Supporting Information

Therapontos et al. 10.1073/pnas.0901505106

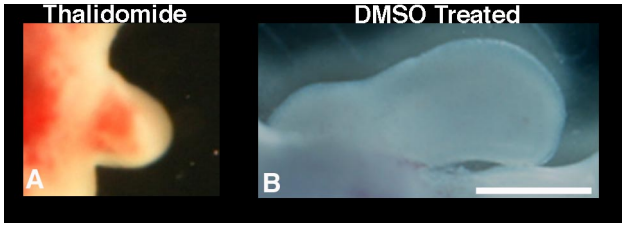

Fig. S1. Thalidomide induces severe defects in chick embryos. (*A*) Application of whole Thalidomide (50 μg) (Sigma) induces severe limb outgrowth failure, when compared with a (*B*) DMSO treated limb (HHst28). (Scale bar, 750 μm.)

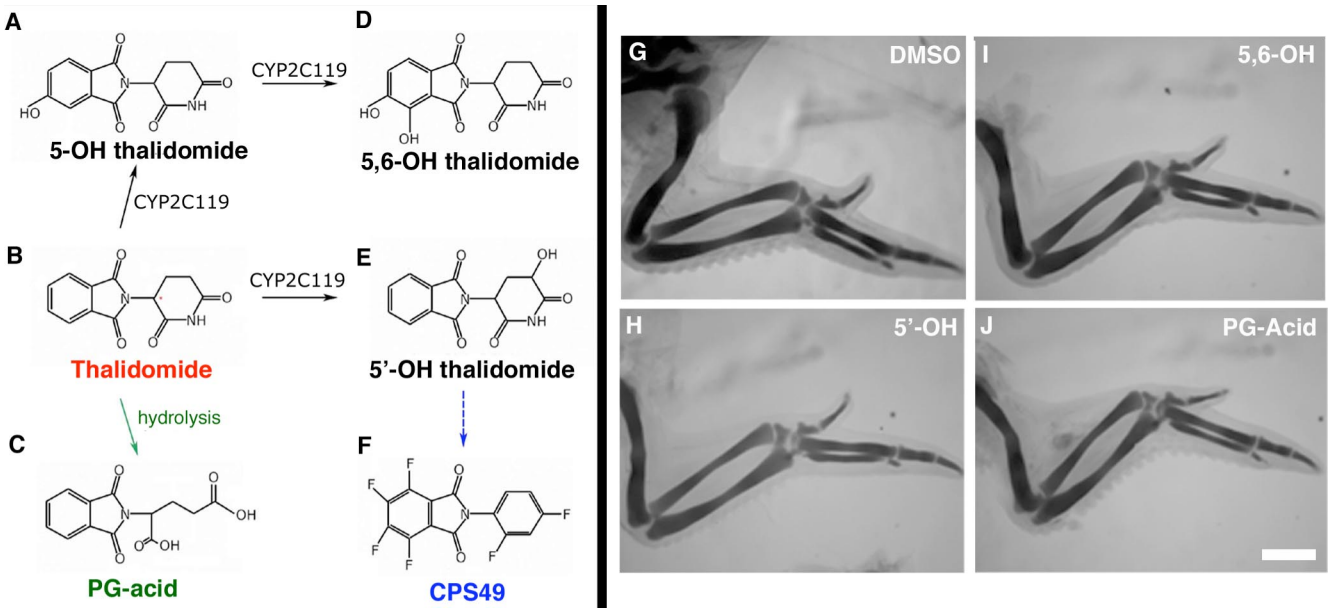

Fig. S2. (*A–F*) Schematic drawings of thalidomide metabolism, hydrolysis products, and analogues. Structure of thalidomide (*B*, chiral centre denoted by asterisk) gives rise to 5-OH thalidomidem (*A*), 5′6′-OH thalidomide (*D*), and 5′-OH thalidomide (*E*) through metabolism by cytochrome P450 enzymes (CYP2C19). PG-Acid (*C*) is a hydrolysis product, whereas CPS49 (*F*) is a tetrafluorinated thalidomide analogue (based on 5′-OH thalidomide). Diagrams were adapted from refs. 1 and 2. (*G–J*) Nonantiangiogenic metabolites of thalidomide have no effect on limb development. Day 10 cartilage patterns of chick embryos treated at HH St18 with DMSO (*G*) or 800 μg/mL of 5,6-OH thalidomide (*I*), 5′OH-thalidomide (*H*), and PG Acid (*J*). No effect on limb development or pattern was observed. (Scale bar, 1,000 μm.)

1. Franks ME, MacPherson GR, Figg WD (2004) Thalidomide. *Lancet* 363:1802–1811.
2. Lepper ER, Smith NF, Cox MC, Scripture CD, Figg WD (2006) Thalidomide metabolism and hydrolysis: Mechanisms and implications. *Curr Drug Metab* 7:677–685.

