Abstract. Several kinds of sesquiterpene lactones have been proven to inhibit NF-κB and to retard atherosclerosis by reducing lesion size and changing plaque composition. The anti-malarial artemisinin (Art) is a pure sesquiterpene lactone extracted from the Chinese herb Artemisia annua (qinghao, sweet wormwood). In the present study, we demonstrate that artemisinin inhibits the secretion and the mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 in a dose-dependent manner in phorbol 12-myristate 13-acetate (PMA)-induced THP-1 human monocytes. We also found that the NF-κB specific inhibitor, Bay 11-7082, inhibited the expression of these pro-inflammatory cytokines, suggesting that the NF-κB pathway may be involved in the decreased cytokine release. At all time-points (1-6 h), artemisinin impeded the phosphorylation of IKKα/β, the phosphorylation and degradation of IκBα and the nuclear translocation of the NF-κB p65 subunit. Additionally, artemisinin inhibited the translocation of the NF-κB p65 subunit as demonstrated by confocal laser scanning microscopic analysis and by NF-κB binding assays. Our data indicate that artemisinin exerts an anti-inflammatory effect on PMA-induced THP-1 monocytes, suggesting the potential role of artemisinin in preventing the inflammatory progression of atherosclerosis.

Introduction

Atherosclerosis is widely recognized as a chronic inflammatory disease. Inflammatory processes not only promote the initiation and evolution of atheroma, but also contribute to the acute thrombotic complications of atherosclerosis (1,2). Monocytes/macrophages play important roles in the development of atherosclerosis. Circulating monocytes migrate under the subendothelial space and differentiate into macrophages as a key inflammatory response in the development of atherosclerotic lesions (3,4). As a result of activation, macrophages release a host of pro-inflammatory mediators, including tumor necrosis factor (TNF), interleukins (IL) and several kinds of proteases, such as the matrix metalloproteinases. Activated macrophages also express scavenger receptors, which mediate the uptake of oxidized low-density lipoprotein and formation of foam cells (5-8). Thus, inflammatory processes not only promote initiation and evolution of atheroma, but also contribute decisively to plaque vulnerability and to the precipitating acute thrombotic complications of atheroma (9).

Artemisinin (C15H22O5) is a natural pure product isolated from the traditional Chinese herb Artemisia annua (qinghao, sweet wormwood) (10). It is a novel sesquiterpene lactone containing an endoperoxide bridge, which is used in China as a treatment for fever and malaria for over 2,000 years. Artemisinin has been recommended by the World Health Organization for malaria control because of its high efficiency and low toxicity (11). Like other sesquiterpene lactones, it also exhibits a wide variety of activities, including immunosuppressive, anti-inflammatory, anti-tumor, anti-angiogenic, and anti-parasitic (12-18). In several kinds of inflammatory reactions, artemisinin has been found to have potent anti-inflammatory effects. It is reported that artemisinin could significantly inhibit the TNF-α and IL-6 release induced by CpG-containing oligodeoxynucleotides (CpG ODN), lipopolysaccharide (LPS) or by heat-killed Escherichia coli in RAW264.7 cells and it could protect mice from a lethal...
challenge by CpG ODN, LPS, or heat-killed E. Coli (14). In addition, artesunate, an artemisinin derivative, decreases TNF-α-induced secretion of IL-1β, IL-6 and IL-8 and NF-κB translocation in rheumatoid arthritis fibroblast-like synoviocytes (15). These data suggest that artemisinin may be effective in the anti-inflammatory process. However, no studies have elucidated the effects of artemisinin on the inflammatory response in atherosclerosis-related monocytes/macrophages.

The present study assessed the novel anti-inflammatory properties of artemisinin with respect to the differentiation of monocytes into macrophages. Phorbol 12-myristate 13-acetate (PMA) was used as an inducer and THP-1 cells were used as the target cells to mimic the differentiation progression of monocytes into macrophages, as recommended by Auwerx (19). Moreover, PMA was able to induce NF-κB activation during the differentiation of monocytes into macrophages (43). The artemisinin involvement in the NF-κB signaling pathway was also demonstrated. Overall, this study suggests a potential role for artemisinin in preventing the inflammatory progression of atherosclerosis.  

