Deaggregators inhibit TNF-α-induced leukocyte adhesion in vitro by breaking up hydrophobic lipophilic interactions

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Aim: Deaggregators (deAgrs) are nontoxic organic molecules that possess the ability to deaggregate simple aggregates formed by hydrophobic lipophilic interactions (HLI). Since HLI-driven organic molecule aggregates may induce leukocyte adhesion, we investigated the influence of deAgrs on TNF-α-mediated leukocyte adhesion in vitro.

Methods: For adhesion studies, vascular endothelial cells or smooth muscle cells monolayers were treated with TNF-α (10 μg/L) and deAgrs for 24 h, followed by addition of monocytes or neutrophils suspension. The non-adherent leukocytes were rinsed, and the number of attached leukocytes was measured using an ELISA plate reader. Simultaneously, fluorescence probes Np-12 and Np-Ch were used to measure the deaggregating efficiencies of these deAgrs.

Results: Among the nine deAgrs tested, eight significantly reduced the cell adhesion rates with the order of efficiencies: 260>160>568>ZPMOP>R68>640>TB6PMOP>CNS, but TBHQ had no effect. The deAgrs for deaggregating an aggregated probe (Np-12 or Np-Ch) exhibited a similar order of efficiencies: 260>160>568>ZPMOP>R68>640>TB6PMOP>CNS>12-AA>11-AA>TBHQ. Spearman correlation coefficient analyses indicated that the adherent rates of leukocytes to endothelial cells or smooth muscle cells treated with deAgrs had significantly negative correlation to their deaggregating abilities.

Conclusion: DeAgrs effectively inhibit TNF-α-mediated leukocyte adhesion in vitro by breaking up hydrophobic lipophilic interactions, thus may be further tested for blocking atherogenesis.

Keywords: deaggregator; hydrophobic lipophilic interaction; arteriosclerosis; TNF-α; leukocyte adhesion; neutrophil; monocytes; endothelial cell; smooth muscle cells

Original Article

Introduction

Leukocyte adhesion to the endothelium is an important event in the earliest stage of arteriosclerotic lesion formation[1]. Cytokines and adhesion molecules may be responsible for the rolling and adherence of monocytes, granulocytes and T cells on the endothelium of the arterial sites that are prone to the formation of atherosclerotic lesions[2–4]. Via the upregulation of cytokines and adhesion molecules, activated mononuclear cells and granulocytes induce cell proliferation and help define and localize the inflammatory response at the lesion sites[5–7]. Once the adhesion of monocytes or neutrophils to the arterial endothelium and smooth muscle layer is blocked, atherogenesis may also be blocked[7].

Although cytokines and adhesion molecules have important chemotactic roles[8], leukocyte adhesion to and aggregation on the endothelium and the smooth muscle layer are the result of the upregulation of cytokines and adhesion molecules. Leukocyte adhesion and aggregation may also be the macroscopic behavior of the aggregation of phospholipids and other lipid components in cell membranes induced by hydrophobic lipophilic interactions (HLI)[9–11]; the phenomenon of aggregation is a prerequisite for the development of life and the ability of cells to function[10, 11].

HLI between apolar molecules or apolar parts of molecules in water or water-organic solvent mixtures are of paramount importance in life processes[10–13]. It was recently discovered that the inherent coaggregating tendencies of organic molecules, e.g., cholesteryl esters, triglycerides, and phospholipids, may be directly related to their behaviors in causing atherogen-
sclerosis\textsuperscript{[10, 11, 14-16]}. If leukocyte adhesion is considered a precipitated aggregate, an extremely effective nontoxic organic molecule called a deaggregator (deAgr), which tends to break up simple aggregates, may be useful in preventing leukocyte adhesion and blocking atherogenesis\textsuperscript{[9]}.

