	β-D-Glu	H	β-D-Glu	H	H
14. Astragaloside VII	H	H	β-D-Glu	H	β-D-Glu
15. Trojanoside A	H	H	β-D-Glu	Ac	H
16. Trojanoside H	α-L-ara	H	β-D-Glu	H	H



	R
17. Cycloastragenol	H
18. Brachyoside B	β-D-Glu



19. Cyclocephaloside I

Fig. 1. Structures of Compounds **1**—**19**

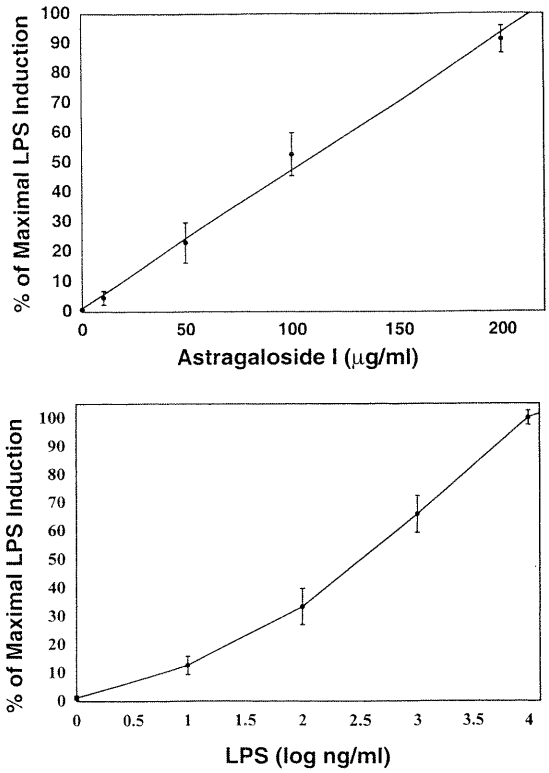


Fig. 2. Dose Response for Astragaloside I and Bacterial Lipopolysaccharide (LPS) Activation of NF-κB in THP-1 Monocytes/Macrophages at 4 h

Samples run in duplicate in 2 separate experiments. Means±S.D.

RESULTS

The 19 pure compounds isolated from *Astragalus* species (Fig. 1) were evaluated at two different dosage levels using the NF-κB reporter gene assay. At the first dosage level all compounds were run at 0.1 μg/ml in combination with 50 ng/ml LPS using THP-1 cells transfected with NF-κB reporter plasmid. All compounds were inactive (results not shown). At the second dosage level, these compounds were evaluated at 100 μg/ml in the absence of LPS. All compounds were inactive except astragaloside I. The EC_{50} (50% B maximal LPS Induction) value for NF-κB directed luciferase expression for astragaloside I was 105 μg/ml. Figure 2 presents a dose response for both LPS and astragaloside I. Addition of polymyxin B (10 μg/ml) did not abrogate NF-κB activation in THP-1 cells by astragaloside I, indicating that the activity is not due to bacterial LPS contamination (data not shown).

To confirm THP-1 activation, mRNA levels for both IL-1β and TNF-α were measured using RT-PCR (Fig. 3). Treatment of THP-1 cells with either LPS (10 μg/ml) or astragaloside I (200 μg/ml) resulted in a dramatic increase of IL-1β mRNA (810 bp) as compared with control. Astragaloside II, included as a representative of the inactive compounds, was similar to the control values. Addition of LPS and astragaloside I also increased levels of TNF-α mRNA (444 bp). The mRNA level of the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH, 1000 bp) was the same for all samples (Fig. 3).