Materials and methods

Cell culture and treatment. A human monocytic cell line (THP-1 cells, obtained from the American Type Culture Collection) was cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS, 10 mM HEPES (Sigma), and 100 U/ml penicillin/streptomycin solution. To induce differentiation of monocytes into macrophages, THP-1 cells were cultured in 100 nM PMA (Calbiochem) for 48 h as previously described (20).

Determination of cell viability (MTT assay). The MTT assay was used to assess the cytotoxicity of artemisinin (Sigma-Aldrich, Taufkirchen, Germany) on PMA-induced macrophages. Cells were seeded in 96-well plates in a medium containing 10% FBS and were pre-treated with increasing concentrations of artemisinin for 4 h (14). PMA was subsequently added to the cells at a final concentration of 100 nM for another 48 h. Cell viability was assessed by measuring the absorbance of the MTT product at 570 nm with a spectrophotometer.

ELISA assay of inflammatory markers. In a previous study, we have confirmed that the appropriate dose range of artemisinin to PMA-induced THP-1 cells is <80 μg/ml (data not shown). On this basis, cells were preincubated in the presence or absence of artemisinin (10-80 μg/ml) for 4 h or 10 μM Bay 11-7082 (NF-κB-specific inhibitor) for 30 min. PMA was added to the cells at a final concentration of 100 nM, and the cells were further incubated for 48 h. Cell viability was assessed by measuring the absorbance of the MTT product at 570 nm with a spectrophotometer.

Real-time PCR analysis. Cells were preincubated in the presence or absence of artemisinin (10-80 μg/ml) for 4 h or 10 μM Bay 11-7082 (NF-κB specific inhibitor) for 30 min (21). PMA was added to the cells at a final concentration of 100 nM, and the cells were further incubated for 48 h. Total RNA was then extracted and reverse-transcribed into cDNA. The mRNA levels were analyzed using the SYBR green reagent kits, with gene-specific primers, on an Applied Biosystems 7500 real-time PCR System, according to the manufacturer’s instructions. Primer sequences are listed in Table I. All results were normalized against GAPDH.

Protein isolation and Western blot analysis. Cells were preincubated in the presence or absence of 60 μg/ml artemisinin for 4 h. PMA was added to the cells at a final concentration of 100 nM, and the cells were further incubated for various time-points (1-6 h). THP-1 cells were washed twice with ice-cold PBS and scraped in 1 ml of the same buffer. After centrifugation at 600 x g, the cell pellet was resuspended in ice-cold lysis buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.2 mM KCl, 0.2 mM phenyl-methyl-sulphonyl-fluoride, 0.5 mM dithiothreitol), vortexed for 10 sec and then centrifuged at 10,000 x g for 5 min. The packed cells were suspended in ice-cold hypotonic lysis buffer in the presence of 50 μl of 8% Nonidet P-40 and then kept on ice for 30 min. The nuclear fraction was precipitated by centrifugation at 10,000 x g for 15 min. The supernatants, corresponding to the cytosolic fraction, were transferred to fresh precooled tubes and assayed for protein content by BCA protein assay (Pierce, Rockford). The nuclei pellet was resuspended in 50-100 μl low salt extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM phenyl-methyl-sulphonyl-fluoride, 0.5 mM dithiothreitol) and added to an equal volume of high salt extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM phenyl-methyl-sulphonyl-fluoride, 0.5 mM dithiothreitol) in a dropwise fashion, and then incubated at 4°C for 45 min under continuous shaking. The sample was centrifuged for 20 min at 10,000 x g. The concentration of the nuclear extract was measured by the BCA protein assay (Pierce). After denaturation, the solubilized proteins (20 μg) were subjected to electrophoresis on 10% polyacrylamide SDS-gels and subsequently transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). This was followed by probing with primary antibodies for rabbit anti-phospho-IKKα/β, rabbit anti-IκBα, rabbit anti-phospho-IκBα, rabbit anti-p65 (Cell Signaling) (diluted 1:1000 in TBST), or mouse anti-actin (diluted 1:5000 in TBST) for 2 h. After incubation with horseradish peroxidase-conjugated goat or mouse anti-rabbit secondary antibody (Cell Signaling) for 2 h, the protein-antibody conjugates were detected by chemiluminescence (Immobilon Western chemiluminescent HRP substrate, Millipore). Densitometric analysis was performed by using the Quantity One software (Bio-Rad) to scan the signals.

