Supplemental Information for

Ref-1/APE1 Inhibition with Novel Small Molecules Blocks Ocular Neovascularization

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Supplemental Methods

Compound Synthesis

General. All synthesis was performed by Cascade Custom Chemistry (Eugene, OR). Iodolawsone (2-iodo-3-hydroxy-1,4 naphthoquinone) is available from Cascade Custom Chemistry. HPLC were performed using an Alltech Alltima column C18 5u, 250 x 5.6 mm, flow 1 ml/min at 40 ºC. Elution was with a mobile phase of 15:10:75 water:A1:methanol where A1 was made using 700 ml of water, 300 ml methanol and 3 ml trimethylamine to which phosphoric acid was added to bring the pH to 3.4.

(*E*)-2-((3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylene)pentanoic acid (3). In a 2L 3-necked flask equipped with a mechanical stirrer and a gas dispersion fritted tube was placed 2-iodo-3-hydroxy-1,4 naphthoquinone (iodolawsone, **1**) (18 g, 0.06 mol) and 2-propylacrylic acid **2** (17.1 g, 0.15 mol) in a solution of potassium carbonate (41.4 g, 0.3 mol) in water (600 ml). The reaction mixture was stirred and sparged with argon for 30 min. Palladium(II) acetate (0.67 g, 0.003 mol) was added and sparging continued for an additional 30 min. The resulting mixture was heated in an oil-bath at 100°C. HPLC analysis showed the reaction was complete after 1 hr. The reaction mixture was cooled to room temperature and the black Pd metal was filtered. The filtrate was placed in a 2L 3-necked flask equipped with a mechanical stirrer, cooled in an ice-methanol bath and acidified with 50% H_3PO_4 (160 ml) to pH 2. After stirring for 1 hr, the solid was collected, washed with water (1L), then a mixture of 20% acetone in water (500 ml), then air dried to give 12.3 g (72%) of **3** as a mustard colored solid. HPLC analysis showed a purity of 98%. NMR (d_6-DMSO) δ 12.6 (br s, 1H), 11.65 (br s, 1H), 8.0 (m, 2H), 7.8(m, 2H), 7.15 (s, 1H), 2.1(m, 2H), 1.4 (m, 2H), 0.8 (m, 3H).

(*E*)-2-((3-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylene)pentanoyl chloride (4). To a suspension of **3** (4.0 g, 0.014 mol) and DMF (0.1 ml) in dichloromethane (75 ml) was added oxalyl chloride (17.5 ml of 2M in CH_2Cl_2, 0.035 mol) over 20 min at room temperature. The resulting mixture was stirred at room temperature overnight and then was concentrated under reduced pressure to give 4.5 g (100%) **4** as a brown solid. This solid was used directly in the next step. NMR ($CDCl_3$) δ 7.8-8.2 (m, 2H), 7.7-7.8 (m, 2H), 7.4 (s, 1H), 2.1-2.4 (m, 2H), 1.2-1.7 (m, 2H), 0.6-1.0 (m, 3H).

(*E*)-*N*,*N*-diethyl-2-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylene)pentanamide (5a). To a solution of crude **4** (9.7 g, 0.03 mol) in dichloromethane (50 ml) was added a solution of diethylamine hydrochloride (4.97 g, 0.045 mol) and diisopropylamine (11.6 g, 0.09 mol) in dichloromethane (50 ml) at room temperature over 45 min. HPLC analysis after 15 min showed the reaction was complete. The reaction mixture was washed with water (100 ml), 1M HCl (2x100 ml), and brine (100 ml). The organic phase was dried with 1PS paper and concentrated to a deep red solid. The solid was flash chromatographed over silica gel (150 g) with anhydrous sodium sulfate (20 g) on top, packed with hexane. The column was eluted with 125 ml portions of 15% ethyl acetate in hexane for fractions 1–4, 25% ethyl acetate in hexane for fractions 5–8, 35% ethyl acetate in hexane for fractions 9–16, and 50%

