Supplementary Material

The TRPM7 inhibitor carvacrol suppresses angiogenesis and vasculogenic mimicry in triple-negative breast cancer

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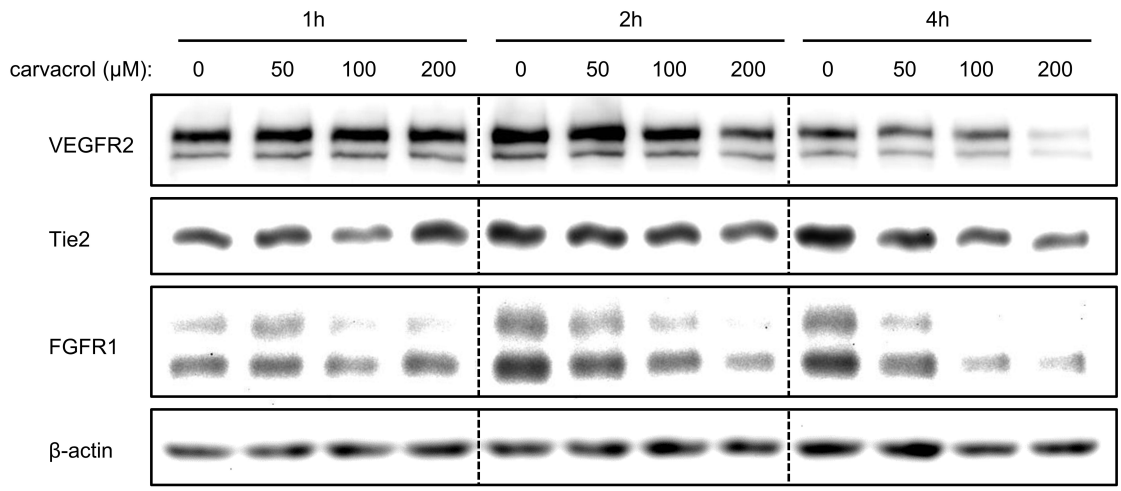
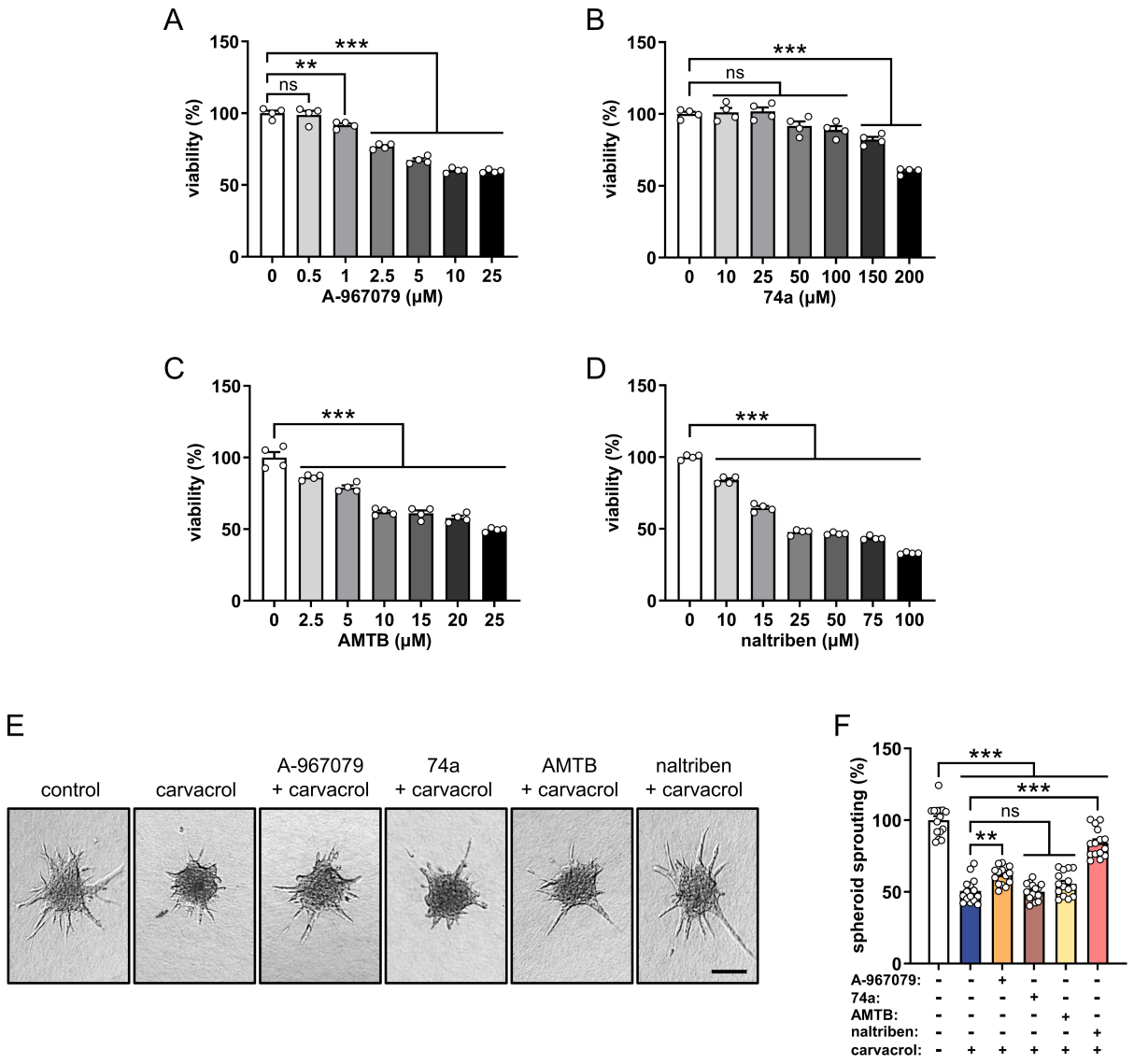
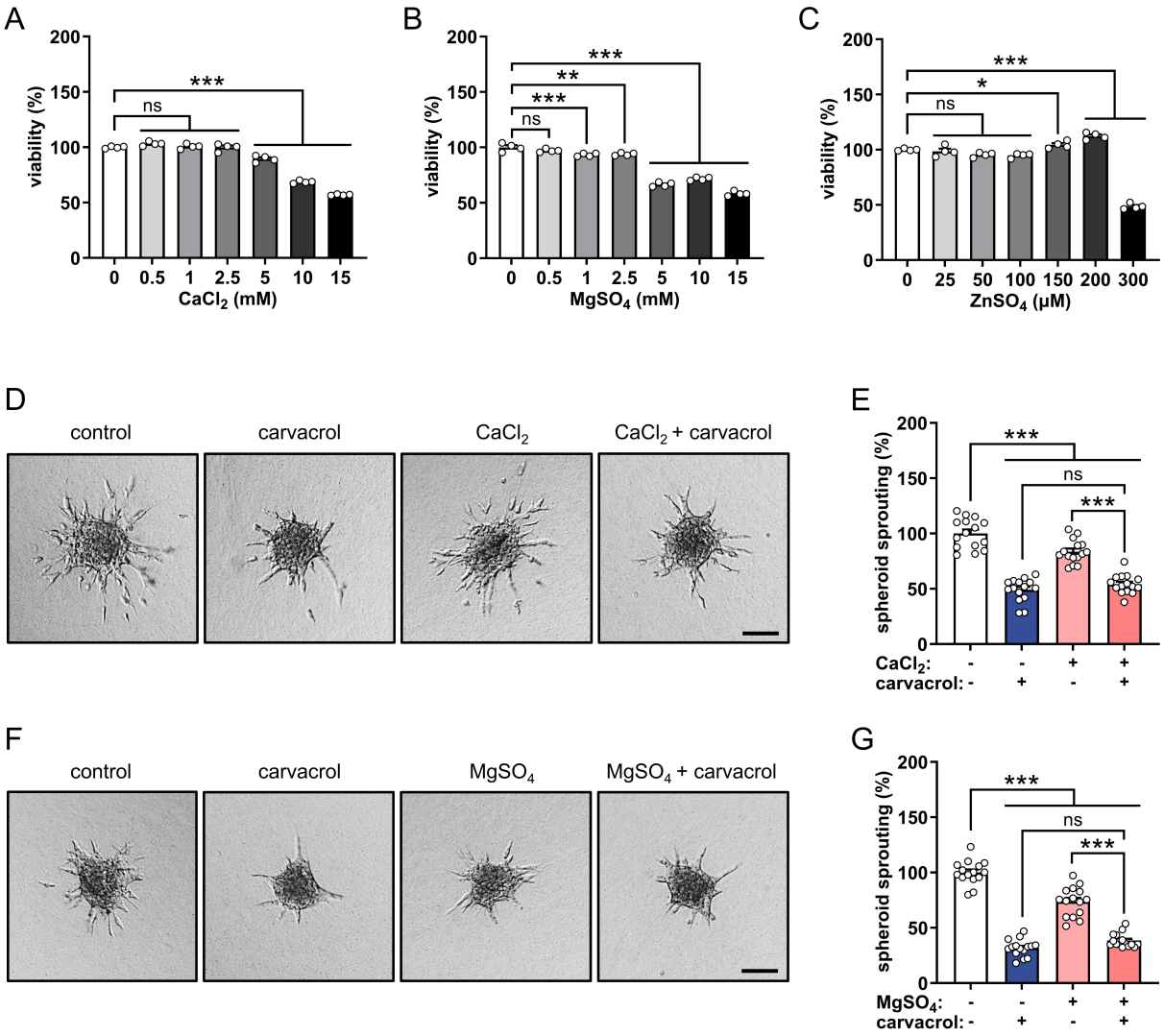