Confocal laser scanning fluorescence microscopy of NF-κB. Cells were seeded onto flame-sterilized coverslip placed in a 6-well tissue culture plate. Cells were preincubated in the presence or absence of 60 μg/ml artemisinin for 4 h. PMA was added to the cells at a final concentration of 100 nM and the cells were further incubated for 3 h. The cells were immunofluorescence-labeled according to the manufacturer’s protocol.
using a cellular NF-κB translocation kit (Beyotime Biotech) by the method of Musa et al. (22). Briefly, after washing and fixing, cells were incubated with a blocking solution at 4°C overnight and then anti-NF-κB p65 antibody for 2 h. After washing 3 times, anti-rabbit IgG antibody conjugated with Cy3 was added and later incubated for 1 h. Cells were then incubated with DAPI for 5 min to stain the nuclei. Activation of NF-κB p65 was visualized with a confocal laser scanning microscope (FluoView™ FV1000; Olympus) at an excitation wavelength of 350 nm for DAPI and 540 nm for Cy3. The red and blue images were overlaid to create a two-color image in which purple fluorescence indicated the areas of co-localization.

**NF-κB binding assay.** Cells were pretreated with 60 μg/ml artemisinin for 4 h and exposed to 100 nM PMA for 3 h. The DNA binding activity of NF-κB (p50/p65) was determined using an ELISA-based non-radioactive NF-κB (p50/p65) transcription factor assay kit (Chemicon, Temecula, CA).

**Statistical analysis.** Results are expressed as means ± SD. Differences were compared by one-way ANOVA, with P<0.05 considered to be statistically significant. All experiments were performed at least three times.

**Results**

**Effects of artemisinin on cell proliferation.** The effect of artemisinin on the proliferation of PMA-stimulated THP-1 monocytes was measured using the MTT assay (Fig. 1), with the concentration ranging from 0-160 μg/ml for 48 h. No significant differences in cell proliferation was observed in response to up to 80 μg/ml artemisinin for 48 h (Fig. 1B). On this basis, we used artemisinin doses ranging from 10 μg/ml to 80 μg/ml for subsequent experiments. The relative cell proliferation of artemisinin-treated monocytes exceeded 80%. The chemical structure and molecular weight of artemisinin are shown in Fig. 1A.

**Artemisinin inhibited the secretion of inflammatory cytokines.** Protein levels of TNF-α, IL-1β, and IL-6 in cell culture supernatants were quantitated using sandwich enzyme immunoassay kits (Fig. 2). Cells were incubated with increasing concentrations (10-80 μg/ml) of artemisinin for 4 h

