Original Article

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

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Aim: Deaggregators (deAgs) 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 deAgs 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 μ g/L) and deAgs 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 deAgs.

Results: Among the nine deAgs 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 deAgs 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 deAgs had significantly negative correlation to their deaggregating abilities.

Conclusion: DeAgs 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

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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, atherogen-

esis 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, *eg*, cholesteryl esters, triglycerides, and phospholipids, may be directly related to their behaviors in causing athero-

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sclerosis^[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^[9].

In this study, we used tumor necrosis factor- α (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-H₂O binary system of different Φ values, *ie*, the volume fraction of the organic component of the aquiorcano 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 Ca²⁺ or Mg²⁺. The pial membranes were removed, and the cerebral cortices were cleaned of white matter, scissor-minced and homogenized in HBSS. The homogenate was centrifuged, and the pellet was resuspended in 15% dextran (M_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 $\times g$. 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 $\times g$ 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- μ m 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% CO₂ 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 μ g/mL epidermal cell growth factor (ECGF, Roche, Austria) was added. The culture dish was incubated at 37°C in a humidified 5% CO₂ 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)^[17, 18].

Figure 1. (A) Images of primary cultured cerebral microvascular endothelial cells under a light scope ($\times 200$). (B) Immunohistochemical images indicating the expression of the Von Willebrand factor to characterize the cultured cells as being endothelial cells from normal growth of the third generation ($\times 100$). Positive cells are more than 95%.

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 $\times$ 1 mm $\times$ 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% CO₂ 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)^[19].

Figure 2. (A) Normal growth of primary cultured vascular smooth muscle cells ($\times 100$). 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 $300\times g$ for 20 min. The platelet-rich plasma layer was carefully aspirated and centrifuged at $2500\times g$ for 15 min to produce platelet-poor plasma (PPP). Five milliliters of 6% dextran (M_w 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 $275\times g$ for 6 min. The pellet was resuspended in 2 mL PPP and transferred to a 10-mL glass graduated 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 centrifuged for 10 min at $275\times g$. Neutrophils in a wider band at the interface of the 42% and 51% Percoll layers were aspirated into a 10-mL glass centrifuge tube using a polyethylene transfer pipette and centrifuged at $275\times g$ for 10 min. One milliliter 0.2% sodium chloride was forcefully added to the pellet with a syringe for lysing the erythrocytes. The mixture was swirled continuously for a maximum of 20 s, and 1 mL of 1.6% sodium chloride was immediately added to stop lysis of the neutrophils and centrifuged at $275\times g$ for 10 min. The pellet was washed once with 0.01 mol/L phosphate buffer saline (PBS). The supernatant was removed, and the pellet was resuspended in DMEM to a final concentration of 2×10^5 /mL to be used for the adhesion assay^[20].

Isolation of monocytes

Twenty milliliters of blood collected from the inferior vena cava of SD rats were transferred to a 50 mL tube containing 200 U heparin, and an equal volume of 0.9% NaCl was used to dilute the blood. Then, 20 mL diluted blood was carefully

layered over 10 mL Lymphoprep^{TM} (Pharmacia) in two new 50 mL centrifuge tubes. Alternatively Lymphoprep^{TM} was underlayered. The tube was capped to prevent the formation of aerosols and centrifuged at $800\times g$ for 20 min at room temperature in a swing-out rotor. If the blood had been stored for more than 2 h, the centrifugation time would be increased to 30 min. After centrifugation, the mononuclear cells formed a distinct band at the sample/medium interface. 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 $250\times g$ and then harvested mononuclear cells containing approximately 20% monocytes were washed twice with PBS and resuspended to concentration of 5×10^6 /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 2×10^5 /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 2×10^5 /mL were seeded in 96-well dishes (200 μ L 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 μ g/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 10^{-4} mol/L of a series of deAgns 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 μ L of 2×10^5 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 μ L 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^[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^[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^[9, 26] (Figure 4). The principle of fluorescence studies of deaggregation is based on the following premise. The formation of aggregates in aqueous or aquiorgano solvents from organic molecules with a long hydrocarbon chain is almost solely driven by HLL. 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 ρ 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^[9, 16, 27] (Figure 5B).

