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ORIGINAL ARTICLE

# Antroquinonol, an Active Pure Compound from *Antrodia Camphorata* Mycelium, Modulates the Development of Atherosclerosis in a Mouse Carotid Artery Ligation Model

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**Background:** Antroquinonol (Antroq) is an active component of Antrodia camphorate. The present study was to validate the preventive effects of Antroq in an atherosclerosis model. **Materials and Methods:** We examined Antroq inhibitory effect on rat aortic smooth muscle cell proliferation and migration and evaluated its effect on neointima formation and inflammation in mouse carotid artery ligation (CAL). **Results:** Our data show that Antroq[1] inhibited the proliferation (Antroq [3.0 μg/ml] + PDGF 41.7 ± 7.3%, vehicle + PDGF 134.5 ± 7.3%) ($p$<0.005) and migration (6h: Antroq [3.0 μg/ml] + PDGF 0.9 ± 0.3%, vehicle + PDGF 25.0 ± 3.4%; 12h: Antroq [3.0 μg/ml] + PDGF 4.0 ± 1.6%, vehicle + PDGF 40.5 ± 2.2%; 24h: Antroq [3.0 μg/ml] + PDGF 14.2 ± 3.0%, vehicle + PDGF 59.8 ± 3.3%) (each, $p$<0.005) of the cultured smooth muscle cells,[2] prevented neointima formation and reduced N/M ratios in CAL mice (900 μm: Antroq + CAL 0.8 ± 0.3, CAL 3.5 ± 1.1; 800 μm: Antroq + CAL 0.6 ± 0.2, CAL 3.5 ± 0.7; 700 μm: Antroq + CAL 0.7 ± 0.2, CAL 3.8 ± 0.4; 600 μm: Antroq + CAL 0.9 ± 0.2, CAL 3.8 ± 0.9; 500 μm: Antroq + CAL 1.3 ± 0.4, CAL 3.9 ± 0.8; 400 μm: Antroq + CAL 1.5 ± 0.5, CAL 4.0 ± 1.0; 300 μm: Antroq + CAL 1.8 ± 0.6, CAL 3.5 ± 0.6; 200 μm: Antroq + CAL 2.3 ± 0.6, CAL 4.6 ± 1.1) (each, $p$<0.01),[3] and prevented inflammatory processes and matrix accumulation/fibrosis in the CAL mice. **Conclusions:** Our data may be useful in developing new and practical strategy for the prevention of atherosclerosis based on the pathogenesis of the disorder.

Key words: Antroquinonol, *Antrodia camphorate*, atherosclerosis, carotid artery ligation, smooth muscle cell

## INTRODUCTION

Atherosclerosis is a leading cause of morbidity and mortality in the world.[1-4] Atherosclerosis occurs commonly at certain areas of the great and medium-sized arteries with disturbed blood flow, such as the branched or curved sites.[5,6] Although various systemic factors such as hyperlipidemia, diabetes, hypertension, and smoking are closely linked to the disease, inflammatory responses have been increasing considered to play a key pathogenic role in the development and progression of atherosclerosis.[1-4] In the early stage of this inflammatory process, inflammatory cells start to approach or adhere to the endothelial cells in locations where turbulent blood flow exists and adhesion molecules are preferentially expressed.[7] Besides, acute inflammation, along with the activation of leukocytes and platelets that circulate in the blood, endothelial cells of the vascular wall as well as the upregulation of cellular adhesion molecules, is also implicated in the early vascular reaction to physical or mechanical injury, whereby resulting in a restenotic lesion.[8,9] As a result, these leukocyte-endothelial interactions incur further cellular activation and production and secretion of cytokines and growth factors, together promoting the migration and proliferation of smooth muscle cells (SMCs) of the artery affected and the development of artherosclerotic lesions.[7,10] Clinically, various therapeutic regimens and strategies for the prevention and treatment of atherosclerosis have been used, but the outcome remains unsatisfactory, and thus to develop novel yet practical therapeutic strategies are warranted.