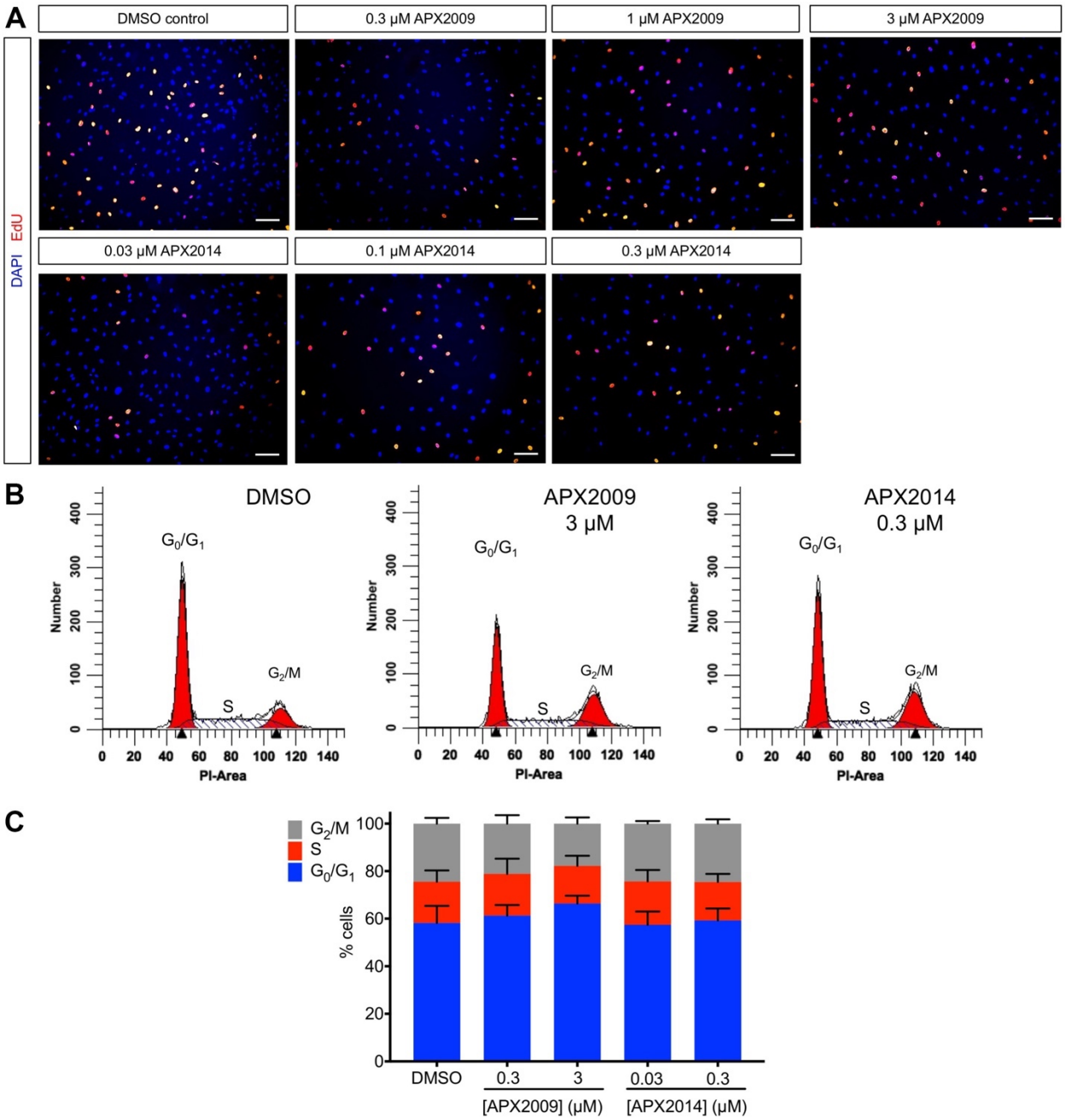
ethyl acetate in hexane for fractions 17–32. All fractions were checked by TLC (ethyl acetate: hexane; 1:1) and some fractions by HPLC. The product was eluted in fractions 21–30. They were combined and concentrated under reduced pressure to give an orange solid. This solid was suspended over 15% ethyl acetate in hexane (50 ml) and stirred for 15 min. The solid was collected and air dried to give 6.7 g (62%) of **5a** as an orange solid. HPLC analysis showed a purity of 99%. NMR ($CDCl_3$) δ 8.1-8.3 (m, 2H), 7.7-7.8 (m, 2H), 6.1 (s, 1H), 3.6 (br d, 4H), 2.2 (t, 2H), 1.45 (m, 2H), 1.25 (br s, (6H), 0.9 (t, 3H).

(*E*)-*N*-methoxy-2-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylene)pentanamide (5b). A solution of crude **4,** prepared from **3** (20.0 g, 0.7 mol) with DMF (0.5 ml) in DCM (300 ml) and oxalyl chloride (2M in DCM, 87.5 ml, 0.0175 mol), in 100 ml DCM was added to a solution of methoxyamine hydrochloride (7.0 g, 0.084 mol) and DIPEA (27.1 g, 0.21 mol) in DCM (100 ml) under argon and cooled in a room temperature water bath over 1 hour. After 30 minutes, HPLC indicated the reaction was complete. The mixture was washed with water (100 ml), 1 M HCl (100 ml), and brine (100 ml). The organic phase was dried with 1PS paper and concentrated to an orange oil. The crude oil was chromatographed on silica gel (350 g) with hexanes/EtOAc. The product eluted with 60% EtOAc/hexanes. The pure fractions were combined to give 19 g of an oil that solidified. The solid was triturated with hexanes (100 ml) and filtered to give 16.6 g of **5b** as a yellow solid (71%) at 98% purity.

