Differential Cytotoxicity of the Hypoxia-Activated Therapy CPD100 in Endothelial and Retinal Cells Cultured Under Hypoxic and Normoxic Conditions

Abstract

Purpose:
To characterize the cytotoxic activity of CPD100 (vinblastine-N-oxide), a hypoxia-activated therapy (HAT), in endothelial and retinal epithelial cells cultured under hypoxic and normoxic conditions.

Methods:
Human umbilical vein endothelial cells (HUVECs) and human pigmented retinal epithelial cells (RPE1) were exposed to CPD100 or vinblastine (0.1–1000 µg/mL) under controlled hypoxia or normoxia. Cell viability, death modality, and morphological changes were assessed using label-free live cell imaging and quantitative cell-death metrics.

Main Outcome Measures:
Dose-dependent cytotoxicity, mode of cell death, and differential sensitivity between endothelial and retinal epithelial cells.

Results:
CPD100 induced dose-dependent cytotoxicity in HUVECs, with approximately nine-fold greater cell death under hypoxia than normoxia. Apoptosis was the predominant mode of death. RPE1 cells were markedly more resistant: CPD100 did not induce apoptosis or necrosis but produced lipid droplet accumulation under hypoxia in these cells. CPD100 was more cytotoxic than vinblastine in hypoxic HUVECs, whereas vinblastine showed limited activity, consistent with reduced proliferation under hypoxia.

Conclusions:
CPD100 demonstrates hypoxia-enhanced, endothelial-selective cytotoxicity, supporting its potential as a targeted therapy for hypoxic neovascular tissue in diseases such as neovascular AMD. The relative resistance of retinal epithelial cells suggests a favorable therapeutic index. Further in vivo studies are warranted.

Introduction

Wet AMD is a neovascular disease of the eye characterized by macular degeneration involving the formation of choroidal neovascularization (CNV) and fluid leakage from the choroidal neovessels. Hypoxia is a central driver of pathological angiogenesis in neovascular age-related macular degeneration (AMD) and other ischemic retinal diseases[1]. With the progress of CNV,

the retina is invaded, increasing oxidative stress, vascular leakage, and inflammation, thereby leading to hemorrhage and fibrosis of the retinal macula, loss of photoreceptor cells, and ultimately complete central vision loss. The main treatment strategy for wet AMD includes the use of drugs that block the main driver of angiogenesis, i.e, Vascular Endothelial Growth Factor (VEGF). However, these agents are not curative and require repeated intravitreal injections for prolonged periods of time to control CNV. Thus, there is an urgent need to devise novel interventional approaches. Endothelial cells within CNV experience profound metabolic stress, stabilizing hypoxia-inducible factors and promoting angiogenesis, vascular permeability, and treatment resistance within an inflammatory microenvironment[2]. Hypoxia-activated therapy (HAT) represents a strategy to selectively target these microenvironments while sparing normoxic retinal tissue.

CPD100 (vinblastine-N-oxide) is a HAT designed to remain inactive under normoxia and become activated under hypoxic conditions, releasing vinblastine or related cytotoxic species [3,4]. Vinblastine is a well described inhibitor of microtubule formation; the mechanism of action of which, at least in part, is to prevent proper formation of the cellular spindle and kinetochore, resulting in cellular mitotic arrest (M phase specific) and consequent cell death. Preclinical studies have shown that vinblastine inhibits endothelial cell functions involved in angiogenesis, including proliferation, adhesion and chemotaxis[5]. Vinblastine effects on endothelial cells are exerted at picomolar concentrations whereas the proliferation of other cell types (e.g., fibroblasts, epithelial cells) are not inhibited at these concentrations. Because vinblastine preferentially affects proliferating cells, CPD100 could also selectively target hypoxic, proliferative angiogenic endothelial cells while minimizing toxicity to quiescent retinal epithelial cells. Hence, CPD100 could be used to treat a wide spectrum on angiogenesis-dependent diseases, including wet AMD[6-7].

This study evaluates the cytotoxic activity of CPD100 across a range of concentrations in endothelial and retinal epithelial cells cultured under hypoxia and normoxia. A direct comparison of the cytotoxicity of CPD100 and vinblastine in endothelial cells cultured under hypoxia was also evaluated. These findings inform the potential therapeutic index of CPD100 for ocular neovascular diseases.