*Antrodia camphorata*, a fungus parasitic on *Cinnamomum*

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*kanehirai* Hay trees that mainly grow in Taiwan,[11] has been used as a folk medicine for the treatment of diarrhea, abdominal pain, hypertension, and dermatitis. This Chinese medicine has several pharmacological effects:

1. Inhibition of inflammation,[12,13]
2. Antioxidant activities,[14] and
3. Cytotoxicity for tumor cells.[15]

In addition, *A. camphorata* mycelial extract can protect the kidney from immunological damage in lupus-prone mice by inhibiting the synthesis and release of interleukin-1β and tumor necrosis factor-α.[16] Our previous studies show that antroquinonol (Antroq), an active pure compound from *A. camphorata* mycelium, can modulate inflammatory responses and reduce oxidative stress in of chronic kidney disease models featuring chronic inflammation and fibrosis. These preliminary results prompted us to examine whether Antroq is a potential agent for the prevention of atherosclerosis. In this study, we demonstrated that a short-term Antroq treatment was preventive from the development of atherosclerosis in a carotid artery ligation (CAL) model in mice.

## MATERIALS AND METHODS

### Antroquinonol preparation

Antroquinonol, pure compound, was provided by the Golden Biotechnology Corp., Taipei, Taiwan. Antroq was originally isolated from the solid-state fermented mycelium of *A. camphorata*, as described previously.[12]

### Culture of smooth muscle cells

A rat aortic SMCs (CRL-1444) was obtained from American Type Culture Collection, and maintained in dulbecco modified eagle medium (Invitrogen, CA, USA) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin, in a humidified atmosphere of 5% $CO_2$ in the air at 37°C. Cell cultures were free of mycoplasma.

### Cytotoxicity assay of smooth muscle cells

Smooth muscle cells ($5 \times 10^3$ cells/well) were plated in 96-well plates. The cells were incubated with the presence or absence of Antroq. (0.1, 0.3, or 3.0 μg/mL) in 24 h. Then, 50 μL cell-free culture supernatant were collected from each well for further assay. Cytotoxicity induced was assessed by lactate dehydrogenase using commercial kits from Promega (WI, USA) according to manufacturer instructions. $n = 3$ each time, triplicated. The absorbance was recorded using a multi-mode enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek, MA, USA). Results were presented as a percentage of positive control values.

### Migration assay of smooth muscle cells

A wound scratch assay[17] was used to evaluate the influence of Antroq on the migration of SMCs. SMCs ($5 \times 10^5$/well) were plated in 6-well plates. The cells were incubated with the presence or absence of Antroq (0.1, 0.3, or 3.0 μg/mL) for 20 h before primed with or without Platelet-derived growth factor (PDGF) (10 ng/mL) (Roche, IN, USA) for 6, 12, or 24 h. $n = 3$ each time, triplicated. The migration ability was expressed by the migration distance of drug-treated cells (μm) divided by the migration distance of untreated cells (mm).

### Proliferation assay of smooth muscle cells

The effect of Antroq on SMCs proliferation was evaluated using MTT proliferation assay. SMCs ($5 \times 10^3$/well) were plated in 96-well culture plates. The cells were incubated the presence or absence of Antroq (0.1, 0.3, or 3.0 μg/mL) for 20 h before primed with or without PDGF-BB (10 ng/mL) (Roche) for 24 h. Methyl thiazoleterazolium (5 mg/ml; Sigma, MO, USA) was added (20 μl/well) and the mixture was incubated for 3 h at 37°C. Dimethy-sulforide (Merck, Darmstadt, Germany) was then added (150 μl/well) for 15 min as described previously.[18] $n = 3$ each time, triplicated. The absorbance at 540 nm was determined using a multi-mode ELISA plate reader (Bio-Tek).