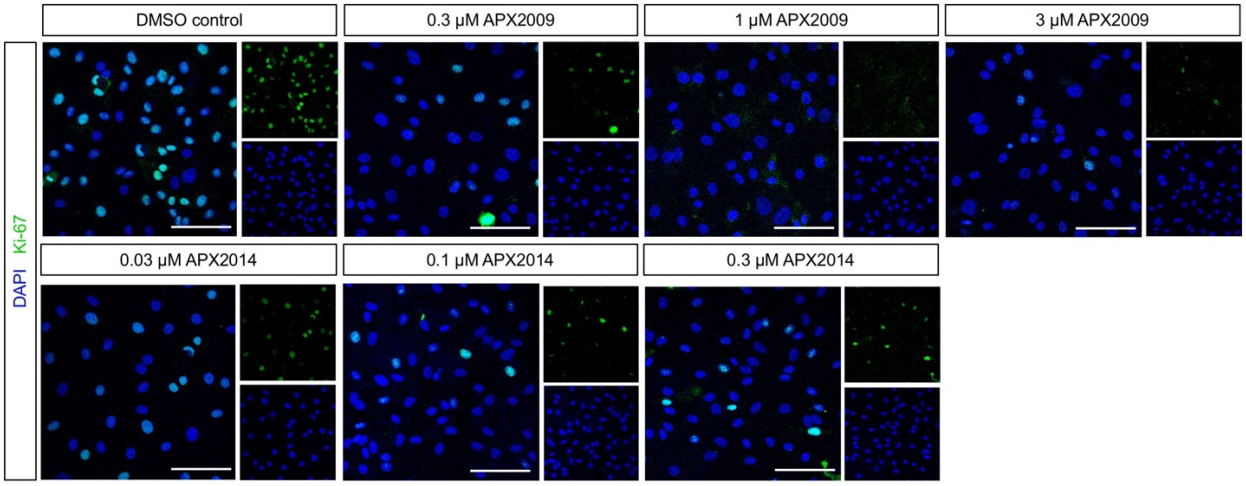
(*E*)-*N*,*N*-diethyl-2-((3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylene)pentanamide (6a). To a solution of **5a** (5.0 g, 0.014 mol) in methanol (100 ml) was added a solution of sodium methoxide in methanol (4.2 ml of 5M in MeOH) in one portion sparged with argon. After 30 min, HPLC indicated the reaction was complete. The reaction mixture was acidified to pH 3 by using 3M HCl (3.5 ml), and then was concentrated under reduced pressure. The resulting residue was dissolved in ethyl acetate (150 ml), washed with water (2x75 ml), and brine (1x100 ml), filtered through 1PS filter paper and concentrated under reduced pressure to give an oil which solidified. This solid was triturated with hexane (50 ml) for 30 min and the solid was collected and air dried to give 4.8 g (96%) of **6a, APX2009,** as a light orange solid. HPLC analysis showed a purity of 99%. NMR ($CDCl_3$) δ 8.15 (m, 2H), 7.75 (m, 2H), 6.2 (s, 1H), 4.1 (s, 3H), 3.6 (br d, 4H), 2.2 (t, 2H), 1.4 (m, 4H), 1.25 (br d, 4H), 0.85 (t, 3H).

(*E*)-*N*-methoxy-2-((3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylene)pentanamide (6b). To a solution of **5b** (10.0 g, 0.03 mol) in methanol (100 ml) was added a solution of sodium methoxide in methanol (9.0 ml of 5M in MeOH) in one portion sparged with argon. After 30 min, HPLC indicated the reaction was complete. The mixture was acidified to pH 2-3 with 3 M HCl. The mixture was concentrated under reduced pressure to a residue. The residue was dissolved in ethyl acetate (150 ml) and washed with water (100 ml) and brine (100 ml). The organic phase was dried over 1PS paper and concentrated under reduced pressure to an oil that solidified. The solid was triturated with hexanes (150 ml) for 30 min and filtered to give 8.7 g (83%) of **6b, APX2014,** as a yellow solid. HPLC analysis showed a purity of 99%.