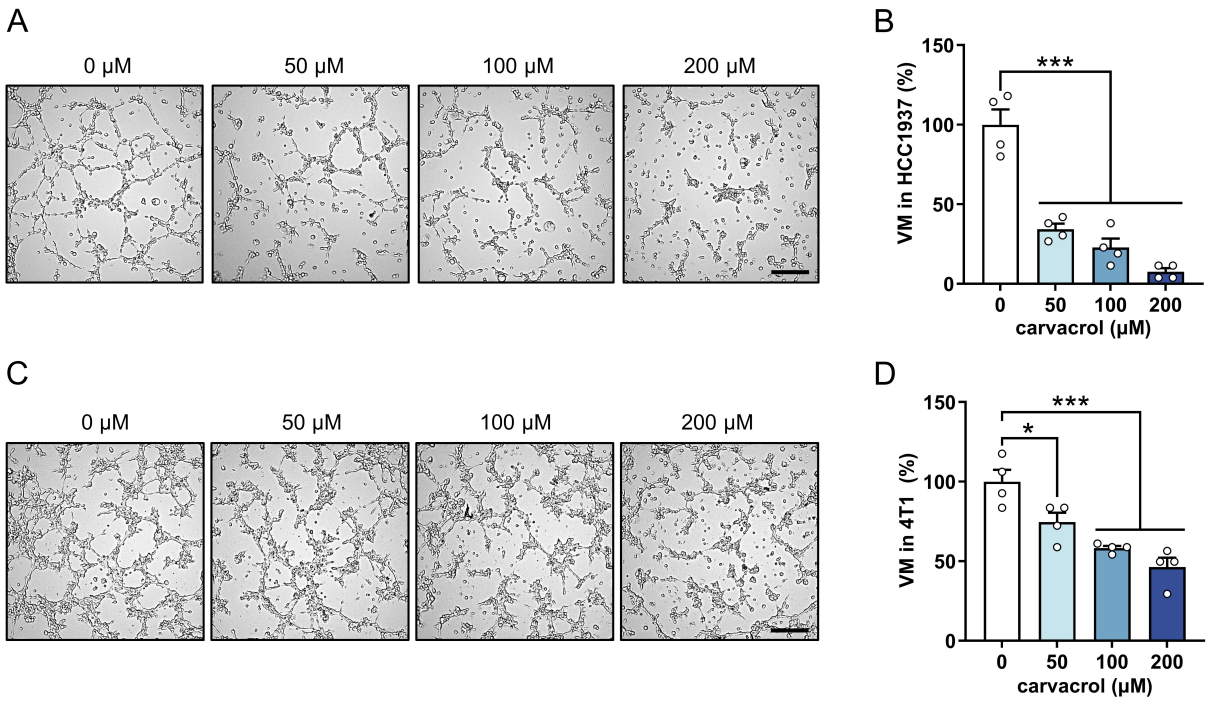
Supplementary Fig. 1 Effects of carvacrol on the protein levels of VEGFR2, Tie2, and FGFR1 in ECs after 1, 2, and 4 h of treatment.