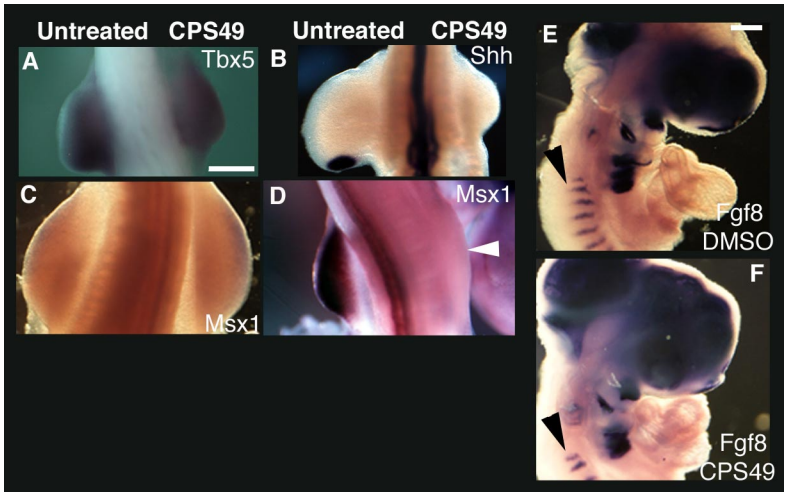

Fig. S3. Expression of signaling molecules in CPS49-treated embryos. Shown is wholemount in situ hybridization of embryos after treatment with CPS49 at HH St18 and fixed at 6 h (*C*) or 24 h (*A*, *B*, *D*, *E*, and *F*). (*A*) *Tbx5* expression remains comparable with control. (*B*) *Shh* expression is gone by 24 h. *Msx1* expression is present at 6 h (*C*) but undetectable after 24 h (*D*, white arrowhead). (*A*–*D*) Treated limb on right, untreated limb on left. (*F*) *Fgf8* expression is normal in the head and somites after 24-h treatment compared with DMSO control (*E*). (*E* and *F*) Arrowheads indicate normal somite expression of *Fgf8*, highlighting that CPS49 is not directly targeting gene expression. (Scale bars: *A*–*D*, 400 μm; *E* and *F*, 350 μm.)

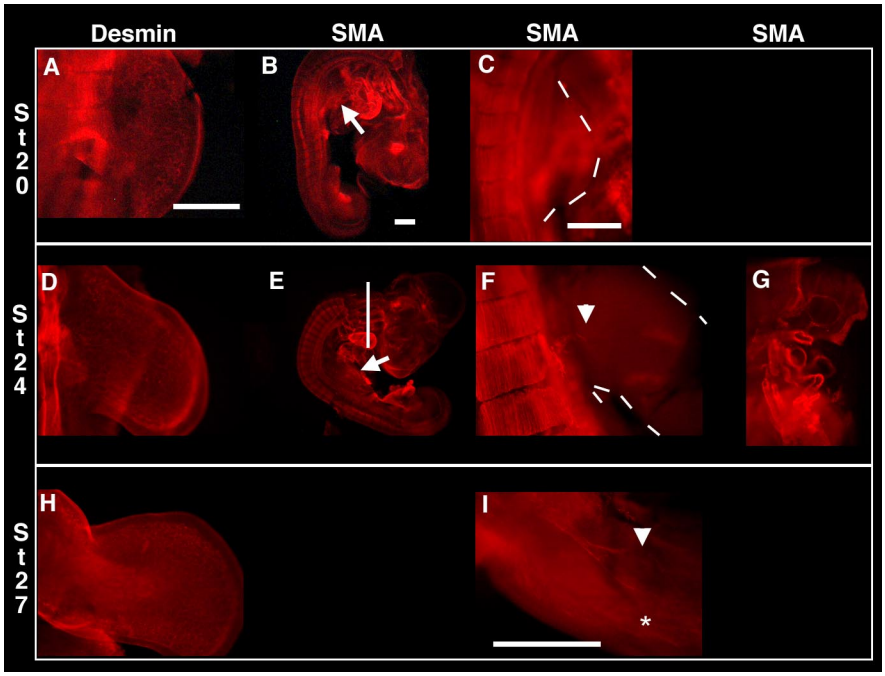

Fig. S4. Developing limbs possess pericytes, but not smooth muscle. Shown is wholemount antibody staining of chick embryos at HHst20 (*A*–*C*), HHst24 (*D*–*G*), and HHs27 (*H* and *I*) for desmin (*A*, *D*, and *H*) or alpha-smooth muscle actin (*B*, *C*, *E*, *F*, *G*, and *I*). Desmin, a pericyte marker, is present on limb vessels at HHst20 (*A*) until at least HHst27 (*H*). Alpha-smooth muscle actin, a marker of maturity and stability, is not present in the limb at HHst20 (*B*, white arrow indicates position of higher power image of limb in *C*). (*E*–*G*) At HHst24, alpha-smooth muscle actin is found only around the subclavian artery, entering the limb (*E*, white arrow indicates position of higher power image of limb in *F*; white arrowhead in *F* indicates subclavian artery), but is found surrounding all of the major vessels of the body (*G*, section through body as indicated by white line in *E*). (*I*) At HHst27, alpha-smooth muscle actin staining (white arrowhead) is seen along the major vessels of the limb bud. (Scale bars: *A*–*I*, 500 μm.)