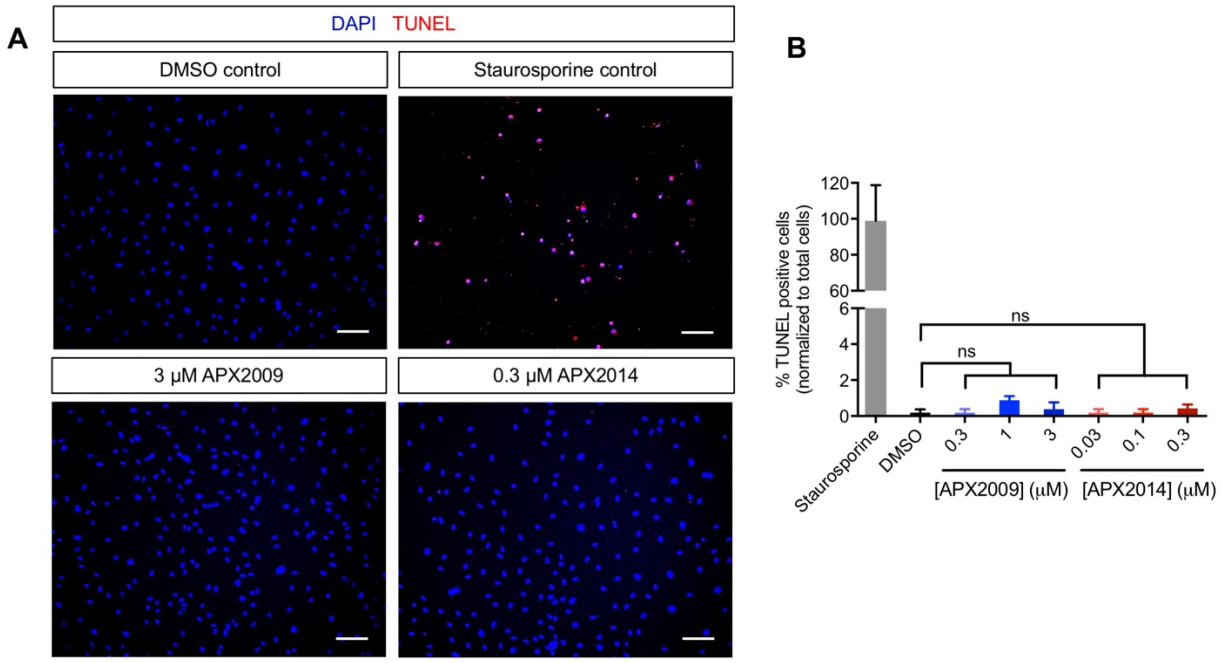
NMR ($CDCl_3$) δ 8.8 (br s,1H), 8.1 (m, 2H), 7.75 (m, 2H), 6.7 (s, 1H), 4.15 (s, 3H), 3.9 (s, 3H), 2.2 (m, 2H), 1.4 (m, 2H), 0.85 (t, 3H).



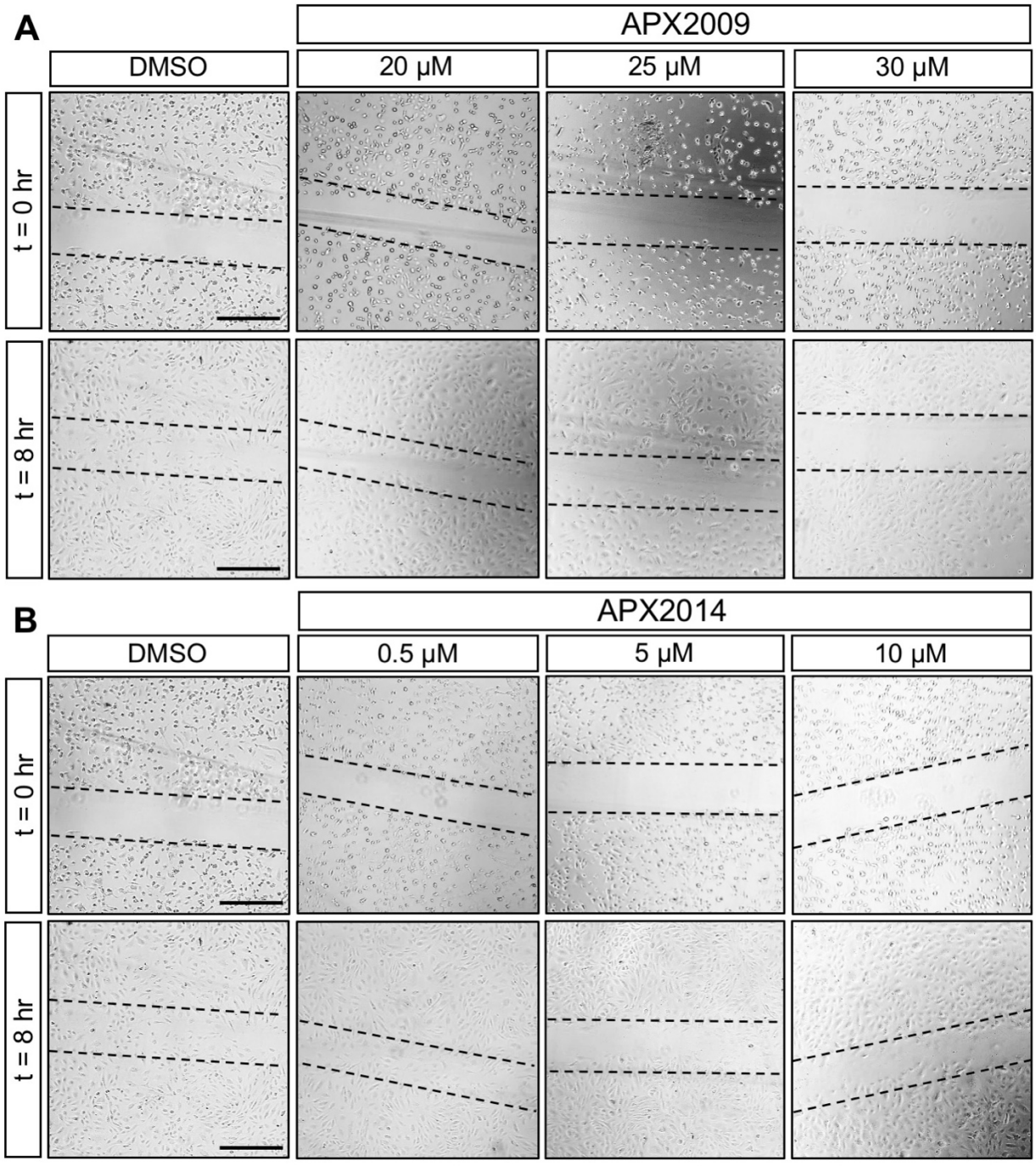
Supplemental Fig. 1. (A) Full fields of the EdU staining for all doses (same experiment as Fig. 3) show that APX2009 and APX2014 decrease DNA synthesis dose dependently in HRECs. Scale bars = 100 μm. (B) Propidium iodide cell cycle profiles for indicated treatments. (C) Quantification of cell cycle phase. Mean ± S.E.M., *n* = 3 independent experiments.