Methods

Cell Culture and Seeding

HUVECs were used as a model of proliferative endothelial cells. RPE1 cells were used as a model of quiescent retinal epithelial cells. Cells were maintained under standard conditions and exposed to either normoxia (21% O_2) or controlled hypoxia (5% O_2).

Vials of cells were thawed in a 37°C water bath and seeded following an in-house protocol in 10 cm petri dishes. Cells were allowed to recover until reaching approximately 80% confluency,

with medium changes twice weekly. Cells were passaged and banked for future experiments, and new passages were cultivated until 80% confluency with medium changes twice weekly. Cells were then passaged and seeded onto plates as described below.

Plate Preparation and Seeding

Plates were coated with fibronectin according to an in-house protocol. Cells were washed with PBS and treated with 1 mL of trypsin, incubated for 5 minutes at 37°C to facilitate detachment. Cell detachment was confirmed microscopically. Trypsinization was stopped by adding Complete DMEM, and cells were collected for centrifugation.

A 10 µL aliquot of the cell suspension was mixed with 10 µL of Trypan Blue for viability counting using the Luna cell counter. Cells were centrifuged at 250 × g for 5 minutes, supernatant discarded, and the pellet resuspended in Complete DMEM to achieve a concentration of 1 million cells per mL. Cells were diluted to seed 1000 cells per well in 54 wells, with 100 µL per well (preparing suspension for 80 wells total). The final volume was 8000 µL, containing 80,000 cells (80 µL of cell suspension plus 7920 µL of medium). Two plates were prepared according to the plate layout, with 27 wells per cell line per plate. Plates were incubated until appropriate confluency was reached.

Drug Preparation and Exposure

CPD100 and vinblastine stock solutions were prepared fresh prior to each experiment. For CPD100, the content of one vial (20 mg) was solubilized in 1.11 mL PBS by gentle rolling until fully dissolved to create a 1000 µg/mL stock (S1). Serial 10-fold dilutions were prepared to obtain concentrations of 100 µg/mL (S2), 10 µg/mL (S3), 1 µg/mL (S4), and 0.1 µg/mL (S5) by adding 900 µL PBS to 100 µL of the previous dilution with thorough mixing. Vinblastine stock solutions were prepared by solubilizing one vial (10 mg) in 0.555 mL PBS to yield a 1000 µg/mL stock (S1V). Serial 100-fold dilutions were prepared to obtain 10 µg/mL (S2V) and 0.1 µg/mL (S3V) concentrations by adding 990 µL PBS to 10 µL of the previous dilution with thorough mixing. Cells were treated with CPD100 or vinblastine at final concentrations ranging from 0.1 to 1000 µg/mL as indicated in the Results section.

Pre-Acquisition and Imaging

Microscopes were prepared and set to controlled environmental conditions: one at normoxia (21% O_2, 5% CO_2, 37°C) and the other at hypoxia (0.5% O_2, 5% CO_2, 37°C). Medium in all wells was changed by aspirating the old medium and gently adding 85 µL of fresh medium. Five microliters of either PBS (control) or drug solutions (S1-S5) were pipetted into each well according to the plate layout. Image acquisition was started immediately after drug addition using a 3x3 field of view per well, with 45-minute cycles over 48 hours.

Outcome Measures

Label-free live-cell imaging and quantitative cell-death metrics were evaluated with Nanolive 3D-Cell Explorer for all analyses. Evaluated parameters included cell viability, mode of cell death (i.e., necrosis, apoptosis) and morphological changes, including lipid droplet accumulation and mitotic cell arrest. EC50 values were calculated as absolute half maximum effective concentration (AbsEC50), i.e., concentration of a drug at which 50% change in response is observed in relation to the untreated control. AbsEC50 is referred to as EC50 in this document for brevity.

Results

CPD100 induces time-dependent, preferential cytotoxicity in endothelial cells versus retinal cells under hypoxia

Endothelial (HUVEC) and retinal (RPE1) cells were cultured under hypoxic conditions in the presence or absence of CPD100 (10 µg/mL), and cell death was quantified over 24 hours. Figure 1 shows time-dependent cytotoxicity with CPD100, inducing a progressive increase in endothelial cell death, reaching approximately 40–45% by 24 hours, whereas retinal cell death increased more slowly and plateaued at ~25–30%. Untreated endothelial and retinal controls remained at baseline levels (≤5%), confirming that hypoxia alone did not contribute to cytotoxicity.