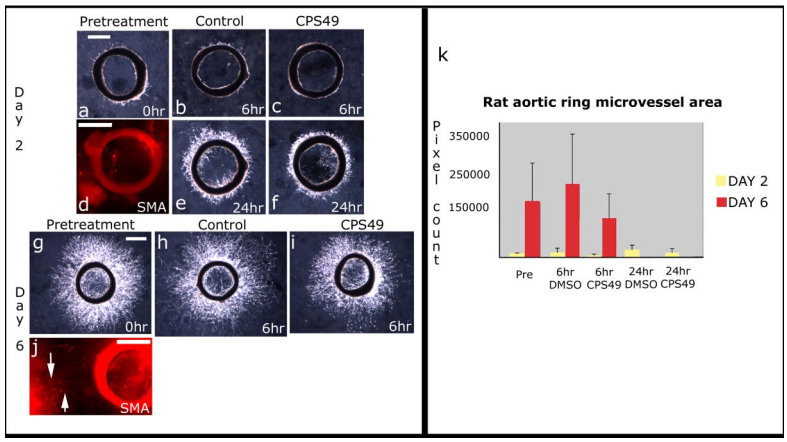

Fig. S5. CPS49 targets angiogenic blood vessels. Shown are rat aortic rings cultured for 2 D.I.V. (*a*–*f*) or 6 D.I.V. (*g*–*j*) before treatment with DMSO (*b*, *e*, and *h*) or PS49 (*c*, *f*, and *i*) and fixed 6 h (*b*, *c*, *h*, and *i*) or 24 h (*e* and *f*) posttreatment. (*d* and *j*) After 6 D.I.V., but not 2 D.I.V., vessels are smooth-muscle-actin positive (white arrows). (*b*, *c*, *e*, *f*, *h*, and *i*) Treatment with CPS49 after 2 D.I.V., but not 6 D.I.V., significantly decreased vessel density and number. (*k*) Mean ± SEM microvessel area of cultures treated after 2 D.I.V. (yellow bars) or 6 D.I.V. (red bars). Aortic ring cultures were carried out as described in ref. 1. (Scale bars: 1,000 μm.)

1. Zhu W-H, Nicosia RF (2002) The thin prep rat aortic ring assay: A modified method for the characterization of angiogenesis in whole mounts. *Angiogenesis* 5:81–86.

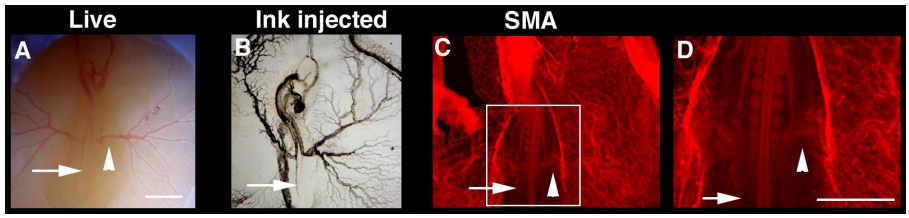

Fig. S6. Blood vessels in HHst15 embryos are immature and susceptible to CPS49. Visualization of blood vessels (*A* and *B*) and their maturity through alpha-smooth muscle actin wholemount antibody staining (*C* and *D*) in HHst15 chicken embryo. (*A*) Live image of embryo. (*B*) Ink-injected embryo, highlighting vascular network. (*C*) Antibody-stained embryo highlighting that embryonic vessels are alpha-smooth-muscle negative (white box is shown at higher magnification in *D*). (*A*–*D*) White arrow indicates embryonic vein, which is alpha-smooth-muscle-actin negative (*C* and *D*). White arrowhead indicates vitteline artery, which is alpha-smooth-muscle positive (*C* and *D*). (Scale bars: *A*–*D*, 250 μm.)

Table S1. Effect of CPS49 on microvessel number in mouse aortic ring cultures after incubation with vehicle or 10 μg/mL CPS49

	time 0	6 h		24 h	
	WT	control	CPS49	control	CPS49
2 D.I.V.					
Neovessel number	67.7 ± 5.7	72.5 ± 6.9	31.1 ± 4.5	93.4 ± 9.3	56.5 ± 5.8
n (cultures)	10	15	15	15	15
5 D.I.V.					
Neovessel number	49.4 ± 6.0	70.8 ± 9.4	52 ± 5.7	56.2 ± 6.8	44.1 ± 9.2
n (cultures)	7	12	12	12	12

Neovessel number = mean ± SEM microvessel count of day 2 and day 5 cultures.