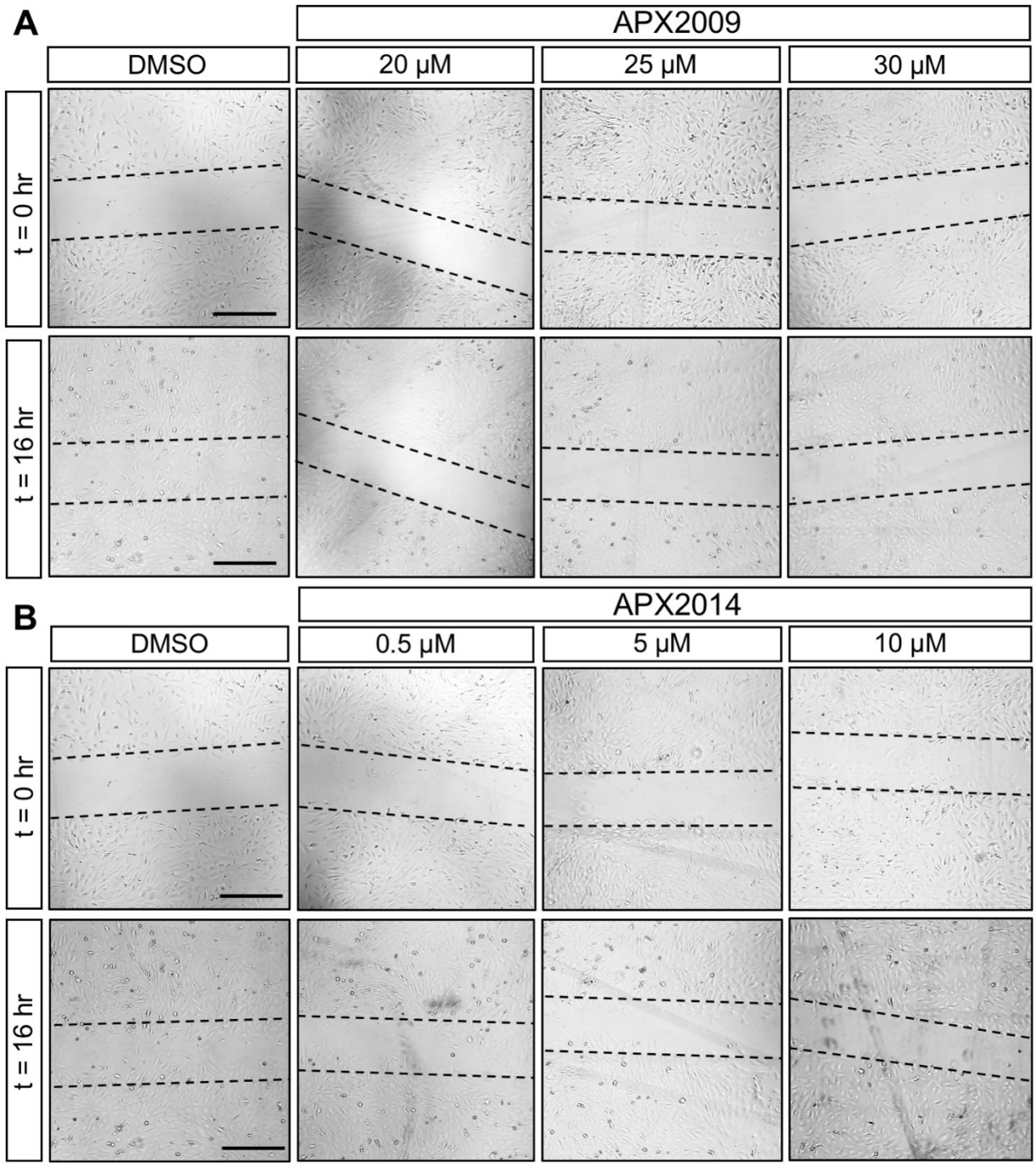
Supplemental Fig. 2. Separate channel images of Ki-67 staining for all doses (same experiment as Fig. 3) show that APX2009 and APX2014 decrease proliferation dose dependently in HRECs. Scale bars = 100 μm.