### Table I. Primers for real-time PCR.

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<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Size (bp)</th>
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<td>TNF-α</td>
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<tr>
<td>NM_000594.2</td>
<td>5’-GTAGGAGCAGCGGCATGC-3’</td>
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</tr>
<tr>
<td>IL-1β</td>
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<tr>
<td>NM_000576.2</td>
<td>5’-ATGAAGGAAAGAAGGTG-3’</td>
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</tr>
<tr>
<td>IL-6</td>
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<td>121</td>
</tr>
<tr>
<td>NM_000600.2</td>
<td>5’-GCCACTACCTTCTTCAAGACG-3’</td>
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<tr>
<td>NM_002046</td>
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**Figure 1.** The effects of artemisinin on the proliferation of PMA-induced THP-1 cells. Chemical structure and molecular weight of artemisinin. THP-1 monocytes were incubated with various concentrations of artemisinin (0-160 μg/ml) for 4 h and exposed to 100 nM PMA for 48 h. Cell proliferation was assessed using the MTT assay. Cells incubated in a medium without artemisinin and PMA were defined as control and were considered to have a 100% proliferation rate.
or with 10 μM of the NF-κB-specific inhibitor, Bay 11-7082, for 30 min and were exposed to 100 nM PMA for 48 h. As shown in Fig. 2, treatment with PMA alone (48 h) markedly increased the secretion of TNF-α, IL-1β, and IL-6 compared to unstimulated cells. Pretreatment with different artemisinin concentrations (10-80 μg/ml) for 4 h dose-dependently antagonized the PMA-induced increase in TNF-α, IL-1β, and IL-6 secretion (Fig. 2A, C and E). In addition, 80 μg/ml artemisinin almost completely inhibited the PMA-induced TNF-α, IL-1β, and IL-6 secretion. To investigate the related mechanism of the artemisinin effect on cytokine release, the cells were pre-treated with 10 μM Bay 11-7082 for 30 min before addition of PMA. When cells were cultured in the presence of Bay 11-7082, complete inhibition of the secretion of all cytokines assayed was observed, which was congruent with the artemisinin-pretreated groups (Fig. 2A, C and E).
Artemisinin inhibited the mRNA expression of inflammatory cytokines. Next, we investigated whether the mRNA levels of inflammatory cytokines were affected by artemisinin. As shown in Fig. 2, there were weak signals for the basal mRNA expression of TNF-α, IL-1β, and IL-6 in quiescent THP-1 cells. In contrast, their mRNA was greatly increased in PMA-alone stimulated THP-1 cells. When artemisinin (10, 20, 40 or 80 μg/ml) was added prior to the stimulators, TNF-α, IL-1β and IL-6, mRNA levels were markedly decreased in a dose-dependent manner (Fig. 2B, D and F). In addition, an almost complete inhibition of the mRNA expression of all the cytokines assayed was observed when the cells were cultured in the presence of 80 μg/ml artemisinin or Bay 11-7082. These results indicate that artemisinin could inhibit the transcription of cytokines such as TNF-α, IL-1β, and IL-6, which may in turn affect protein production and release, in which the NF-κB canonical activation pathway might play a pivotal role.

Artemisinin inhibited activation of the NF-κB signaling pathway. NF-κB is a key transcription factor for the expression of inflammatory genes, including TNF-α, IL-1β, and IL-6 (23-26). To further elucidate the mechanism of action of artemisinin, the key protein levels in the activated NF-κB signaling pathway at different time-points (1, 3 or 6 h) were examined by Western blot analysis. As shown in Fig. 3, PMA-induced phosphorylation of both IKKα/β (Fig. 3A and B) and IκBα protein (Fig. 3A and C) was significantly blocked by artemisinin treatment. In parallel, subsequent IκBα degradation in the cytoplasm of macrophages was decreased, which led to an increased IκBα protein level (Fig. 3A and D). The protein level of NF-κB p65 in the nucleus was decreased, which is a direct proof of the reduced translocation of NF-κB (Fig. 3A and E). p-IKKα/β, p-IκBα, and p65 were found to be
inhibited as early as at 1 h, and this inhibitory effect reached its peak at 3 h. p-IKKβ, p-IκBα, and p65 were still lower than the PMA-alone group at 6 h. As compared to the PMA-alone group, IκBα was also found to be higher as early as at 1 h, reached its peak at 3 h and gradually declined but was still higher than the PMA-alone group at 6 h. Accordingly, the time-point of 3 h was chosen for subsequent experiments.