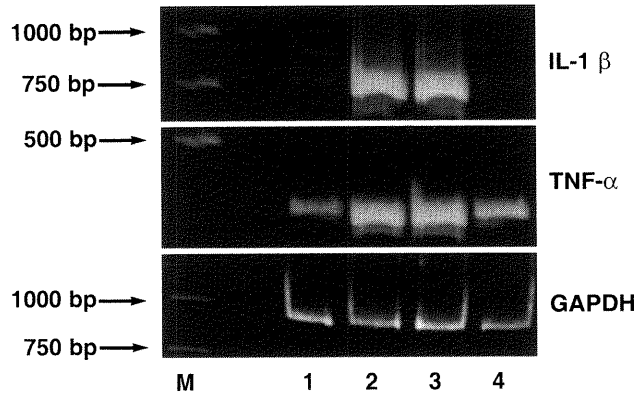


Fig. 3. Astragaloside I Enhances Cytokine Production

RT-PCR Results for IL-1β mRNA, TNF-α mRNA and GAPDH mRNA in THP-1 Cells at 2 h: (M) PCR Marker, (1) Control, (2) LPS at 10 μg/ml, (3) Astragaloside I at 200 μg/ml, and (4) Astragaloside II at 200 μg/ml

DISCUSSION

There appears to be at least several classes of chemical compounds that are responsible for the immune enhancing properties of *Astragalus* species. Over the last two decades a number of papers have been published on the antitumor, antiviral and immune stimulating effects of polysaccharides isolated from this plant.[3—5] In comparison, relatively few articles have been published on the immunostimulatory effects of saponins. For example, two studies have indicated that administration of astraglaoside IV to mice increases macrophage phagocytosis and proliferation of β-lymphocytes.[19,20] In concanavalin A-treated human lymphocytes, saponins have been shown to effect Ca^{2+} activated K^+ channels. However, saponins alone did not show any effect.[21] Clearly more research is needed to establish the mechanism of action of immunostimulation and how saponin compounds function *in vitro*.

Earlier research from our group in Turkey reported moderate stimulation of concanavalin A treated human lymphocytes against 8 saponins isolated from *Astragalus melanophrurius* between the concentration range of 0.01—10 μg/ml. At higher concentrations (100 μg/ml and 200 μg/ml) lymphocyte stimulation was inhibited.[13] These results differ from the results reported in this study. At low concentrations we observed no effect on macrophage activation with any compound in the presence of 50 ng/ml LPS. However, at higher dosages astragaloside I was able to stimulate NF-κB expression in macrophages. The observed stimulation was achieved by astragaloside I alone, in the absence of any LPS. The difference between the previous and current findings is difficult to explain due to the complexity of mechanisms involved in immune activation. It may simply be due to differences in the immune cells used to run the bioassays.

In addition to increased NF-κB directed luciferase expression by astragaloside I, we also observed enhanced mRNA expression of IL-1β and TNF-α cytokines. Therefore, it appears that observed effects by astragaloside I are relavent to immune stimulation. Although all 19 cycloartane-type compounds tested were from the same chemical class, only astragaloside I was active. This implies that there are critical structural features responsible for macrophage activation by *Astragalus* saponins.

At least two structural features appear to be crucial to the immunstimulatory effects of these saponins. First, the presence of two acetyl groups on the second and third carbon atoms of the β-D-xylopyranose moiety. Neither astragaloside IV or its monoacetylated derivative astragaloside II (acetyl function on C-2(O) of β-D-xylose moiety) showed any effect on macrophage activation. In contrast, astragaloside I with two acetyl groups on C-2(O) and C-3(O) of the β-D-xylopyranose moiety was able to increase macrophage activation. The second crucial structural feature is the presence of a β-D-glucopyranose unit on the sixth carbon atom of the aglycon moiety. The difference between astragaloside I and astrasieversianin II is the sugar moiety on C-6(O) of the aglycon part (β-D-xylopyranose for astrasieversianin II, and β-D-glucopyranose for astragaloside I). In terms of biological activity, astragaloside I was active and astrasieversianin II was inactive in our bioassay system.

Further studies are now in progress to confirm the above assumptions regarding structure–activity relationship of cycloartane-type compounds from *Astragalus* species.

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