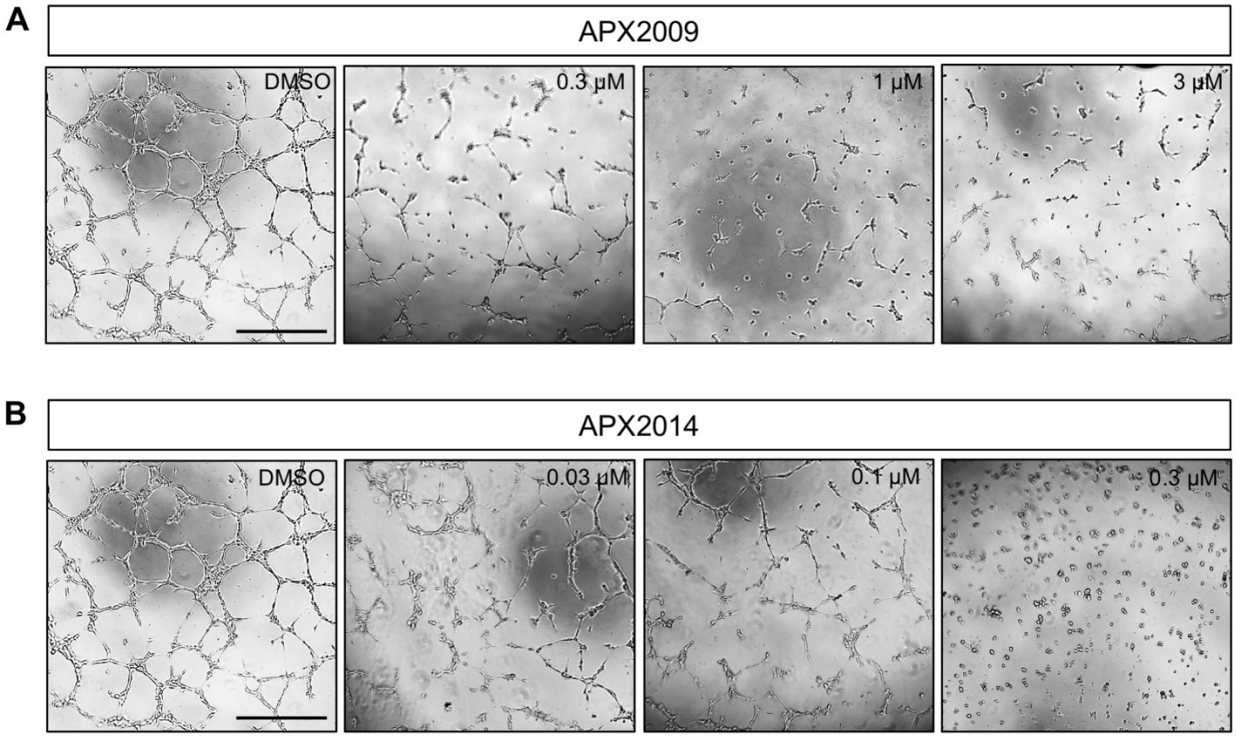
Supplemental Fig. 3. APX2009 and APX2014 do not induce cell death in HRECs. (A) TUNEL staining (red) for cell death and DAPI (blue) for nuclear staining. No TUNEL-positive cells are observed in these images. Staurosporine acts as a positive control. Scale bar = 100 μm. (B) Quantification data shows the percentage of TUNEL positive cells upon various treatments. Mean ± S.E.M., *n* = 3. ns, non-significant (one-way ANOVA with Dunnett's post hoc tests). Representative data from two independent experiments.



