Xanthohumol Blocks Proliferation and Migration of Vascular Smooth Muscle Cells in Vitro and Reduces Neointima Formation in Vivo

Rongxia Liu,† Elke H. Heiss,*‡ Daniel Schachner,‡ Baohong Jiang,§ Wanhui Liu,† Johannes M. Breuss,† Verena M. Dirsch,‡ and Atanas G. Atanasov*,‡∥

†School of Pharmacy, Key Laboratory of Molecular Pharmacology and Drug Evaluation (Yantai University), Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Yantai University, Yantai, 264005, People’s Republic of China
‡Department of Pharmacognosy, University of Vienna, Vienna, 1090, Austria
§State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, 201203, People’s Republic of China
∥Center for Physiology and Pharmacology, Institute for Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, 1090, Austria

ABSTRACT: Xanthohumol (1) is a principal prenylated chalcone found in hops. The aim of this study was to examine its influence on platelet-derived growth factor (PDGF)-BB-triggered vascular smooth muscle cell (VSMC) proliferation and migration in vitro and on experimentally induced neointima formation in vivo. Quantification of resazurin conversion indicated that 1 can inhibit PDGF-BB-induced VSMC proliferation concentration-dependently (IC₅₀ = 3.49 μM). Furthermore, in a wound-healing assay 1 potently suppresses PDGF-BB-induced VSMC migration at 15 μM. Tested in a mouse femoral artery cuff model, 1 significantly reduces neointima formation. Taken together, we show that 1 represses PDGF-BB-induced VSMC proliferation and migration in vitro as well as neointima formation in vivo. This novel activity suggests 1 as an interesting candidate for further studies addressing a possible therapeutic application to counteract vascular proliferative disease.

Cardiovascular disease (CVD) remains a leading cause of morbidity and mortality in the world, despite recent improvements in therapeutic approaches.1,2 It is primarily caused by atherosclerosis and kept in check by cardiovascular interventions including angioplasty, stenting, and bypass, which are already widely used in clinics.3 However, restenosis (renarrowing) of surgically dilated arteries occurs with high rates and is a very serious complication of these interventions. The pathology of atherosclerosis and restenosis involves primarily intimal hyperplasia, due to proliferation and migration of vascular smooth muscle cells (VSMCs).4 This pathological process is started by vessel injury, which triggers a series of inflammatory responses controlled by various circulating growth factors and inflammatory cytokines.5–7 Of the involved injury-induced factors, platelet-derived growth factor-BB (PDGF-BB) has been reported as the most potent inducer of VSMC proliferation and migration.7 The abnormal VSMC proliferation and migration lead to neointima formation and eventually result in arterial lumen narrowing.8,9 Thus, to prevent neointima hyperplasia, one good strategy is to inhibit PDGF-BB-induced proliferation and migration of VSMCs.

Plant-derived natural products proved to be an excellent resource for the identification of new lead compounds.10,11 Hops, the cones from Humulus lupulus L., are used in the brewing industry to flavor beer and also in folk medicine. Many pharmacological activities have been reported for this plant, including sedative, estrogenic, anticancer, antibacterial, and cardioprotective properties.12,13 Xanthohumol (1; Figure 1), a major bioactive constituent of hops, possesses anti-inflammatory, antioxidant, anticancer, antiobesity, and hepatoprotective properties.

Figure 1. Chemical structure of xanthohumol.
activities. Regarding an influence on the (cardio)vascular system, 1 hinders angiogenesis presumably by inhibition of nuclear factor-kB- and growth factor-induced AKT signaling. It also prevents platelet activation and aggregation, with an apparent contribution of diminished AKT, p38, and PLCγ2/PKC signaling as well as reduced thromboxane 2 formation and [Ca2+]i. A modulated calcium signaling may also account for potential antiarrhythmic properties of 1. Furthermore, inhibition of cholesteryl ester transfer protein and promotion of reverse cholesterol transport afford protection from atherosclerosis by 1. Despite these promising data in relevant test systems, the potential impact of 1 on intimal hyperplasia has not been the focus of any study so far.

Thus, the present study aimed to investigate the effects of 1 on PDGF-BB-induced VSMC proliferation and migration in vitro, as well as its influence on neointima formation in vivo.