### Carotid artery ligation model

C57BL/6 mice (8 weeks) were obtained from the National Laboratory Animal Center (Taipei, Taiwan, R.O.C), and maintained at the animal center of the National Defense Medical Center, Taipei, Taiwan. The mice were treated daily with 60 mg/kg of Antroq or with corn oil (vehicle) by oral gavage for 4 weeks, the first dose being given 1 day before CAL induction ($n = 10$ mice per group). CAL model was induced by ligation of left common carotid artery near the carotid bifurcation with a 6-0 silk under anesthesia, as described previously.[19] All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taiwan, and complied with the NIH Guide for the Care and Use of Laboratory Animals.

### Pathology evaluation

Tissues were fixed in 10% buffered formalin and embedded in paraffin, and then sections (4 μm) were cut at 100 mm intervals and stained with hematoxylin and eosin (H & E). The neointima was evaluated by subtracting the luminal area from the internal elastic lamina (IEL) area, and the media area was calculated by subtracting the IEL area from the external elastic lamina (EEL) area. The ratio of neointima to media area (N/M ratio) was measured using the Adobe Photoshop software program with mild modification as described previously.[19,20]

The areas of the 10 sections were analyzed and average in each animal. N/M ratio= (IEL area - luminal area) / (EEL area-IEL area). All pictures were taken under light microscope attached with a digital camera.

### Immunohistochemistry and detection of apoptosis

Paraffin-embedded tissue sections were prepared for immunohistochemistry (IHC) with rat anti-F4/80$^+$ (Serotec, NC, USA) antibodies as described previously.[21] ApopTag Plus Peroxidase *in situ* Apoptosis Detection kits (Chemicon, CA, USA) were used for detection of apoptosis according to the manufacturer's instructions. Scoring of the IHC or TUNEL results was performed using Pax-it quantitative image analysis software (Paxcam, IL, USA) as described previously.[21]

### Statistical analysis

Data were presented as the mean ± SE. Comparisons of the semi-quantitative measurements from the culture cell proliferation, cytotoxicity, and migration activity assays among groups were made with analysis of variance (ANOVA) method with least significant difference *post-hoc*. Comparisons of IHC and TUNEL measurements were also performed using ANOVA method. Regression model was adopted for prediction of N/M ratio with a range of 200-900 μm away from the site of ligation. $P < 0.05$ was considered as statistically significant.

## RESULTS

### *In vitro* experiments in smooth muscle cells

Cytotoxicity of antroquinonol

The optimal doses of Antroq for *in vitro* study were determined in a dose dependent manner. As shown in Figure 1, the percentages of cytotoxicity of different doses of Antroq treatment in SMCs were 0.1% for 0.1 μg/ml, 0.5% for 0.3 μg/ml, 1.0% for 3 μg/ml, respectively. There were no significantly toxic effects to confluent SMCs in different doses of Antroq on the cells.

### Antroquinonol retards migration and proliferation

The dedifferentiation of contractile and quiescent SMCs to migratory, proliferative, and synthetic phenotype in the intima has been implicated in the development of atherosclerosis.[10,22] As shown in Figure 2, the inhibitory effects of Antroq arose as early as 6 h after the incubation with Antroq in the PDGF-treated SMCs, and this effect persisted until 24 h in a dose-dependent manner, as demonstrated by wound scratch assay (6 h: Antroq [0.1 μg/ml] + PDGF 8.0 ± 4.1%, Antroq [0.3 μg/ml] + PDGF 7.7 ± 3.8%, Antroq [3.0 μg/ml] + PDGF 0.9 ± 0.3%, vehicle + PDGF 25.0 ± 3.4%; 12 h: Antroq [0.1 μg/ml] + PDGF 32.8 ± 1.4%, Antroq [0.3 μg/ml] + PDGF 28.8 ± 1.6%, Antroq [3.0 μg/ml] + PDGF 4.0 ± 1.6%, vehicle + PDGF 40.5 ± 2.2%; 24 h: Antroq [0.1 μg/ml] + PDGF 46.8 ± 1.2%, Antroq [0.3 μg/ml] + PDGF 43.9 ± 2.9%, Antroq [3.0 μg/ml] + PDGF 14.2 ± 3.0%, vehicle + PDGF 59.8 ± 3.3%) (each, $P < 0.05$). To test inhibitory effects of Antroq on the proliferation