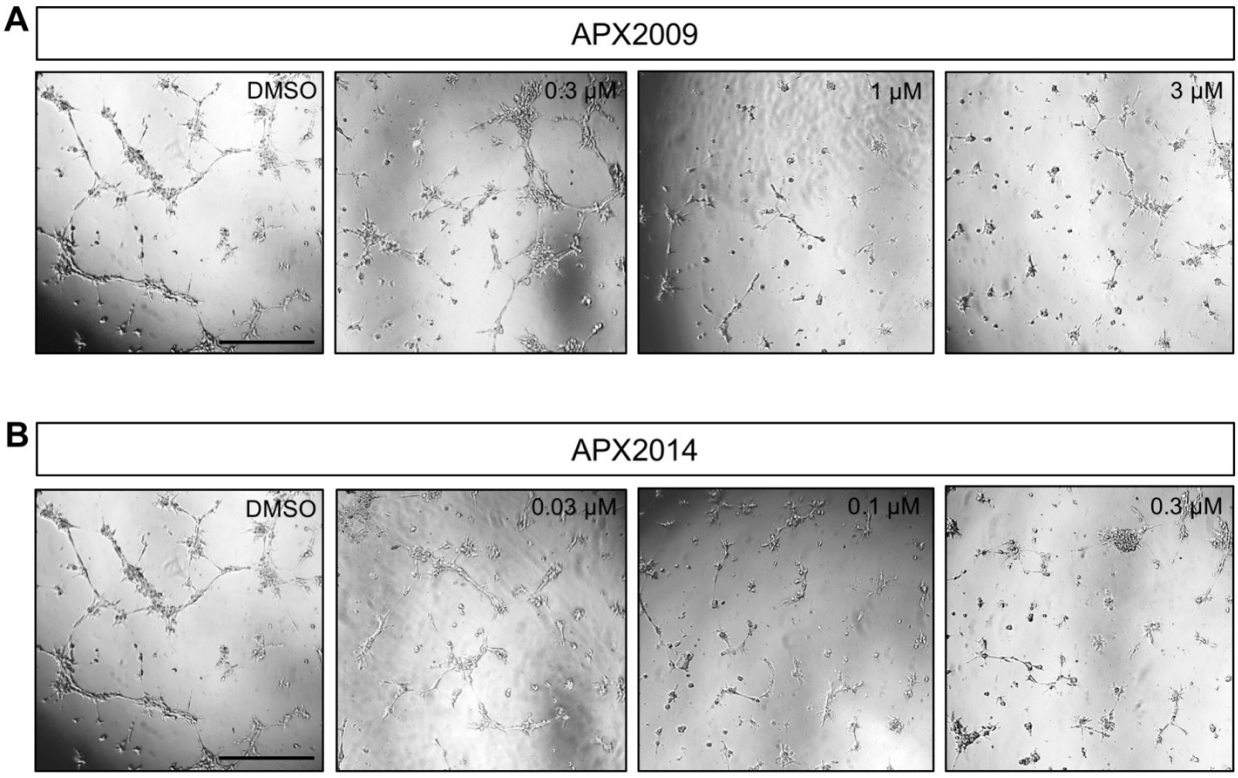
Supplemental Fig. 4. APX2009 and APX2014 inhibit migration of HRECs in vitro. Effect of (A) APX2009 and (B) APX2014 on cell migration in HRECs. A confluent monolayer of HRECs treated with various concentrations of each compound was wounded and wound closure was monitored for 8 hours. Scale bars = 500 μm.