In this study, we used tumor necrosis factor-alpha (TNF-α) to induce increased adhesion between monocytes or polymorphonuclear leukocytes and vascular endothelial cells or smooth muscle cells to simulate the earliest type of atherosclerotic lesion in vitro. This process allowed the observation and measurement of the inhibitory effects of different types of deAgrs on leukocyte adhesion. At the same time, we studied the deaggregation abilities of these deAgrs in a MeOH-H\textsubscript{2}O binary system of different Φ values, \( \text{ie} \), the volume fraction of the organic component of the aquiorgano mixture, by employing naphthylethyl lauryl ether (Np-12) and cholesteryl β-naphthylacetic ester (Np-Ch) as the fluorescent probes. We investigated the relationship between deaggregating abilities and inhibitory efficiencies on the leukocyte adhesion of these deAgrs. The results may provide some insight into using deAgrs to reduce (or even reverse) arteriosclerosis in the future.

**Materials and methods**

**Animals**

The experimental protocols complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Academy of Sciences of China. Sprague-Dawley rats were purchased from the Experimental Animal Center of the Shanghai branch, Chinese Academy of Sciences, China. They were divided into three groups. The rats in the first group weighed approximately 20 g and were used to isolate and culture cerebral microvascular endothelial cells. In the second group, rats weighing approximately 150 g were used to obtain a primary culture of vascular smooth muscle cells. In the third group, rats weighing 300–350 g were used to isolate and purify neutrophils and monocytes.

**Isolation and culture of vascular endothelial cells (VECs)**

One-week-old rats were utilized for these studies. For each preparation, 5–7 rats were used. Rat brains devoid of cerebellum were placed in cold Hanks’ balanced salt solution (HBSS) without Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. The pial membranes were removed, and the cerebral cortices were cleaned of white matter, scissors-minced and homogenized in HBSS. The homogenate was centrifuged, and the pellet was resuspended in 15% dextran (M\textsubscript{w} 70 000–80 000) (Amersham Pharmacia Biotech, Uppsala, Sweden) containing 5% fetal bovine serum (FBS) (HyClone Lab, Inc Logan, UT, USA) and recentrifuged at 3000×g. The pellet was suspended in 3–5 mL 0.1% type II collagenase (Sigma, Chemical Co, St Louis, MO, USA) for 30 min at 37°C after being ground continuously for 15–20 min using a Pasteur pipette tube. The suspension was centrifuged at 200×g for 10 min. The upper layer of water was removed, and the pellet was resuspended in 33 mL Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) (pH=7.2) supplemented with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L pyruvic acid sodium, 3.5 g/L glucose, 100 U/mL penicillin, and 100 mg/mL streptomycin. The suspension containing many single and dispersed endothelial cells was sifted using a 74-μm nylon mesh sieve, and the filtrate was collected into a 100 mm tissue culture dish (Corning Co, Corning, NY, USA) and incubated at 37°C in a humidified 5% CO\textsubscript{2} 95% air atmosphere. After a 4-h incubation, the culture medium was removed, the culture dish was carefully washed once with serum-free DMEM, and fresh complete medium containing 15 μg/mL epidermal cell growth factor (ECGF, Roche, Austria) was added. The culture dish was incubated at 37°C in a humidified 5% CO\textsubscript{2} 95% air atmosphere. The medium was changed every four days. After five days, the cells began to proliferate, and by 7–9 d, they were nearly confluent. The cells were harvested using 0.25% trypsin and subcultured in 100 mm culture dishes at a 1:2 ratio. Passage 4–6 was used for the adhesion assay (Figure 1)\textsuperscript{[17, 18]}.