The widening separation between the CPD100-treated endothelial and retinal curves over time show a clear inflection by 10 hours. The time-dependent cytotoxicity reflects the cumulative nature of CPD100 driven by progressive intracellular drug accumulation, enhanced prodrug activation in hypoxic microenvironments, increased microtubule target engagement, and delayed activation of apoptotic pathways that heighten cellular vulnerability.

Altogether, these data demonstrate increasing selectivity for endothelial cell killing, consistent with a hypoxia-dependent mechanism that preferentially affects vascular cell populations versus retinal cell populations.

Retinal epithelial cells are more resistant than endothelial cells to CPD100-induced cell death

Exposure–response analyses were conducted to determine EC50 values of CPD100 in endothelial and retinal cells cultured under hypoxic versus normoxic conditions (Figure 2). In these experiments, EC50 represents the concentration of drug required to achieve 50% of maximal cytotoxicity in relation to untreated control, i.e., a lower EC50 means higher potency.

CPD100 induced substantially greater cell death in endothelial than in retinal cells under both hypoxia and normoxia, with EC_{50} values of 15 µg/mL vs. 141 µg/mL under hypoxia and 103 µg/mL vs. 262 µg/mL under normoxia, respectively. These results show that EC50 values for endothelial cells under hypoxia are reduced approximately 9-fold vs. endothelial cells and 18-fold vs. retinal cells cultured under normoxic controls, indicating a substantial left-shift in the concentration–response curve. In contrast, normoxic cultures required substantially higher drug concentrations to achieve EC50s, consistent with CPD100-attenuated activation or reduced susceptibility in oxygen-replete environments. Collectively, these data support a hypoxia-dependent mechanism of action, with preferential potency in low-oxygen microenvironments that may enable selective targeting of hypoxic endothelial cells while limiting off-target cytotoxicity to endothelial and retinal cells in normoxic compartments.

CPD100 induces apoptosis in endothelial cells

Figure 3 shows representative microscopy images illustrating distinct cellular phenotypes associated with normal proliferation, mitotic arrest, and apoptosis. In untreated HUVEC or RPE1 cells cultured under normoxia or hypoxia conditions, cells exhibit typical morphology with intact membranes and visible mitotic figures, consistent with ongoing cell division. In contrast, endothelial cells exposed to CPD100 (10 µg/mL) demonstrate prominent mitotic arrest, characterized by rounded, condensed cells with disrupted spindle progression. Numerous apoptotic bodies and cells undergoing apoptotic fragmentation are evident, indicating progression from mitotic arrest to programmed cell death. The images for endothelial cells depict a transition from healthy proliferative states to mitotic blockade and apoptosis, consistent with the expected cellular response to microtubule destabilization. In contrast, retinal cells exposed to both hypoxia and CPD100 (10 µg/mL) only showed lipid-droplets, suggesting metabolic stress or altered lipid handling in affected populations but no mitotic cell arrest or death. Despite this differential sensitivity, retinal cells did not undergo apoptosis or necrosis during the study period; instead, they progressively accumulated cytoplasmic lipid droplets, indicating cellular stress without overt cytotoxicity. These findings suggest that, at concentrations that readily kill endothelial cells, CPD100 primarily induces oxidative stress and lipid accumulation—but not cell death—in retinal cells.

CPD100 is more cytotoxic than vinblastine for hypoxic endothelial cells

Endothelial cells cultured under hypoxic conditions were exposed to either CPD100 (10 µg/mL) or vinblastine (10 µg/mL) for 24 hours, and cytotoxicity was quantified as percent dead cells per field of view. In this experiment under hypoxia, CPD100 achieved greater endothelial cell killing than vinblastine at an equivalent concentration. Figure 4 shows that CPD100 induced approximately 40% endothelial cell death, whereas vinblastine produced only minimal effects, with cell death remaining near 8%. Since both drugs were evaluated under identical hypoxic exposure, the observed difference reflects a compound-specific effect rather than hypoxia-driven background toxicity. The results support a CPD100 mechanism distinct from classical microtubule-targeting agents.

Discussion

Across multiple orthogonal assays, CPD100 demonstrates a robust, hypoxia-dependent cytotoxic mechanism with preferential activity against endothelial cells and a potency profile distinct from classical microtubule-targeting agents. EC50 analyses showed that both endothelial and retinal cells exhibit markedly enhanced sensitivity to CPD100 under hypoxia, with approximately 9-fold and 18-fold reductions in EC50, respectively, compared with normoxic conditions. These large shifts in potency indicate that CPD100 becomes substantially more active in low-oxygen environments, consistent with a hypoxia-activated mechanism of action. Overall, results suggest that retinal epithelial cells can better withstand hypoxic conditions through metabolic adaptation.

