**LETTER**

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**Aiaphanol, a native compound, suppresses angiogenesis via dual-targeting VEGFR2 and COX2**

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**Dear Editor,**

Pathological neo-vascularization is a hallmark of cancer and several diseases. Accumulating evidence supports the notion that antiangiogenic treatment can abolish tumor angiogenesis to achieve longer disease-free survival. Although growth factors and their receptors function as the main drivers in angiogenesis, the involvement of other regulators, e.g., Cyclooxygenase-2 (COX2), should also be considered, especially for managing the resistance to therapies against receptor tyrosine kinases (RTKs). Hence, utilizing distinct inhibitors and developing multitargeting agents could be desired and practical approaches in conquering tumor angiogenesis.

Plants are rich in compounds with diverse biological functions. The anti-tumor and anti-inflammatory potentials of *Sarsaparilla*, aka *Smilax Glabra Rhizome* (SGR), were extensively studied, but its influence on angiogenesis has not been explored. Herein we found that SGR inhibited proliferation and motility of primary human umbilical vein endothelial cells (HUVECs) (Supplementary Fig. S1a–c). Importantly, SGR inhibited both basal and growth factors-stimulated angiogenesis in tube formation assay (Supplementary Fig. S1d), and this effect was dose-dependent (Supplementary Fig. S1e). We noticed that SGR contains some compounds with antiangiogenic capabilities. Aiaphanol, originally separated from the seeds of *Aiphanes aculeata*, was also identified in SGR. Aiaphanol represents an unprecedented scaffold of stilbenolignan in which one stilbene unit is connected with one phenylpropane moiety by a 1,4-dioxane bridge (Fig. 1a). To date, the biological effects of stilbenolignans, including Aiaphanol, are largely unclear. Aiaphanol was reported to inhibit angiogenesis in the rat aortic ring assay; however, the mechanism and its role in regulating tumor angiogenesis remain to be determined.

Aiaphanol could inhibit COX1/2 activities, but the mechanism and the biological significance are elusive. We verified Aiaphanol-antagonized COX2 activity (IC$_{50}$ = 2.7 μM) (Supplementary Fig. S5a). Microscale thermophoresis measurements demonstrated a direct Aiaphanol-COX2 binding with the dissociation constant (Kd) of 36.6 μM (Fig. 1e). Structural simulation revealed that Aiaphanol binds to the catalytic domain of COX2 (docking score = −8.118). Driven by the phenylpropane unit and the dioxane bridge, Aiaphanol might inhibit COX2 activity through occupying its substrate binding pocket (Supplementary Fig. S5b, S5c). In Aiaphanol-treated HUVECs, protein levels of COX2 remained unaffected (Supplementary Fig. S5d), but COX2’s enzymatic product, the inflammatory mediator Prostaglandin E2 (PGE2), were reduced in the conditioned medium. After COX2 knockdown (Supplementary Fig. S5e), the inhibitory effects of Aiaphanol on PGE2 and VEGF levels were markedly counteracted (Fig. 1f, g), validating Aiaphanol-inhibited COX2 activity in cells. Compared with Celecoxib, a selective COX2 inhibitor, Aiaphanol displayed stronger activity in blocking tube formation (Fig. 1h).

The stilbenes could repress kinase activities due to their structural characteristics. We hypothesized that Aiaphanol may also inhibit kinase activity through its stilbene unit. Results of cell-ELISA showed that global phospho-serine/threonine and phospho-tyrosine signals were transiently diminished in Aiaphanol-treated HUVECs (Supplementary Fig. S6a). By screening 201 diseases-related kinases, we found that Aiaphanol strongly inhibited the activities of lymphangiogenesis-related kinase VEGFR3/FLT4, angiogenesis-related kinases VEGFR2/KDR and VEGFR1/FLT1. Meanwhile it weakly antagonized COX2 activity (IC$_{50}$ = 2.7 μM) (Fig. 1e). Structural simulation revealed that Aiaphanol binds to the catalytic domain of COX2 (docking score = −8.118). Aiaphanol-COX2 binding with the dissociation constant (Kd) of 36.6 μM (Fig. 1e). Structural simulation predicted a docking score of −10.576 for this complex. Contributed by the stilbene and the phenylpropane unit, Aiaphanol might target the VEGFR2’s ATP-binding domain, thereby preventing its activation (Supplementary Fig. S6f, S6g). Unlike VEGF-targeting antibody Bevacizumab, Aiaphanol did not prevent VEGF-VEGFR2 interaction (Supplementary Fig. S6h). Nevertheless, Aiaphanol treatment in HUVECs reduced VEGF-induced phosphorylations of VEGFR2, AKT and MAPK pathways (Fig. 1i and Supplementary Table S1). Because VEGFR1 deletion failed to override Aiaphanol-inhibited tube formation, viability, or motility (Supplementary Fig. S6e–w), we then focused on the roles of VEGFR2. We validated Aiaphanol-imposed inhibition on VEGFR2 kinase activity (IC$_{50}$ = 0.92 μM) (Fig. 1j) and showed a direct VEGF-Aiaphanol interaction (Kd = 9.76 μM) (Fig. 1k). Structural simulation predicted a docking score of −10.576 for this complex. Contributed by the stilbene and the phenylpropane unit, Aiaphanol might target the VEGFR2’s ATP-binding domain, thereby preventing its activation (Supplementary Fig. S6f, S6g). Unlike VEGF-targeting antibody Bevacizumab, Aiaphanol did not prevent VEGF-VEGFR2 interaction (Supplementary Fig. S6h). Nevertheless, Aiaphanol treatment in HUVECs reduced VEGF-induced phosphorylations of VEGFR2, AKT, and ERK in a time- and dose-dependent manner (Supplementary Fig. S6i, S6j). COX2 silencing partially reversed the inhibitory effect of Aiaphanol on tube formation, but failed to ameliorate Aiaphanol-inhibited viability or motility. However, VEGFR2 depletion significantly antagonized Aiaphanol-inhibited tube formation, viability, and motility, and the effects of dual-silencing against VEGFR2 plus COX2 were similar to those of VEGFR2 knockdown (Fig. 1i and Supplementary