Pure, analytical grade NaCl, Na₂HPO₄, and NaH₂PO₄ were used without further purification. The fluorescent probe Np-12 and Np-Ch were prepared^[16, 27]. All of the synthesized substrates were identified by ¹H 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 $45:55 \text{ v/v}$ ($\Phi=0.45$) and $60:40 \text{ v/v}$ ($\Phi=0.60$) MeOH-H₂O systems at 35°C using a 280 nm excitation wavelength and the intensities at the 330 nm monomer emission wavelength and at $400\text{--}420 \text{ nm}$ excimer emission were monitored.

Statistical analysis

The results are expressed as the mean values $\pm$ SD. Student's

Figure 3. The chemical structure of the organic molecules in this study.

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 ($\times 10^{-4}$ mol/L) at the fixed concentrations of Np-12 and Np-Ch ($\times 10^{-6}$ mol/L).

Organic molecule	Ede ($\times 10^{-4}$ mol/L) (Np-12)	Ede ($\times 10^{-4}$ 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^[28], sodium nitroprusside^[22], nitric oxide^[22, 29, 30] and the antioxidants^[31] 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^[9]. 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^[24]. The formation of aggregates in water or water-organic solvent mixtures from organic molecules with a long hydrocarbon chain is almost solely driven by HLI^[9]. HLI depends on the hydrophilic groups and lipophilic groups in the organic compounds^[25]. If some organic molecules possess the ability to deaggregate 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 I_e/I_m vs the concentration of deAgrs^[25]. 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^[9, 27]. 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 Agr molecules and (2) carrying the Agr out of the Ag (k_{out}) into the bulk of the solvent; the Agr 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} ($K_{in}=k_{in}/k_{in}^{-1}$) is larger than K_{out} ($K_{out}=k_{out}/k_{out}^{-1}$), 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^[9]. 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.

Figure 7. Hypothetical mechanism of deaggregation. K_{in} and K_{out} denote the ability of deAgrs to get into the Ag and go out of the Ag with grabbed aggregated molecules, respectively. Initially, a deAgr gets into the Ag to form CoAg by its long hydrocarbon chain(s) driven by HLI. Then, it carries one or two aggregating molecule(s) out of the Ag and releases the aggregating molecules in their monomeric form, by virtue of their hydrophilicity. The deaggregating abilities of the deAgr are closely dependent on K_{in} and K_{out} . Only when the two interactions reach an optimal balance can a deAgr exert the desirable pharmacological effects.

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 $\mu\text{g/L}$) 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 conglutinated 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.