Artemisinin blocked NF-κB translocation. It was tested whether artemisinin inhibits PMA-induced stimulation of TNF-α, IL-1β, and IL-6 secretion by interfering with the translocation of the transcription factor NF-κB. Intracellular localization of NF-κB p65 in THP-1 cells was evaluated by a confocal laser scanning microscope using NF-κB p65-specific antibody and Cy3-conjugated secondary antibody (Fig. 4). DAPI was used to mark the nucleus. Cytoplasmic red area (representative of the area that contains p65) was observed in PMA-free cells (Fig. 4A), while nuclear blue staining in PMA-alone-exposed cells was observed (Fig. 4B), indicative of nuclear localization of activated NF-κB p65 at

**Figure 4.** Representative microphotographs showing effect of artemisinin on the translocation of NF-κB p65 in PMA-treated THP-1 monocytes. The NF-κB localization was visualized by binding with a Cy3-conjugated secondary antibody. Cells were then incubated with DAPI to stain the nuclei. Microscopic images were obtained using a confocal laser scanning microscope (three independent experiments) and the red area (representative of the area that contains p65) and blue area (representative of the nucleus part that is DAPI-conjugated) images were overlaid to create a purple fluorescence in areas of co-localization. In quiescent THP-1 cells, the NF-κB p65 subunit was predominantly localized in the cytoplasm (A). Cells stimulated with PMA for 3 h showed significant translocation of p65 to the cell nucleus (B). In cells pretreated with 60 μg/ml artemisinin for 4 h and exposed to 100 nM PMA for 3 h, NF-κB p65 was significantly retained in the cytoplasm (C).

**Figure 5.** The effect of artemisinin (Art) on the PMA-induced increase of p50/p65 DNA binding activity. THP-1 cells were pretreated with 60 μg/ml artemisinin for 4 h, then induced with PMA for 3 h. Nuclear extracts were prepared. The DNA binding activity assay showed a marked decrease in p50/p65 DNA binding activity in nuclear fractions from THP-1 monocytes treated with PMA and artemisinin compared to cells treated only with PMA. Results are expressed as the mean ± SD. **P<0.01, relative to PMA-alone incubation.
single cell level. In contrast, artemisinin- and PMA-treated cells had diminished staining levels of nuclear p65 (Fig. 4C). As shown in Fig. 5, treatment of cells with PMA led to a robust activation of p50 and p65. This activation was partially blocked by 60 µg/ml artemisinin.

Discussion

The present study demonstrates for the first time that the anti-malarial agent artemisinin could effectively inhibit the production of TNF-α, IL-1β, and IL-6 in PMA-induced monocyte-derived macrophages in a dose-dependent manner. The results also provide evidence that the inhibitory effect of artemisinin on cytokines is mediated by the NF-κB canonical activation pathway. Therefore, these findings strongly support that artemisinin has a unique anti-inflammatory effect in atherosclerosis-related monocytes/macrophages, and may provide novel insights into the protection against atherosclerosis inflammation.

Inflammation plays a fundamental role in mediating all stages of atherosclerosis, from initiation to progression, and the associated thrombotic complications. Pro-inflammatory cytokines play a strict proatherogenic role in atherosclerosis, which has been demonstrated in numerous animal studies and has been suggested by their expression in atherosclerotic human plaques. For example, TNF-α, produced by THP-1 cells, macrophages and NK cells, could activate NF-κB and inhibit key metabolic enzymes including lipoprotein lipase. IL-1β, also produced mainly by macrophages and the action of which overlaps partly with TNF, induces metalloproteinases, nitric oxide synthase, and adhesion molecules. Moreover, IL-6 and TNF-α have been proven to be clinical markers to identify and track inflammation (27-30). Targeting inflammatory cytokines has been prosperously developed and been proven to successfully protect against atherosclerosis. Pentoxifylline, a TNF-α antagonist, inhibits plaque formation in apoE-/- mice and the production of the atheroprotective cytokine IL-10 (31). Blockade of TNF-α ameliorates atherosclerosis progression in apoE-/- mice (32). Therefore, the inhibitory effect of artemisinin on pro-inflammatory cytokines indicates that artemisinin might prove to be a novel and promising natural medicine for preventing atherosclerosis.