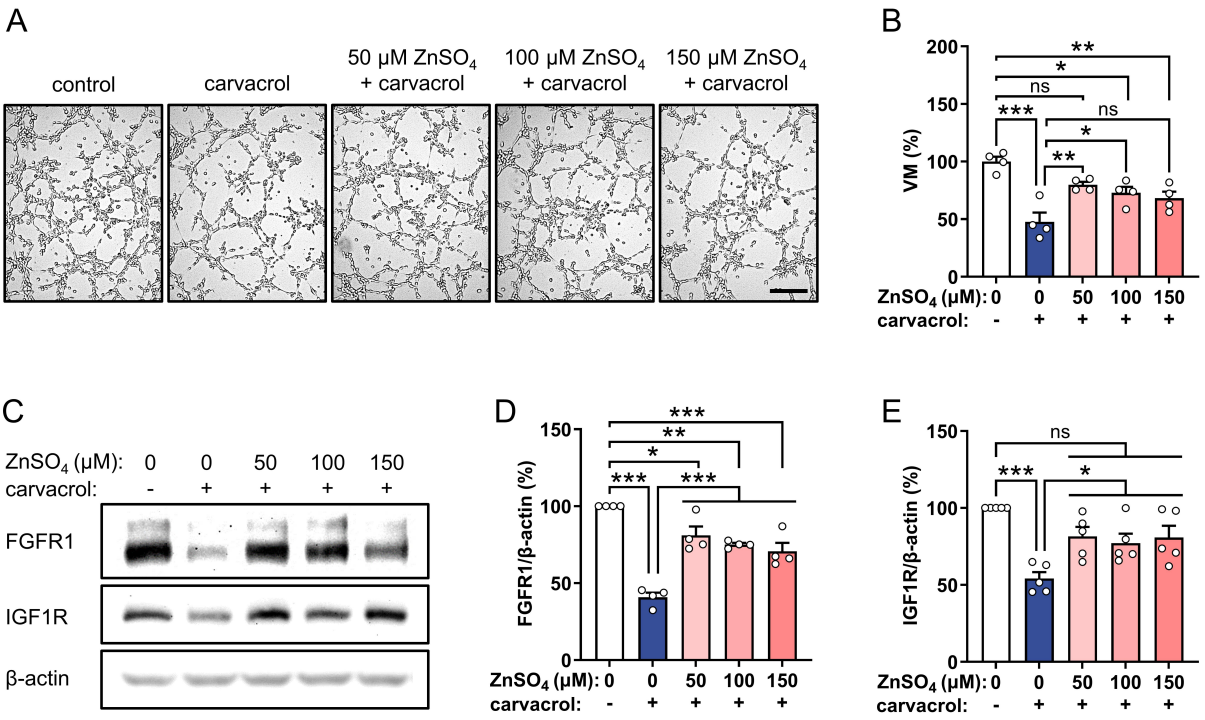
Supplementary Fig. 2 Carvacrol inhibits angiogenesis mainly through TRPM7 antagonism in ECs. **A-D:** Viability (% of 0 µM) of HUVECs that were treated for 24 h with a serial dilution of A-967079 (TRPA1 inhibitor; A), 74a (TRPV3 inhibitor; B), AMTB (TRPM8 inhibitor; C), or naltriben (TRPM7 channel activator; D), as assessed by WST-1 assay (n = 4). **E:** Representative images of HUVEC spheroids that were treated for 24 h with 0.1% DMSO (vehicle) or 200 µM carvacrol in the absence or presence of 2.5 µM A-967079, 150 µM 74a, 5 µM AMTB, or 10 µM naltriben. Scale bar: 65 µm. **F:** Sprouting (% of control) of treated HUVEC spheroids depicted in (E), as assessed by spheroid sprouting assay (n = 15). Data are presented as means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant.



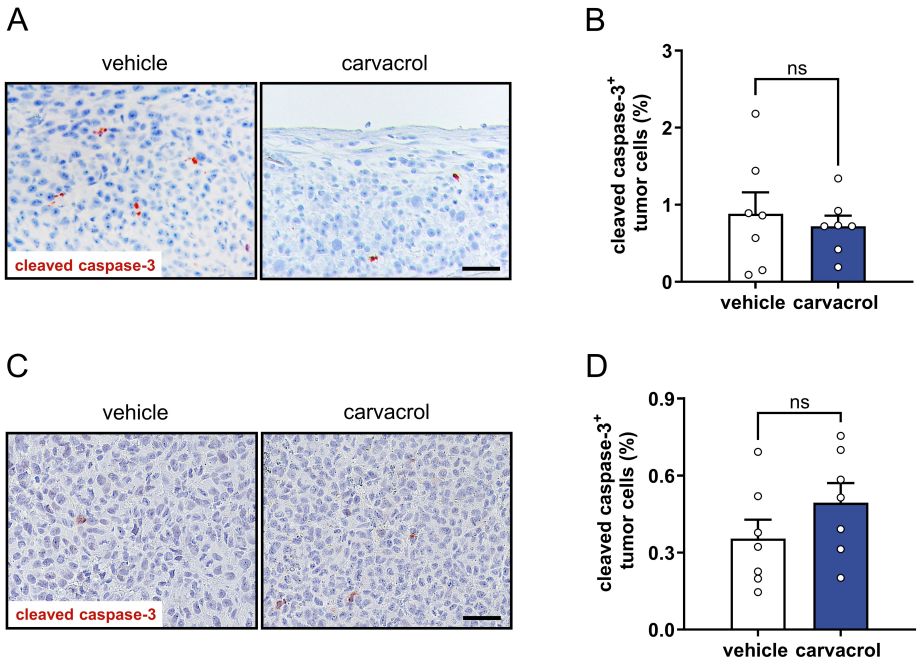
Supplementary Fig. 3 Carvacrol inhibits angiogenesis independently of TRPM7-mediated Ca^{2+} or Mg^{2+} influx in ECs. **A-C:** Viability (% of 0 mM or µM) of HUVECs that were treated for 24 h with a serial dilution of $CaCl_2$ (A), $MgSO_4$ (B), or $ZnSO_4$ (C), as assessed by WST-1 assay (n = 4). **D:** Representative images of HUVEC spheroids that were treated for 24 h with 0.1% DMSO (vehicle) or 200 µM carvacrol in the absence or presence of 5 mM $CaCl_2$. Scale bar: 60 µm. **E:** Sprouting (% of control) of treated HUVEC spheroids depicted in (D), as assessed by spheroid sprouting assay (n = 15). **F:** Representative images of HUVEC spheroids that were treated for 24 h with 0.1% DMSO (vehicle) or 200 µM carvacrol in the absence or presence of 2.5 mM $MgSO_4$. Scale bar: 60 µm. **G:** Sprouting (% of control) of treated HUVEC spheroids depicted in (F), as assessed by spheroid sprouting assay (n = 15). Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.