**Culture of vascular smooth muscle cells (VSMCs)**

The main thoracic arteries of three SD male rats that weighed approximately 150 g were dissected and carefully excised. The fat, adventitia, and connective tissue surrounding the arteries were removed by blunt dissection. The arteries were washed three times in cold HBSS, and the ectoblast was removed. The artery wall was sliced using an ophthalmic scissor, and the vascular endothelium was scratched softly with the back of a surgical knife. The vascular tissue was then washed twice with HBSS and cut into fine tissue pieces, 1 mm×1 mm×1 mm in size. These pieces at the density of 1 piece per square centimeter were placed onto the bottom of a 100 mm culture dish, and a small amount of medium was added to the culture dish. These small tissue pieces were incubated at 37°C for 3–4 h until they adhered to the bottom of culture dish. Then, 3 mL DMEM+10% FCS was gently added to the culture dish from the edge of the dish. After incubating 5–7 d in a humidified 5% CO\textsubscript{2} 95% air atmosphere, cells began to move away from the edges of the tissue pieces, and a compact cell layer formed after 2–3 weeks. The medium was changed every four days. Vascular smooth muscle cells were harvested using 0.25%
trypsin containing 0.02% EDTA, passaged when they reached 80%–90% confluence and used for the adhesion assay after 4–6 passages (Figure 2).[20]

Figure 2. (A) Normal growth of primary cultured vascular smooth muscle cells (×100). The dark portion is a vascular tissue mass, and smooth muscle cells are creeping at the edge of the tissue mass. (B) The third generation of vascular smooth muscle cells. The cells are spindle-shaped or in fascicular arrangement.

Isolation of neutrophils
Using sterile procedures, approximately 20 mL of blood was collected from the inferior vena cava of SD rats in 50 mL sterile polyethylene tubes containing approximately 10 U of heparin per 1 mL of blood. The tubes were centrifuged at 300×g for 20 min. The platelet-rich plasma layer was carefully aspirated and centrifuged at 2500×g for 15 min to produce platelet-poor plasma (PPP). Five milliliters of 6% dextran (Mₐ 500 000) (Pharmacia) was added to the remaining contents of each tube, and the volume was brought to 50 mL with 0.9% saline, gently and thoroughly mixed, and allowed to stand for 30 min for erythrocyte sedimentation to occur. The leukocyte-rich plasma was aspirated and centrifuged at 275×g for 6 min. The pellet was resuspended in 2 mL PPP and transferred to a 10-mL glass centrifuge tube where it was underlayered with 2 mL freshly prepared 42% Percoll in PPP, which was in turn underlayered with 2 mL freshly prepared 51% Percoll in PPP using a Pasteur pipette. The gradients were layered into a 10-mL glass centrifuge tube using a polyethylene transfer pipette and centrifuged at 275×g for 10 min. One milliliter 0.2% sodium chloride was forcefully added to the upper layer into a tube. The cells were carefully removed from the interface using a Pasteur pipette without removing the upper layer into a tube. The cells were pelleted by centrifugation for 10 min at 250×g and then harvested mononuclear cells containing approximately 20% monocytes were washed twice with PBS and resuspended to concentration of 5×10⁶/mL in DMEM+10% FCS.

Ten milliliters of a 30 mg/mL solution of gelatin was added to each 100 mm tissue culture dish. After the dishes were incubated for 2 h in a 37 °C drying oven, the gelatin was removed by suction. The dishes were put in a 55 °C drying oven overnight and then stored at room temperature. Ten milliliters of medium was added to each gelatin-coated dish. The dishes were incubated at room temperature for at least 1 h. The medium was removed by suction and the dishes were washed gently two times with PBS. Then, 15 mL of mononuclear cell suspension was added to each dish. The flasks and cells were incubated for 90 min at 37 °C, 5% CO₂. Non-adherent cells were then removed by suction and discarded. After the dishes were washed gently three times with DMEM+10% FCS, 10 mL of cold 10 mmol/L disodium EDTA in HBSS was added to each dish. The dishes were incubated at 4 °C for 10 min. The dishes were hit on the heel of a hand to dislodge adherent cells, and the cells were harvested, washed and resuspended to a concentration of 2×10⁶/mL for the adhesion assay. By this method, the purity of the monocytes was greater than 80% and the viability was greater than 90%.[21]