In the present study, we found that artemisinin inhibited the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in atherosclerosis-related monocytes/macrophages in a dose-dependent manner. In fact, artemisinin and its derivatives have been reported to possess anti-inflammatory effects, acting through suppression of several inflammatory cytokines (13-15). Artemisinin was found to suppress CpG ODN- and LPS-induced TNF-α and IL-6 release in RAW264.7 cells, which has also been demonstrated in animal models of sepsis (14). Another study reported that artemisinin inhibits nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells (33). Hou et al showed that artesunate, an artemisinin derivative, could decrease TNF-α-induced secretion of IL-1β, IL-6 and IL-8 and NF-κB translocation in rheumatoid arthritis fibroblast-like synoviocytes (16). A novel derivative of artemisinin, SM933, has been shown to be effective in the treatment of autoimmune diseases and inflammation (13). Furthermore, it has been reported that artether exhibited potent immunosuppressive activity on T cells both in vitro and in vivo (12). Our findings, coupled with other studies on the anti-inflammatory effect of artemisinin, unveiled the possible role of artemisinin in preventing atherosclerosis-related inflammation.

Secondly and more importantly, we demonstrated that artemisinin exerts anti-inflammatory actions in monocytes/macrophages through its unique inhibition of NF-κB signaling pathways. NF-κB is one of the key regulators of inflammation and oxidative stress, which controls the transcription of many genes with an established role in atherosclerosis. The classical NF-κB activation pathway involves the activation of the IKK complex with the subsequent degradation of IκBα and nuclear translocation of the NF-κB dimer (34). A number of factors implicated in the development of atherosclerosis, such as cytokines, oxidized lipids, hemodynamic forces, angiotensin II, and integrin/matrix signaling, have all been shown to activate NF-κB (35-38). NF-κB regulates the expression of many genes with an established role in atherosclerosis, such as cytokines (e.g., TNF, IL-1β, and IL-6), chemokines (e.g., MCP-1), adhesion molecules (e.g., VCAM-1 and ICAM-1), proteases (e.g., MMP-9) and antiapoptotic proteins (e.g., c-IAP and Bcl-2) (39-43). Several studies have demonstrated that TNF-α, IL-1β, and IL-6 are the target genes of NF-κB (23-26). It has been shown that several kinds of sesquiterpene lactones, such as parthenolide, could inhibit NF-κB and retard atherosclerosis by reducing lesion size and changing plaque composition in apoE-/- mice (44). Aldieri et al reported that artemisinin blocks the LPS/cytokine-induced activation of NF-κB by electrophoretic mobility shift assay in T67 human astrocytoma cells, and Western blotting showed an increased IκBα level as compared to artemisinin-untreated cells (33). In our present study, a significant inhibition of the NF-κB canonical activation pathway at different time-points (1, 3, or 6 h) was observed as compared to the artemisinin-untreated groups. Additionally, the inhibitory effect of artemisinin reached its peak in 3 hours. Furthermore, a direct view of the impeded nuclear translocation of NF-κB p65 was observed using a confocal laser scanning microscope. The DNA binding activity assay also determined a marked decrease in p50/p65 DNA binding activity in nuclear fractions from THP-1 monocytes treated with PMA and artemisinin compared to cells treated only with PMA. The NF-κB specific inhibitor, Bay 11-7082, reversed the PMA induced pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) production in the current study. These results suggest that the NF-κB canonical activation pathway is at least partly responsible for PMA-induced TNF-α, IL-1β, and IL-6 production. Artemisinin blocked PMA-induced TNF-α, IL-1β, and IL-6 production via the NF-κB canonical activation pathway in THP-1 monocytes differentiated into macrophages.

In summary, our findings provide strong evidence for the first time that artemisinin attenuates PMA-induced production of TNF-α, IL-1β, and IL-6 via inhibition of the NF-κB canonical activation pathway in PMA-induced human THP-1 monocytes. Considering the critical role of the suppression of the inflammation process in protecting the development of atherosclerotic plaque, our study suggests that artemisinin may have a potential role in the inhibition of the inflammatory progression of atherosclerosis.
Acknowledgements

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