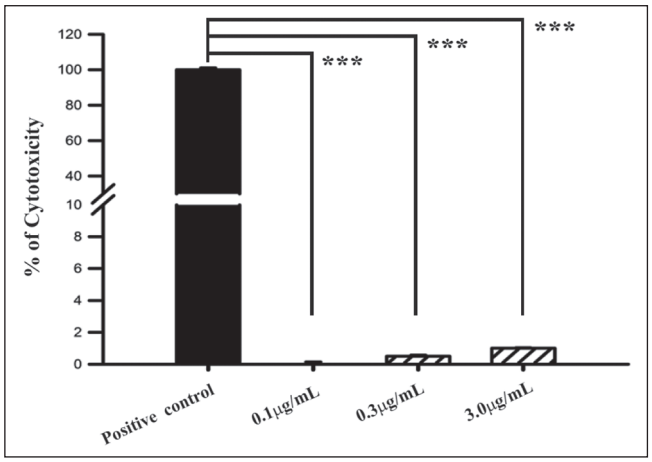


**Figure 1.** Cytoxicity test. The percentages of cytotoxicity in different doses of Antroq (0.1, 0.3, or 3.0 μg/mL) treatment in smooth muscle cells. ***$P < 0.005$. $n = 3$ each time, triplicated. Analysis of variance method with least significant difference *post-hoc* were used

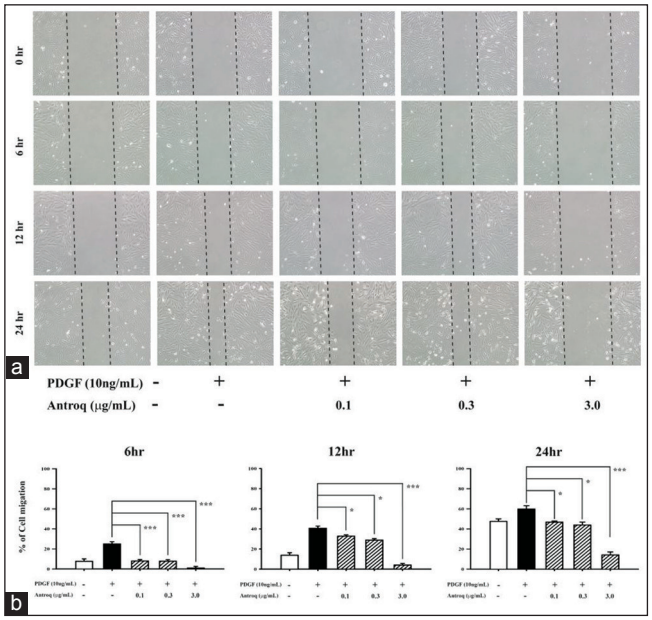


**Figure 2.** Inhibitory effects of Antroq on migration of smooth muscle cells (SMCs). (a) Representative photographs of SMCs for migration assay. The cells incubated with or without Antroq (0.1, 0.3, or 3.0 μg/mL) before primed with or without platelet-derived growth factor (10 ng/mL). Original magnification, each, ×400. (b) Quantitative analysis. *$P < 0.05$, ***$P < 0.005$. $n = 3$ each time, triplicated. Analysis of variance method with least significant difference *post-hoc* were used

of SMCs, PDGF-treated SMCs were incubated with Antroq in a dose dependent manner. As shown by MTT assay, the percentage of proliferation was significantly increased in PDGF-treated SMC (134.5 ± 7.3%) compared with saline-treated cells (100 ± 1.0%) ($P < 0.005$), and this effect was markedly inhibited in the cells incubated with Antroq at 3 μg/ml (41.7 ± 7.3%) ($P < 0.005$) [Figure 3].