Adhesion assay
For adhesion studies, VECs or VSMCs at the concentration of 2×10⁵/mL were seeded in 96-well dishes (200 μL each well) and allowed to proliferate to confluence for approximately 48 h. After 24 h of growth in serum-free DMEM, EC and SMC monolayers were stimulated with 10 μg/L recombinant rat TNF-α (Peprotech, Rocky Hill, NJ, USA) and incubated in a humidified 5% CO₂ 95% air atmosphere for 24 h. The cells incubated with 10⁴ mol/L of a series of deAgrs for 24 h were the treated groups, and those incubated with medium alone for 24 h served as negative controls. After rinsing twice with warm, serum-free medium, 100 μL of 2×10⁵ monocyte or neutrophil suspension was added to each well. After 2 h of incubation, non-adherent leukocytes were rinsed twice with 0.9% saline at 37 °C. Then, 200 μL of 2% Rose Bengal liquid was added to each well and incubated for 20 min at room temperature. The dyeing liquid was removed, and each well was washed gently with 0.9% saline twice. Two hundred microliters of 0.01 mol/L PBS-ethanol solution (1:1 v/v) was added to each well and decolorized for 60 min at room temperature. Finally, The OD value of each well was measured by an ELISA
plate reader, and the results were determined as the mean total absorbance at 570 nm. The number of attached leukocytes was counted using the following formula\cite{22, 23}:

\[
\text{Cell adhesion rate} = \frac{\text{treated.well (OD)} - \text{control.well (OD)}}{\text{PMNs.or.Monocytes.well (OD)}} \times 100\% 
\]

Fluorescent studies on deaggregation

The chemical structures of the deAgrs including 260, 160, 568, R68, 640, ZPMOP, TB6PMOP, TBHQ, and CNS used in this study are shown in Figure 3. All compounds were synthesized and identified as previous description\cite{24, 25}.

The fluorescence method is widely used for measuring the deaggregating efficiencies of organic molecules. Naphthylethyl lauryl ether (Np-12) was used as the long chain fluorescent probe, and cholesteryl β-naphthylacetic ester (Np-Ch) was used as the plate fluorescent probe\cite{9, 26} (Figure 4). The principle of fluorescence studies of deaggregation is based on the following premise. The formation of aggregates in aqueous or aqiuorgano solvents from organic molecules with a long hydrocarbon chain is almost solely driven by HLI. Thus, aggregates may serve as one of the simplest models for such studies. Aggregation can affect the chemical reactivity or the spectroscopic behavior of the studied molecules. Fluorescence spectroscopy is a useful tool for evaluating the coaggregating tendencies of organic molecules that possess fluorophores. In an aggregating media, the aggregation of the long chain fluorescent probe molecules, eg, Np-12, and the plate fluorescence probe, eg, Np-Ch, may bring the chromophores close enough to form excimers. Given that aggregators can coaggregate with the monomeric probe molecules, eg, Np-12 or Np-Ch, deAgrs can also break up the aggregated Np-12 or Np-Ch. Np-12 or Np-Ch is then released into the bulk of the solvent and causes the changes in the fluorescence spectra of the aggregating media, ie, the excimer emission (I_m) decreases and the monomer emission (I_e) increases (Figure 5A). The slope \( \rho \) of the plot of \( I_e/I_m \) vs the deAgr concentration represents the qualitative deaggregating ability in which larger \( P \) values reflect greater deaggregating abilities\cite{9, 16, 27} (Figure 5B).

Pure, analytical grade NaCl, Na_2 HPO_4, and NaH_2 PO_4 were used without further purification. The fluorescent probe Np-12 and Np-Ch were prepared\cite{16, 27}. All of the synthesized substrates were identified by \(^1\)H NMR, IR, and elemental analysis. Water was distilled twice, and methanol was redistilled from the methanol used for HPLC. Spectral experiments were performed in the \( \Phi = 0.45 \) and 0.60 mixtures of MeOH and water containing 0.37 mol/L NaCl.