In order to test whether 1 is able to inhibit growth-factor-induced proliferation of VSMCs, its influence on the total cellular metabolic activity of VSMCs was measured by the resazurin conversion method. PDGF-BB was used as a mitogenic stimulus in this study. Total metabolic activity was measured 48 h after PDGF-BB treatment of VSMCs, which...
were already preincubated with vehicle or 1 (1–15 μM) for 30 min. Indeed, 1 inhibited PDGF-BB-induced VSMC proliferation concentration dependently, with an IC_{50} value of 3.49 μM (Figure 2A). To ensure that the decreased VSMC number upon treatment with 1 is due to proliferation inhibition and not cytotoxicity, we quantified cytosolic lactate dehydrogenase (LDH) inside the cells and in cell supernatants upon exposure to 1. No significant cytotoxicity was detected in the investigated concentration range (Figure 2B).

To investigate whether 1 can also inhibit PDGF-BB-induced VSMC migration, we performed a wound-healing assay in the presence of 1. After preincubation with vehicle or 1 (5 or 15 μM) for 30 min, the scratched VSMCs were treated with PDGF-BB and incubated for the next 20 h. Photos of the "wound" in the VSMC monolayer at the beginning (t = 0 h) and the end (t = 20 h) were taken, and the cell recolonization rate was analyzed. Upon stimulation with PDGF-BB, control cells were able to significantly recover the cell-free area (~3.5-fold compared to unstimulated cells). Co-treatment with 15 μM 1 showed potent inhibition of PDGF-induced VSMC migration, allowing cell motility only in the range of the basal unstimulated control group (Figure 3).

We then examined whether 1 can also prevent neointima formation in vivo. The mouse femoral artery cuff model represents an animal model used to examine vessel responses to injury that are relevant for restenosis.\textsuperscript{26–28} The femoral artery cuff placement leads to predictable neointima formation in mice over a 14-day period.\textsuperscript{28} To assess the effect of locally applied 1 on the cuff-induced restenosis, 1 was dissolved in F-127 gel and placed in the cuff surrounding the femoral artery. Compared to sham-operated vessels, injured vessels displayed significant neointima formation 14 days after the cuff placement, which could be significantly attenuated by treatment with 1 (Figure 4).

**Figure 4.** Xanthohumol (XN) suppresses neointima formation in vivo. A nonconstructive polyethylene cuff filled with F-127 pluronic gel containing xanthohumol or vehicle was placed loosely around the right femoral artery of mice. For the sham-operated group, the right femoral artery was dissected from surrounding tissues, but a cuff was not placed. (A) Representative photomicrograph of hematoxylin and eosin staining cross sections in femoral arteries at day 14: sham-operated control, cuff injured control, and cuff injured with XN treatment. (B–D) Quantitative analysis of the neointima cross-sectional area, the media cross-sectional area, and the neointima to media cross-sectional ratio. Data are shown as the mean ± SD, at least six experimented mice per group, and Student’s t-test was used to compare the difference between groups. **p < 0.001; n.s., not significant; vs cuff-injured control. Scale bars, 50 μm.

Aberrant VSMC proliferation and migration are major events in atherosclerosis and restenosis progression. Therefore, identification of phytochemicals able to suppress VSMC proliferation and migration is of potential therapeutic relevance. Hops have been widely used since ancient times not only in the manufacturing of beer, but also as a medicinal plant. As the most abundant prenylated flavonoid in hops, 1 attracts an increasing amount of attention due to a variety of newly described health-promoting activities. To our knowledge, the present study is the first to show that 1 also suppresses proliferation and migration of PDGF-BB-induced VSMCs in vitro and reduces neointima formation in vivo. Further studies will be necessary to dissect the underlying molecular mode of action. Due to its electrophilic nature, 1 directly affects several cellular targets and signaling pathways\textsuperscript{29} and modulates activity of kinases, such as Akt and AMPK, and of transcription factors, such as Nrf2, NF-kB, or STAT3.\textsuperscript{19,30,31} All those signaling molecules may conceivably contribute to the reduced neointima formation. Therefore, it is highly likely that the antihyperplastic effect of 1 observed in this study cannot be pinned down to one single molecular target or pathway but rather exploits the pronounced polypharmacology of 1.

**EXPERIMENTAL SECTION**

**General Experimental Procedures.** In this study, rat aortic VSMCs, growth media, and cell culture supplements were purchased from Lonza (Basel, Switzerland). Serum for cell culture was obtained from Gibco Life Technologies (Darmstadt, Germany), and PDGF-BB was supplied from Bachem (Weilheim, Germany). Compound 1 and the other used reagents were obtained from Sigma-Aldrich (Shanghai, China).