### *In vivo* experiment

Antroquinonol prevents neointima formation

Intimal injury causes vascular repair by the overgrowth of endothelial and vascular SMCs[22-24] and can mimic an acute induction of atherosclerosis. We used CAL model to assess the effects of Antroq on the development and evolution of neointima. The Antroq-treated CAL mice showed a greatly suppressed neointima formation when compared to corn oil-treated CAL (disease control) mice [Figure 4a]. Besides, Antroq also prevented the development of focal necrosis, fibrin deposits, prominent leukocyte infiltration, apoptotic figures, and focal matrix accumulation/fibrosis along the arterial wall in the CAL mice. Further analyses with serial sections of the carotid artery affected showed that there were significantly reduced N/M ratios in the Antroq-treated CAL mice compared to those of corn oil-treated (disease control), with a range of 200 - 900 mm away from the site of ligation (900 μm: Antroq + CAL 0.8 ± 0.3, CAL 3.5 ± 1.1; 800 μm: Antroq + CAL 0.6 ± 0.2, CAL 3.5 ± 0.7; 700 μm: Antroq + CAL 0.7 ± 0.2, CAL 3.8 ± 0.4; 600 μm: Antroq + CAL 0.9 ± 0.2, CAL 3.8 ± 0.9; 500 μm: Antroq + CAL 1.3 ± 0.4, CAL 3.9 ± 0.8; 400 μm: Antroq + CAL 1.5 ± 0.5, CAL 4.0 ± 1.0; 300 μm: Antroq + CAL 1.8 ± 0.6, CAL 3.5 ± 0.6; 200 μm: Antroq + CAL 2.3 ± 0.6, CAL 4.6 ± 1.1) (each, $P < 0.01$) [Figure 4b]. These findings support that Antroq prevents neointima formation in CAL model. Collectively, our results suggest that inhibition of inflammatory pathways may account for the preventive effects of Antroq on the mouse model of atherosclerosis.

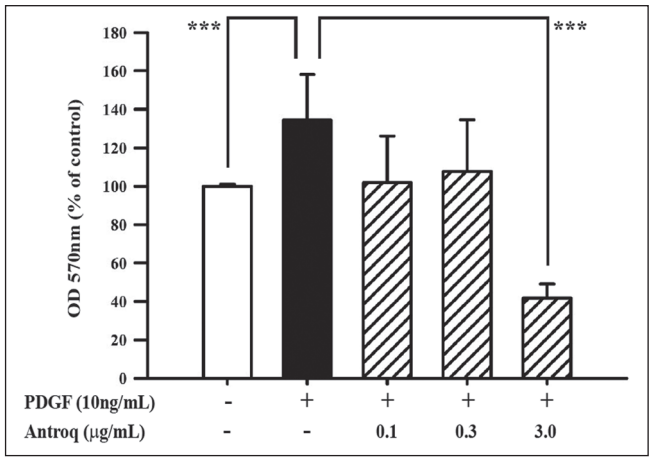


**Figure 3.** Inhibitory effects of antroquinonol (Antroq) on proliferation of smooth muscle cells (SMCs). The percentages of proliferation in different doses of Antroq (0.1, 0.3, or 3.0 μg/mL) treatment before primed with or without platelet-derived growth factor (10 ng/mL) in SMCs. ***$P < 0.005$. $n = 3$ each time, triplicated. Analysis of variance method with least significant difference *post-hoc* were used