The steady state fluorescence spectra of Np-12 were measured on a Perkin-Elmer Luminescence Spectrometer LS 55 in the 45:55 v/v (\( \Phi = 0.45 \)) and 60:40 v/v (\( \Phi = 0.60 \)) MeOH-H_2O systems at 35 °C using a 280 nm excitation wavelength and the intensities at the 330 nm monomer emission wavelength and at 400–420 nm excimer emission were monitored.

Statistical analysis

The results are expressed as the mean values±SD. Student’s
**Results**

**Effects of deAgrs on cell adhesion**

The organic molecules including 260, 160, 568, R68, 640, ZPMOP, TB6PMOP, CNS, and TBHQ were diluted to the concentration of $10^{-4}$ mol/L and added to each well (3 wells each group) coated by monolayer endothelial cells or vascular smooth muscle cells and simultaneously induced by 10 μg/L TNF-α for 24 h. The cell adhesion rate was measured, and the results are shown in the Table 1. Significant declines in the cell adhesion rate occurred in each group pretreated with each deAgr (excluding the TBHQ) compared with those cells pretreated with TNF-α. The 260 treated group produced the greatest leukocyte adhesion inhibitory abilities, and the 160, 568, R68, 640, ZPMOP, TB6PMOP, CNS, and TBHQ groups also had reductions of leukocyte adhesion rate.

In this study, when the probe molecules Np-12 or Np-Ch were in aggregated form, the studied molecules were added to the probe aggregates. The concentration of the fluorescent probes was determined to be larger than their critical aggregate concentration (CAgC) values (from $4\times10^{-6}$ to $20\times10^{-6}$ mol/L). Graded concentrations of the organic molecules were then added to the system. If the deaggregating phenomenon occurred, the excimer emission ($I_e$) at 330 nm decreased, and the monomer emission ($I_m$) at 420 nm increased (Figure 5A). These results indicate that the aggregated probe Np-12 was broken up by the added organic molecules, and the monomeric probe was released into the bulk of the solvent. If we plot the $I_e/I_m$ vs (studied organic molecules), the absolute value (Ede) of slope $\rho$ may be used as an indicator of the deaggregating ability of these compounds (Figure 5B). The results for the probe aggregates at $10^{-6}$ mol/L are summarized in Table 2, in which larger Ede values represent greater deaggregating abilities.

![Figure 4. The chemical structure of Np-12 and Np-Ch.](image)

![Figure 5. (A) The fluorescence spectra of aggregated Np-12 ($10^{-6}$ mol/L): 1, 1.0; 2, 2.0; 3, 3.0; 4, 4.0; 5, 6.0; 6, 8.0; 7, 10.0. When the deAgrs were added to the aggregated Np-12, the excimer emission ($I_e$) at 420 nm decreased and the monomer emission ($I_m$) at 337 nm increased. (B) We plotted the $I_e/I_m$ vs the concentration of deAgr. The slope $\rho$ may be used as an indicator of the deaggregating abilities of these compounds. Larger $\rho$ values reflect greater deaggregating abilities.](image)
The examination of Table 2 reveals that the 260 compound possesses the highest deaggregating ability, whereas TBHQ has the least deaggregating ability in this study. The order of decreasing deaggregating abilities is: 260>160>568>ZPMOP>R68>640>TB6PMOP>CNS>12-AA>11-AA>TBHQ (Table 2).

Table 2. Deaggregating efficiencies of the organic molecules in terms of Ede values (×10⁻⁴ mol/L) at the fixed concentrations of Np-12 and Np-Ch (×10⁻⁵ mol/L).

| Organic molecule | Ede (×10⁻⁴ mol/L) (Np-12) | Ede (×10⁻⁴ mol/L) (Np-Ch) |
|------------------|--------------------------|---------------------------|
| TBHQ             | 1.55                     | 0.72                      |
| CNS              | 3.00                     | 1.51                      |
| TB6PMOP          | 3.43                     | 1.92                      |
| 640              | 4.02                     | 2.80                      |
| R68              | 4.81                     | 2.96                      |
| ZPMOP            | 5.02                     | 3.46                      |
| 568              | 5.24                     | 3.77                      |
| 160              | 6.89                     | 4.65                      |
| 260              | 8.74                     | 6.28                      |