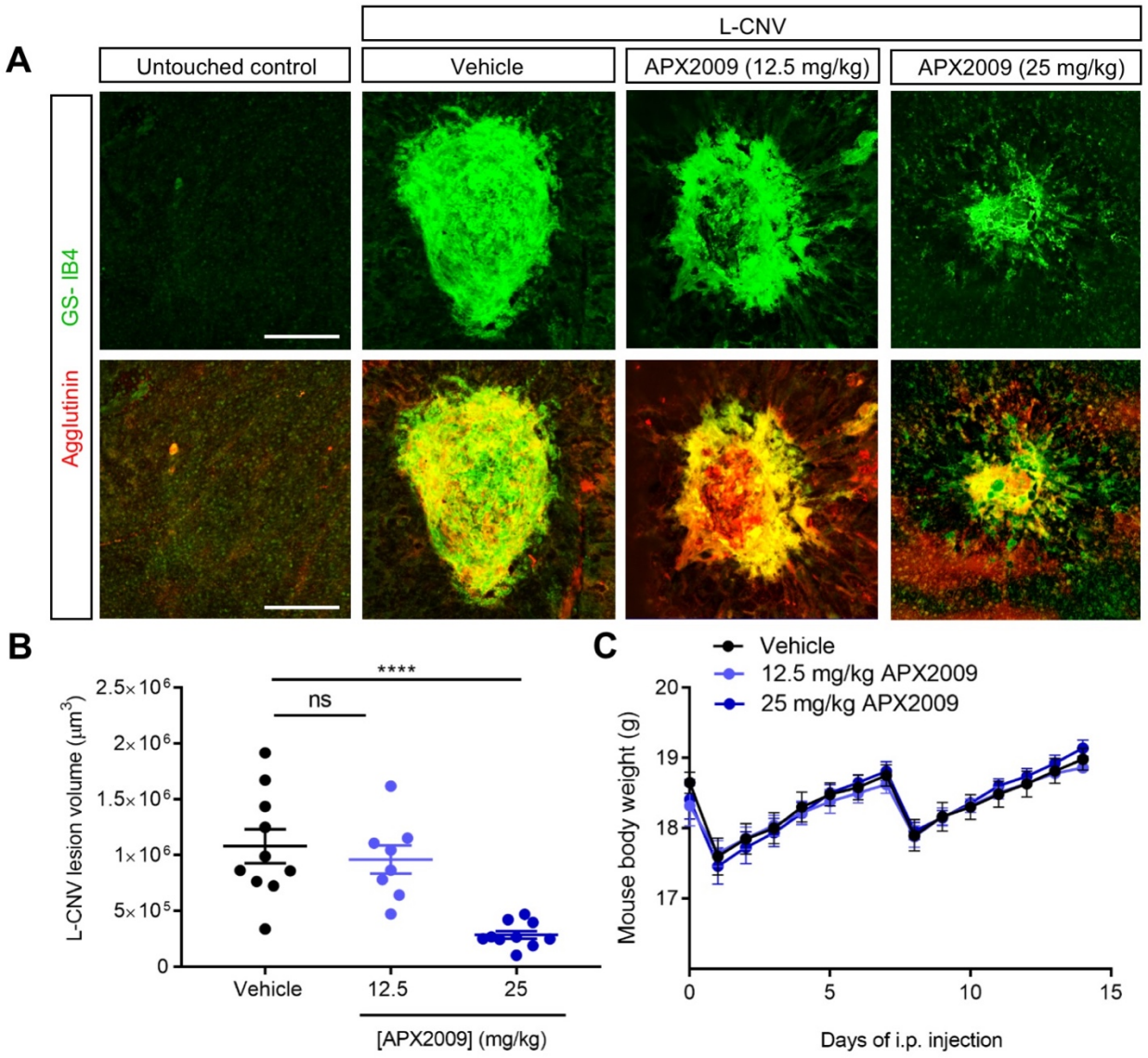
Supplemental Fig. 5. APX2009 and APX2014 inhibit migration of Rf/6a cells in vitro. Effect of (A) APX2009 and (B) APX2014 on cell migration in Rf/6a cells. A confluent monolayer of Rf/6a cells treated with various concentrations of each compound was wounded and wound closure was monitored for 16 hours. Scale bars = 500 μm.



Supplemental Fig. 6. APX2009 and APX2014 inhibit endothelial tube formation in HRECs in vitro. (A) Tube formation on Matrigel by HRECs in the presence of the indicated concentrations of APX2009. (B) Tube formation on Matrigel by HRECs in the presence of the indicated concentrations of APX2014. Scale bars = 500 μm.



Supplemental Fig. 7. APX2009 and APX2014 inhibit endothelial tube formation in Rf/6a cells in vitro. (A) Tube formation on Matrigel by Rf/6a cells in the presence of the indicated concentrations of APX2009. (B) Tube formation on Matrigel by Rf/6a cells in the presence of the indicated concentrations of APX2014. Scale bars = 500 μm.



Supplemental Fig. 8. APX2009 inhibits choroidal neovascularization in the L-CNV mouse model. (A) Double-stained Agglutinin and *Griffonia simplicifolia* isolectin B4 (GS-IB4) confocal images in the L-CNV lesions 14 days post-laser treatment. (B) Quantification of CNV lesion vascular volumes from Z-stack of GS-IB4-stained images at day 14 using ImageJ software. ns, non-significant; ****, $P < 0.0001$ (one-way ANOVA with Tukey's post hoc tests). (C) Quantification of mouse body weight of vehicle and APX2009 injected groups over 14 days. No significant difference in weight between treatments was observed at any time point (repeated measures two-way ANOVA). Mean ± S.E.M., n = 8–10 eyes/treatment. Scale bars = 100 µm.