**Cell Culture and VSMC Proliferation.** VSMCs were cultivated in Dulbecco’s modified essential medium (DMEM)–F12 (1:1) supplemented with 20% fetal calf serum (FCS), 30 μg/mL gentamicin, and...
15 ng/mL amphotericin B at 37 °C in an incubator with 5% CO₂ flow in a humidified atmosphere.

Cell proliferation was assessed by resazurin conversion assay, as described previously. Briefly, VSMCs were seeded at a density of 5 × 10³ cells/well in 96-well plates. After 24 h, cells were serum-starved for another 24 h to render them quiescent. Quiescent cells were pretreated for 30 min with 0.1% DMSO or 1 μM of the indicated concentrations and subsequently stimulated with PDGF-BB (20 ng/mL) for 48 h. Then, after washing the cells with phosphate-buffered saline (PBS), they were incubated in serum-free medium containing resazurin (10 μg/mL) for 2 h. The amount of metabolically active VSMCs was determined by the increased fluorescence yield from the conversion of resazurin. Samples were measured by monitoring the increase in fluorescence at 590 nm using an excitation wavelength of 535 nm in a 96-well plate reader (Tecan GENios Pro) from Tecan Group Ltd. (Männedorf, Switzerland).

Assessment of Cytotoxicity. Cytotoxicity was evaluated by detection of the release of LDH. VSMCs were incubated and treated with the same procedure as for the cell proliferation assay. The supernatant of the treated cells was measured for the released soluble LDH activity. For determination of the total LDH activity, identically treated samples were incubated for 45 min with 1% Triton X-100. The released and total LDH enzyme activity was determined for 30 min in the dark in the presence of 4.5 mg/mL lactate, 0.56 mg/mL NAD+, and 0.5 mM 2-p-iodophenyl-3-nitrophenoxytetrazolium chloride. The absorbance was detected at 490 nm after the enzyme reaction was stopped with 1.78 mg/mL Oxybate. Percentage of extracellular LDH enzyme activity was calculated to estimate the potential effects on cell viability. The cytotoxic compound digitonin (50 μg/mL) was used as a positive control.

Wound-Healing Assay (Scratch Assay). The scratch assay was performed as previously described. Briefly, VSMCs were seeded in six-well plates, grown to confluence, and starved for 24 h. The cell monolayer was scratched using a sterile pipet tip (1000 μL) with the scratch width around 1 mm. Cells were recovered for the next 24 h in freshly exchanged starvation medium (0.1% serum supplemented DMEM-F12). Then, 0.1% DMSO or 1 μM of the indicated concentrations was applied to the cells, and 30 min later, PDGF-BB (10 ng/mL) was also applied. VSMCs were then incubated for the next 20 h, and potential influence on migration was monitored. Photographs of the scratch under the light microscope (Olympus CKX41, Tokyo, Japan, magnification 1000×) were taken at 0 and 2 h after the treatment. Perpendicular lines were predrawn at the bottom side of the plates to ensure that the very same area of each scratch is captured at 0 and 20 h. The cell recolonization rate was recorded by measuring the cell-free area of each scratch, using Cell Profiler software (www.cellprofiler.org, Broad Institute, Cambridge, MA, USA). Relative migration capacity was calculated as the change in the area occupied by cells between 20 and 0 h, normalized by the vehicle control.

Femoral Artery Cuff Model. Male C57BL/6 mice 10 to 12 weeks old were obtained from Shanghai Center of Experimental Animals (Shanghai, China). All the mice were housed in temperature- and humidity-controlled rooms with a 12 h dark/light cycle throughout the study. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at YanTai University (IACUC number: 2015-DA-25). The National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals was followed throughout. Neointima formation was induced by means of vascular injury through cuff placement around the femoral artery of mice, as described previously. In brief, mice were randomly assigned into three groups: sham-operated control, cuff-injured control, and cuff injured with 1 treatment. For the cuff-injured groups, a nonconstrictive polyethylene cuff, 2 mm long (PE-50; Becton-Dickinson, MD, USA), was cut longitudinally, placed loosely around the right femoral artery, and filled with F-127 pluronic gel (Sigma-Aldrich, Shanghai, China) containing or not containing 1 μg/mL. This gel was prepared in advance by mixing 300 μL of cold 40% (wt/vol) F-127 pluronic gel dissolved in sterile water with 100 μL of 20 mM DMSO. Cuffs filled with F-127 pluronic gel containing the solvent vehicle (DMSO), but lacking 1, were used in cuff-injured control animals. For the mice with noncuff treatment, the right femoral arteries were dissected from surrounding tissues, and the respective animals were used as sham-operated control. The cuff is larger than the vessel and does not obstruct blood flow. Two weeks after cuff placement, the cuffed femoral artery was removed after perfusion, fixed in 10% formalin for 24 h, dehydrated, and embedded in paraffin. Cross sections were subjected to standard hematoxylin and eosin staining. Photomicrographs were taken using an Olympus BX51 microscope with an Olympus DP71 CCD camera (Olympus Corporation, Tokyo, Japan). Quantification analysis was performed with the software Image-Pro Plus version 6.0 (Media Cybernetics Inc.).