Antroquinonol inhibits inflammation and apoptosis

CAL mice showed significantly increased infiltration of macrophages (3.3 ± 1.2 cells) (F4/80$^+$) compared with sham mice (0.2 ± 0.1 cells), and this effect was markedly inhibited in Antroq-treated CAL mice (1.0 ± 0.5 cells) ($P < 0.01$) [Figure 5a and c]. Meanwhile, as shown by TUNEL staining, the CAL mice showed greatly increased levels of apoptosis (6.3 ± 2.0 cells) compared to sham mice (0.6 ± 0.2), and this effect was significantly inhibited

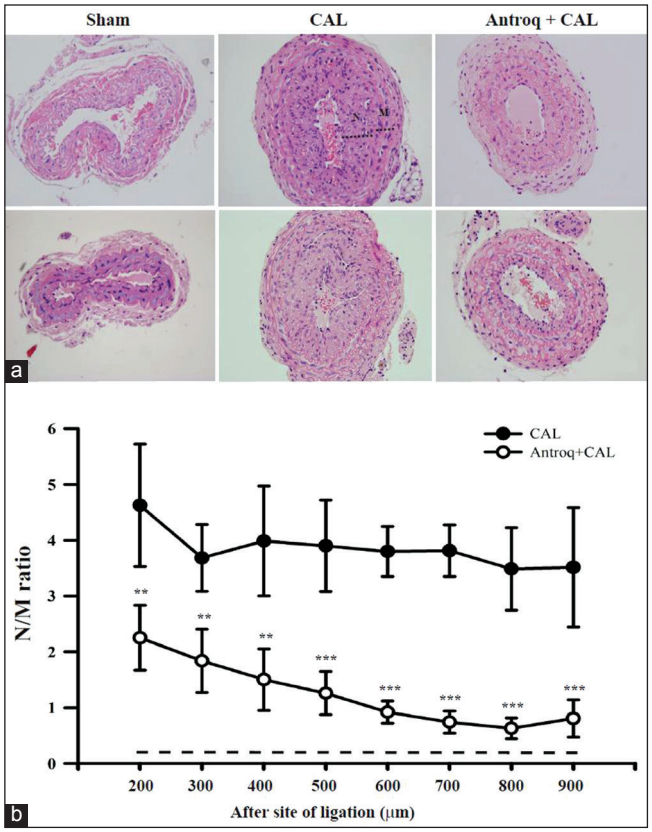


**Figure 4.** Pathology of the arterial atherosclerotic lesion. (a) H and E staining. Original magnification, each, ×400; (b) N/M ratio. Antroquinonol (Antroq)-treated carotid artery ligation (CAL) mice showed significantly reduced N/M ratios compared to those of corn oil-treated (disease control), from 200 to 900 μm away from the site of ligation. The horizontal dashed line indicates the mean of levels from normal control mice. CAL, carotid artery ligation model; N/M ratio, ratio of neointima to media area. The data are the mean ± SE for eight mice per group. **$P < 0.001$, ***$P < 0.005$. $n = 10$ mice per group. Prediction of N/M ratio with the CAL (X1) and Antroq-treated CAL mice (X2) using regression model to fit the explanatory formulas of $Y = \beta0 + \beta1X1 + \beta2X2$, intercept = 4.403, β1: −0.213, β2: −2.257, $R^2 = 0.924$