Leukocyte adhesion rates correlate with deaggregating abilities of the deaggregators

Spearman correlation coefficient analyses indicated that the adherent rates of PMNs or MNCs to ECs treated with the deAgRs had highly negative correlation to their deaggregating abilities (correlation coefficient<0.001, P=-0.945; correlation coefficient<0.001, P=-0.925, respectively, Figure 6A, 6B) and the adherent rates of PMNs or MNCs to SMCs treated with deAgRs had highly negative correlation to their deaggregating efficiencies (correlation coefficient<0.001, P=-0.882; correlation coefficient<0.001, P=-0.949, respectively, Figure 6C, 6D) in the fluorescent experiments that Np-12 as the fluorescent probe.

Similarly, there was highly negative correlation between adherent rates of PMNs or MNCs to ECs treated with the deAgRs and their deaggregating abilities (correlation coefficient<0.001, P=-0.995, Figure 6E; correlation coefficient<0.001, P=-0.940, Figure 6F) and between adherent rates of PMNs or MNCs to SMCs treated with the deAgRs and their deaggregating abilities (correlation coefficient<0.001, P=-0.883, Figure 6G; correlation coefficient<0.001, P=-0.944, Figure 6H) in the studies that Np-Ch as fluorescent probe.

Discussion

Increased leukocyte adhesion is considered to be the result of the upregulation of cytokines and adhesion molecules. Some compounds, such as dimethyl sulfoxide, sodium nitroprusside, nitric oxide and the antioxidants may inhibit the adhesion between monocytes or lymphocytes and VECs or VSMCs induced by IL-1 and TNF-α. They accomplish this by inhibiting the expression of adhesion molecules or cytokines that may induce cell adhesion.

Previous studies have demonstrated that organic molecules, eg. the phospholipid and other lipids on the cell membrane, have the ability to form aggregates via HLI. Leukocyte adhesion may be considered the macroscopic behavior of the aggregation of some organic molecules, eg. phospholipids and other lipid components of cell membranes, driven by HLI. The aggregation of organic molecules in a long hydrocarbon chain is almost solely driven by HLI. HLI depends on the hydrophilic groups and lipophilic groups in the organic compounds. If some organic molecules possess the ability to deagregate an aggregated probe (Np-12 or Np-Ch), the coaggregation of deAgr with probe will be restrained, the size of the aggregate will be reduced, and the number of aggregated molecules in the aggregate will be decreased. The deaggregating abilities were evaluated using the following parameters: a) measuring the coaggregating tendencies (CoCAgC) of organic compounds with a monomeric probe, b) measuring the aggregation number of the coaggregate calculated using a single photon count experiment; c) evaluating the deaggregation efficiencies in terms of a plot of L/L0 vs the concentration of deAgrs. In this study, we adopted the latter to evaluate the deaggregating efficiencies (Figure 5B).

An effective HLI-driven deAgr may operate primarily by a mechanism depicted in a greatly simplified manner in Figure 7. The deAgr molecule can break up or reduce the size of an aggregation (Ag) or coaggregation (CoAg) mainly by two steps: (1) getting into the Ag (k in) by virtue of the HLI between the long hydrocarbon chain(s) of the deAgr and Ag molecules and (2) carrying the Ag out of the Ag (k out) into the bulk of the solvent; the Ag molecules are released in their monomeric form, by virtue of their hydrophilicity.

Based on the suggested mechanism of deaggregation described above (Figure 7), all species incorporated in the deaggregating process are in a dynamic equilibrium. The most effective deAgr should possess the best balance among its four rate constants. Otherwise, when K in (K in=k in/k out) is larger than K out (K out=k out/k in), it is easy for the deAgr to get into the Ag but difficult for it to get out; the net effect is coaggregation. When K in is much less than K out, the deAgr cannot readily get into the Ag to grab the aggregated molecules; then, deaggregation is also not realized. The four rate constants are expected to be affected by multiple factors, eg. the length and foldability of the chain, the hydrophilicity and lipophilicity, as well as the solvent aggregating power of the system.