References

- Okada M, Sugita O, Miida T, Matsuto T, Inano K. Effects of modified low density lipoprotein and hypoxia on the expression of endothelial leukocyte adhesion molecule-1. *Presse Med* 1995; 24: 483–8.
- Li JJ. Inflammation in coronary artery diseases. *Chin Med J (Engl)* 2011; 124: 3568–75.
- Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *New Engl J Med* 2005; 352: 1685–95.
- Russell R. Atherosclerosis-an inflammatory disease. *New Engl J Med* 1999; 340: 115–26.
- Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R. Up regulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol* 1998; 18: 842–51.
- Boring L, Gosling J, Chensue SW, Kunkel SL, Farese RV Jr, Broxmeyer HE, et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 1997; 100: 2552–61.
- Simpson PJ, Todd RF 3rd, Fantone JC, Mickelson JK, Griffin JD, Lucchesi R. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. *J Clin Invest* 1988; 81: 624–9.
- Granger DN, Kubes P. The microcirculation and inflammation: modulation of leukocyte-endothelial cell adhesion. *J Leukoc Biol* 1994; 55: 662–75.
- Ji GZ, Long YQ, Jiang XK. Study on the aggregation and deaggregation behaviors of phosphatidylcholines. *Chin J Chem* 2003; 21: 731–8.
- Jiang XK. Hydrophobic-lipophilic interactions, aggregation and self-coiling of organic molecules. *Acc Chem Res* 1988; 21: 362–7.
- Jiang XK. New concepts for studying and understanding the aggregation, coaggregation, deaggregation and self-coiling of organic molecules. Why are cholesterol and triglycerides culprits of arteriosclerosis? *Pure Appl Chem* 1994; 66: 1621–8.
- Blokziji W, Engberts Jan BFN. Hydrophobic effects. Opinions and facts. *Angew Chem Int Ed Engl* 1993; 32: 1545–79.
- Jiang XK, Li XY, Huang BZ. The effect of hydrophobic-lipophilic interactions on chemical reactivity 9: Putting the spotlight on lipophilic forces in solvent-effect studies. *Proc Indian Acad Sci (Chem Sci)* 1987; 98: 409–21.
- Wu Z, Hofman FM, Zlokovic BV. A simple method for isolation and characterization of mouse brain microvascular endothelial cells. *J Neurosci Methods* 2003; 130: 53–63.
- Jiang XK, Wang JS. Chain-foldability effect on aggregation. Evidents derived from time-resolved fluorescence spectroscopy. *Chin J Chem* 1993; 11: 187–9.
- Zhang JT, Ji GZ, Jiang XK. Fluorescence study on the coaggregating tendencies of the cholesterol-the culprits of arteriosclerosis. *Chin J Chem* 1994; 12: 277–82.
- Ji GZ, Zhang GM, Tang L, Jiang XK. Deaggregation behavior of saponin compounds. *Acta Chim Sin* 2003; 61: 970–5.
- Diglio CA, Grammas P, Giacomelli F, Wiener J. Primary culture of rat cerebral microvascular endothelial cells. Isolation, growth, and characterization. *Lab Invest* 1982; 46: 554–63.
- Rothman A, Kulik TJ, Taubman MB, Berk BC, Smith CW, Nadal-Ginard B.

- Development and characterization of a cloned rat pulmonary arterial smooth muscle cell line that maintains differentiated properties through multiple subcultures. *Circulation* 1992; 86: 1977–86.
- 20 Haslett C, Guthrie LA, Kopaniak MM, Johnston RB Jr, Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* 1985; 119: 101–10.
- 21 Jones BM, Nicholson JK, Holman RC, Hubbard M. Comparison of monocyte separation methods using flow cytometric analysis. *J Immunol Methods* 1989; 125: 41–7.
- 22 Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF- α -activated vascular endothelium under flow. *Blood* 2005; 106: 584–92.
- 23 Issekutz TB, Wykretowicz A. Effect of a new monoclonal antibody, TA-2, that inhibits lymphocyte adherence to cytokine stimulated endothelium in the rat. *J Immunol* 1991;147: 109–16.
- 24 Chen J, Yang, F, Ji G, Yang H, Shen ZW, Zhou XJ. Deaggregation behavior of the phenol gemini derivatives. *Langmuir* 2005; 21: 10931–40.
- 25 Chen J, Yang H, Shen ZW, Zhou XJ, Yang F, Ji GZ. The deaggregation behavior of the phenol derivatives. *Chin J Chem* 2005; 23: 1552–60
- 26 Jiang XK, Ji GZ, Tu B, Zhang XY, Shi JL, Chen X. Shape and aggregating tendency. The aggregating behavior of eight esters of eight-carbon carboxylic acids. *J Am Chem Soc* 1995; 117: 12679–82.
- 27 Jiang XK, Ji GZ, Zhang JT. Effective neutral deaggregators. *Langmuir* 1994; 10: 122–5.
- 28 Sekizuka E, Benoit JN, Grisham MB, Granger DN. Dimethylsulfoxide presents chemoattractant-induced leukocyte adherence. *Am J Physiol* 1989; 256: H594–7.
- 29 De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, et al. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 1995; 96: 60–8.
- 30 Gauthier TW, Scalia R, Murohara T, Guo JP, Lefer AM. Nitric oxide protects against leukocyte endothelium interactions in the early stages of hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 1995; 15: 1652–9.
- 31 Cominacini L, Garbin U, Pasini AF, Davoli A, Campagnola M, Contessi GB, et al. Antioxidants inhibit the expression of intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 induced by oxidized LDL on human umbilical vein endothelial cells. *Free Radic Biol Med* 1997; 22: 117–27.