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Fig. S7a–d). Aiphanol-induced apoptosis of HUVECs was associated with upregulation of P53 and BAX (Supplementary Fig. S3d), which was weakened by VEGFR2 silencing (Supplementary Fig. S7e, S7f). Next, we examined Aiphanol’s effect on tumor angiogenesis. Unlike HUVECs, MC38 murine colon cancer cells were deficient in VEGFR2 expression and insensitive to Aiphanol-induced caspase3 cleavage (Supplementary Fig. S8a). Besides, Aiphanol did not affect soft-agar colony/plate colony formation (Fig. 1m and Supplementary Fig. S8b), likely due to low Aiphanol uptake by MC38 cells (Supplementary Fig. S8c). However, MC38 tumor growth was retarded by oral administration of Aiphanol in the syngeneic mouse model (Fig. 1n and Supplementary Fig. S8d), correlating with enhanced apoptosis and decreased phosphorylations of VEGFR2, AKT, and ERK in tumor tissues (Fig. 1o, p, and Supplementary Fig. S8e). MC38 cells had no potential of vasculogenic mimicry (Supplementary Fig. S8f), but the levels of vascular markers, CD31...
and Factor VIII, were reduced by Aiphanol (Fig. 1o–q and Supplementary Fig. S8e, S8g). We then concluded that Aiphanol’s inhibition on MC38 tumor growth was resulted from diminished angiogenesis. Additionally, PGE2 levels in the plasma and VEGF levels in tumor tissues were lowered by Aiphanol (Fig. 1r), signifying that COX2 activity was inhibited in vivo. Meanwhile, no significant changes in body weight or the morphologies of major organs of Aiphanol-treated mice were detected (Supplementary Fig. S8h, S8i), highlighting the safety of Aiphanol in vivo.

Collectively, we demonstrated that a naturally occurring stilbene-lignan, Aiphanol, can directly target and inhibit VEGFR2 and COX2, thereby blocking angiogenesis and tumor growth (Fig. 1s). The structural characteristics of Aiphanol license a potent activity against angiogenesis through the cooperation among its stilbene unit, phenylpropane moiety, and dioxane bridge, which is distinct from the mechanisms of stilbenes or lignans. A combination of agents respectively inhibiting VEGFR2 and COX2 was shown to be effective in animal models of antiangiogenic therapy.1 Although inhibition of VEGFR2 mainly contributes to Aiphanol’s antiangiogenic function in vitro, the concomitant inhibition of COX2 in vivo may reprogram the proangiogenic microenvironment by declining the levels of PGE2 and VEGF. Our study supports Aiphanol as a potential antiangiogenic lead compound in cancer therapy.

DATA AVAILABILITY
All data generated or analyzed during this study are included either in this article or in the supplementary information files.

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AUTHOR CONTRIBUTIONS
C.S. supervised this study and designed experiments; S. Chen carried out most of the experiments, analyzed data, and wrote the manuscript; L.Q. and C.Z. designed experiments, analyzed data, and wrote the manuscript; J.F. and L.W. participated in part of experiments; L.M. and C.L. provided laboratory assistance; S. Cai. identified Aiphanol from SGR; Y.J. synthesized Aiphanol.

ADDITIONAL INFORMATION
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Competing interests: The authors declare no competing interests.

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