From Table 2, we discovered that each studied molecule reduced and broke the probe aggregates to some extent, and their deaggregating abilities decreased in this order: 260>160>568>ZPMOP>R68>640>TB6PMOP>CNS>TBHQ. Previous studies have demonstrated that the organic molecules, eg. the phospholipid and other lipids on the cell membrane, have the ability to form aggregates by HLI. If leukocyte adhesion is considered the macroscopic behavior of the aggregation of some organic molecules, eg. phospholipids and other lipid components of cell membranes, driven by HLI, the organic molecules possessing deaggregating abilities capable...
Figure 6. Leukocyte adhesion rates correlate with deaggregating abilities of 8 deaggregators in vitro. (A) A change in PMN adherent to EC treated by deAgrs (y-axis) compared to the deaggregating efficiencies measured by fluorescence probe Np-12 of these deAgrs (x-axis) and E. Np-Ch as fluorescence probe. (B) MNC-EC adhesion rates compared to the deaggregating efficiencies determined by probe Np-12 and probe Np-Ch (F). (C) PMN-SMC adhesion rates compared to the deaggregating efficiencies used by Probe Np-12 and Np-Ch (G). (D) MNC-SMC adhesion rates to the deaggregating efficiencies used by probe Np-12 and Np-Ch (H). PMN: polymorphonuclear leukocytes; MNC: monocytes; EC: endothelial cells; SMC: smooth muscle cells. The concentrations of the deAgrs are 1×10^{-4} mol/L. Spearman correlation coefficients for leukocyte adhesion ratios treated by deAgrs and their deaggregating efficiencies.
of breaking up the Np-12 and Np-Ch fluorescent probe aggregates, also driven by HLI, may also be able to inhibit leukocyte adhesion. In Table 1, TNF-α (10 μg/L) induced significantly increased leukocyte adhesion to ECs and SMCs. The studied molecules, excluding TBHQ, significantly inhibited TNF-α-induced leukocyte adhesion. At the same time, their ability to inhibit leukocyte adhesion in each group is parallel to their deaggregating abilities: the more effectively the organic molecules break up the probe aggregates, the higher their inhibiting rates of leukocyte adhesion (Table 1). Further, Spearman correlation coefficient analyses indicate that the adherent rates of either neutrophils or monocytes to ECs or SMCs treated by the above deAgrs had significantly negative correlation to their deaggregating abilities (Figure 6). Our research definitely tests the inherent relationship between the deaggregating efficiencies of these deAgrs and their role in inhibiting conglomerated cells. The organic molecules possessing the deaggregating ability to break up the Np-12 and Np-Ch fluorescent probe aggregates driven by HLI may also inhibit leukocyte adhesion. However, we have not demonstrated that the studied organic molecules have the ability to suppress the expression of some cytokines or adhesion molecules.

Our results show that the deAgrs (excluding TBHQ) in this study could markedly inhibit leukocyte adhesion, especially ZPMOP, 568, 160, 260. Moreover, the strength of inhibition exhibited by the compounds in this study are highly correlated to their deaggregating tendencies, which clearly shows deAgrs can effectively inhibit TNF-α-mediated leukocyte adhesion in vitro by breaking up hydrophobic lipophilic interactions.

In summary, the fact that deAgrs inhibit leukocyte adhesion and that their inhibitory abilities increased as their deaggregating efficiencies increased suggests that deAgrs may be used to block atherogenesis by inhibiting leukocyte adhesion.

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Author contribution
Hui YANG and Jian CHEN performed the experiments; Zheng-wu SHEN, Xiu-jia ZHOU, and Guo-zhen JI provided guidance and critical review of the methods; Hui YANG and Guo-zhen JI wrote the manuscript.

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