Supplementary Fig. 4 Carvacrol inhibits VM in HCC1937 and 4T1 cells. **A:** Representative images of tube-forming HCC1937 cells that were treated for 18 h with 0, 50, 100, and 200 μM carvacrol. Scale bar: 200 μm. **B:** VM (% of 0 μM) in treated HCC1937 cells depicted in (A), as assessed by tube formation assay (n = 4). **C:** Representative images of tube-forming 4T1 cells that were treated for 18 h with 0, 50, 100, and 200 μM carvacrol. Scale bar: 200 μm. **D:** VM (% of 0 μM) in treated 4T1 cells depicted in (C), as assessed by tube formation assay (n = 4). Data are presented as means ± SEM. *$P < 0.05$, ***$P < 0.001$.



Supplementary Fig. 5 Carvacrol inhibits VM through blocking TRPM7-mediated Zn^{2+} influx in TNBC cells. **A:** Representative images of tube-forming MDA-MB-231 cells that were treated for 18 h with 0.1% DMSO (vehicle) or 50 μM carvacrol in the presence of 0, 50, 100, or 150 μM $ZnSO_4$. Scale bar: 200 μm. **B:** VM (% of control) of treated MDA-MB-231 cells depicted in (A), as assessed by tube formation assay (n = 4). **C:** Representative Western blots showing FGFR1, IGF1R, and β-actin expression in MDA-MB-231 cells that were pre-treated with 0, 50, 100, or 150 μM $ZnSO_4$ for 2 h and then exposed to 0.1% DMSO (vehicle) or 200 μM carvacrol for another 4 h. **D, E:** Expression levels (% of control) of FGFR1 (D) and IGF1R (E) normalized to β-actin in treated MDA-MB-231 cells depicted in (C), as assessed by Western blotting (n = 4-5 independent experiments). Data are presented as means ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant.



Supplementary Fig. 6 Carvacrol shows no effects on cell apoptosis of 4T1 tumors and MDA-MB-231 tumors. **A:** Representative images of cleaved caspase-3-stained sections of 4T1 tumors from vehicle- and carvacrol-treated mice on day 14 after spheroid transplantation. Scale bars: 40 µm. **B:** Cleaved caspase-3$^+$ tumor cells (% of total cell number) in 4T1 tumors depicted in (A), as assessed by immunohistochemical staining of cleaved caspase-3 (n = 7). **C:** Representative images of cleaved caspase-3-stained sections of MDA-MB-231 tumors from vehicle- and carvacrol-treated mice on day 42 after tumor inoculation. Scale bars: 40 µm. **D:** Cleaved caspase-3$^+$ tumor cells (% of total cell number) in MDA-MB-231 tumors depicted in (C), as assessed by immunohistochemical staining of cleaved caspase-3 (n = 7). Data are presented as means ± SEM. ns, not significant.