Statistical Analysis. Statistical analysis was performed using the ANOVA/Bonferroni test or Student’s t-test (when comparing just two experimental groups). The number of experiments is given in the figure legends, and all the data presented in this study are expressed as mean ± SD. All statistical tests were performed using the GraphPad Prism 4 software (GraphPad Software Inc.), and a probability value of <0.05 was considered significant.

AUTHOR INFORMATION

Corresponding Authors
*Tel: +43-1-4277-55993. Fax: +43-1-4277-855270. E-mail: elke.heiss@univie.ac.at (E. H. Heiss).
*Tel: +43-1-4277-55231. Fax: +43-1-4277-55969. E-mail: atanas.atanasyov@univie.ac.at (A. G. Atanasov).

ORCID
Verena M. Dirsch: 0000-0002-9261-5293
Atanas G. Atanasov: 0000-0003-2545-0967

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support by the National Natural Science Foundation of China (No. 81603326), by the European Union Seventh Framework Program (EU-FP7) Marie Curie Fellowship (No. 252881 and No. 909881), by the Austrian Science Fund (FWF) project P25971-B23, and by the Polish KNOW (Leading National Research Centre) Scientific Consortium “Healthy Animal—Safe Food”, decision of the Ministry of Science and Higher Education No. 05-1/KNOW2/2015.

REFERENCES

(1) Piepoli, M. F.; Hoes, A. W.; Agewall, S.; Albus, C.; Brotons, C.; Catapano, A. L.; Connroy, M. T.; Corra, U.; Cosyns, B.; Deaton, C.; Graham, I.; Hall, M. S.; Hobbs, F. D.; Lachen, M. L.; Lollgen, H.; Marques-Vidal, P.; Perk, J.; Prescott, E.; Redon, J.; Richter, D. J.; Sattar, N.; Smulders, Y.; Tiberi, M.; van der Worp, H.; van Dis, I.; Verschuren, W. M. Atherosclerosis 2016, 252, 207–274.
(2) Moran, A. E.; Forouzanfar, M. H.; Both, G. A.; Mensah, G. A.; Ezzati, M.; Murray, C. J.; Naghavi, M. Circulation 2014, 129, 1483–1492.
(3) Rader, D. J.; Daugherty, A. Nature 2008, 451, 904–913.
(4) Mills, B.; Robb, T.; Larson, D. F. Persuasion 2012, 27, 520–528.
(5) Libby, P. Nature 2002, 420, 868–874.
(6) Li, B.; Li, W.; Li, X.; Zhou, H. Curr. Pharm. Des. 2016, 22, 1–12.
(7) Raines, E. W. Cytokine Growth Factor Rev. 2004, 15, 237–254.
(8) Johnson, J. L. Cardiovasc. Res. 2014, 103, 452–460.
(9) Inoue, T.; Croce, K.; Morooka, T.; Sakuma, M.; Node, K.; Simon, D. J. JACC. Cardiovasc. Interv. 2011, 4, 1057–1066.
(10) Atanasov, A. G.; Waltenberger, B.; Pflersch-Wenzig, E. M.; Linder, T.; Wawrosch, C.; Uhrin, P.; Temml, V.; Wang, L.; Schwaiger, S.; Heiss, E. H.; Rollinger, J. M.; Schuster, D.; Breus, J. M.; Bochkov, V.; Mihovilovic, M. D.; Kopp, B.; Bauer, R.; Dirsch, V. M.; Stupner, H. Biotechnol. Adv. 2015, 33, 1582–1614.