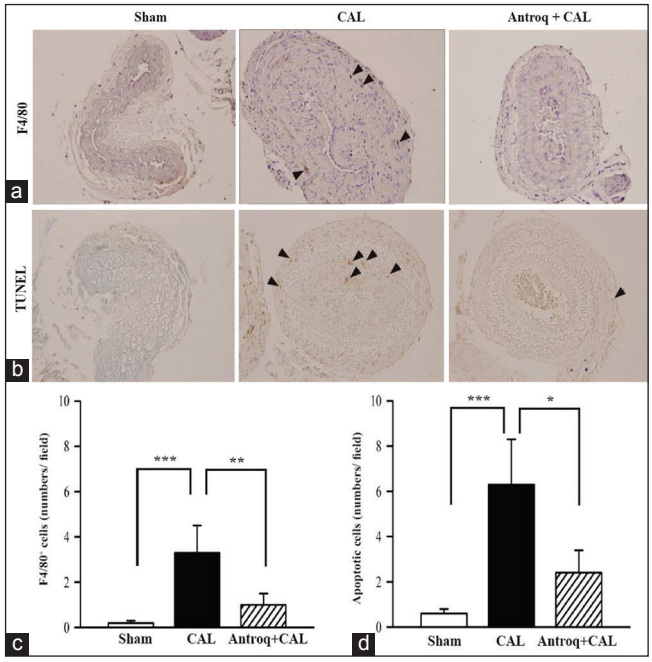


**Figure 5.** Expression of macrophage and apoptosis. (a) Immunofluorescence staining of F4/80+. (b) TUNEL staining. (c and d) Scoring of positive cells. Original magnification, ×400. Arrows indicate positive staining. *$P < 0.05$, **$P < 0.001$. $n = 10$ mice per group. Analysis of variance method was used

in Antroq-treated CAL mice (2.4 ± 1.0 cells) ($P < 0.05$) [Figure 5b and d].

## DISCUSSION

Although many therapeutic approaches for atherosclerosis have been used over decades, no specific treatment has yet been established. In this study, we showed that Antroq which is pure compound isolated from a pure active compound from *A*. *camphorata* mycelium was capable of preventing the development of atherosclerosis in a SMC model and a mouse CAL model as demonstrated, respectively. In SMCs, Antroq inhibited both the proliferation and migration of the cultured cells, while, *in vivo*, it prevented neointima formation and reduced N/M ratios in CAL mice. Although focal necrosis, fibrin deposits, prominent leukocyte infiltration, apoptotic figures and marked thickening of the intima as well as focal matrix accumulation/fibrosis along the arterial wall in CAL mice, Antroq administration was shown to greatly ameliorate the histopathological alterations. To the best of our knowledge, this is the first presentation of using Antroq to treat a mouse atherosclerosis model shortly induced by ligation of carotid artery.

It is suggested that the inhibitory effects on migration and proliferation of SMCs might partly account for the attenuated severity of the atherosclerotic lesion in the CAL model. Besides, our previous studies[16,21,25] showed that Antroq can inhibit renal inflammation, reduce oxidative stress and/or inhibit apoptosis in mouse models of IgA nephropathy, focal segmental glomerulosclerosis and lupus nephritis. Since inflammatory processes are highly considered to play a key pathogenic pathway in atherosclerosis,[2,3,5] we infer that the mechanistic events involved in the mode of action of Antroq that benefited this CAL model might consist of (1) antiinflammation, (2) antioxidative stress and (3) antiapoptosis, although other systemic effects and related pathways might be relevant and need to be further investigated.

In this study, the CAL mice that Antroq + CAL mice showed no detectable physical difference compared to those without the treatment throughout the study. This finding supports that Antroq might be a good choice for being considered as a candidate of good lead compounds in terms of drug development, although other experiments such as pharmacological analyses and toxicity need to be performed.

In summary, Antroq was able to prevent the development of atherosclerosis in CAL mice through potential mechanistic pathways, such as inhibiting the activation and migration of SMCs of the arterial wall to the intima, antiinflammation [Figure 5a and c], antioxidative stress and antiapoptosis [Figure 5b and d], although it couldn't restore the pathological changes of the arterial lesion to nearly normal. This effect suggests that in addition to the mechanistic events we proposed responsible for its effects on the atherosclerosis model, there might be other pathways involved in the mode of action of Antroq. Our data may be useful in developing new and practical strategy for the prevention and treatment of atherosclerosis based on the pathogenesis of the vascular disorder. Besides, Antroq can prove to be complementary ingredient for the management of atherosclerosis in the near future.

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## DISCLOSURE

All authors declare no competing financial interests.

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