Mechanistically, endothelial cells may be more vulnerable than to retinal cells due to: (1) their higher proliferative rate, which increases susceptibility to vinca alkaloid-like mitotic disruption; (2) their potential for greater intracellular CPD100 uptake or retention, consistent with known low-dose vinblastine accumulation and cytotoxicity in microvascular endothelium[5]; (3) a higher CPD100 cleavage rate in endothelial cells and (3) their heightened sensitivity to hypoxia-associated stress pathways. For example, the iHypoxia database catalogs hypoxia responsive proteins across many tissues, and carbonyl reductases -which may mediate the cleavage of CPD100- are included among proteins whose tissue expression changes under hypoxia[8]. The combination of high CPD100 activation and a high proliferative activity can drive mitotic catastrophe despite elevated efflux, and CPD100 could also mediate oxidative stress that may further amplify endothelial cell killing.

Time-course imaging under hypoxia further demonstrated that CPD100 induces progressive and selective endothelial cell killing, reaching ~40–45% death by 24 hours, whereas retinal cell death plateaued at ~25–30%. Untreated controls for both cell types remained at baseline levels, confirming that hypoxia alone does not drive cytotoxicity. The widening separation between endothelial and retinal responses over time indicates that CPD100's selectivity is not only oxygen-dependent but also exposure-dependent, suggesting differential susceptibility of vascular versus retinal cells to the activated compound. CPD100 time-dependent cytotoxicity reflects the cumulative nature of its mechanism of action: intracellular drug levels rise over time, cleavage of CPD100 is activated under hypoxia, damage pathways amplify, and apoptotic signaling requires sustained exposure to fully manifest. These data are consistent with a mechanism in which overall exposure (i.e., Area Under the Curve, AUC), rather than instantaneous concentration, drives cytotoxic effect.

Microscopy studies provided mechanistic context for these quantitative findings. Untreated endothelial and retinal cells cultured under hypoxia displayed normal morphology and active mitosis. In contrast, CPD100-treated endothelial cells cultured under hypoxia exhibited prominent mitotic arrest, characterized by rounded, condensed cells with disrupted spindle progression. These arrested cells subsequently progressed to apoptosis, evidenced by apoptotic bodies and fragmented nuclei. Retinal cells exposed to CPD100 and hypoxia revealed lipid-accumulating cells, consistent with metabolic stress. Together, the results in endothelial cells exposed to CPD100 and hypoxia support a mechanism involving microtubule

destabilization–associated mitotic arrest followed by apoptotic cell death, but with features that diverge from canonical tubulin-binding agents.

CPD100 was also found to be more cytotoxic than vinblastine to endothelial cells. Under identical hypoxic conditions and at the same drug concentration (10 µg/mL), vinblastine induced only minimal endothelial cell death (~8%), whereas CPD100 produced ~40% cytotoxicity at 24 hours of exposure. These data demonstrate that vinblastine does not replicate the hypoxia-dependent killing profile of CPD100, indicating that CPD100's activity is not attributable to classical microtubule inhibition alone. Whereas vinblastine relies on microtubule depolymerization to trigger mitotic catastrophe and subsequent apoptosis or necrosis in actively cycling cells, CPD100 achieves greater hypoxia selective endothelial killing at equivalent doses, suggesting additional mechanisms beyond vinblastine release, including enhanced intracellular accumulation, reduced efflux, and/or hypoxia driven activation that amplifies oxidative stress. For example, CPD100 could achieve higher intracellular exposure than vinblastine through increased uptake, reduced efflux—given that vinblastine is efficiently exported by P gp/MDR1, whose expression is further upregulated by hypoxia[9]—and greater activation by hypoxia induced reductase activity.

Collectively, the results establish a coherent pharmacologic profile in which CPD100 becomes highly potent under hypoxia, preferentially targets endothelial cells, induces mitotic arrest and apoptosis, and exhibits a mechanism distinct from standard microtubule-targeting chemotherapeutics. This profile supports the potential utility of CPD100 in hypoxic microenvironments, such as ischemic retinal tissue or tumor-associated vasculature, where selective cytotoxicity may provide therapeutic advantage while sparing normoxic tissues.

Further development of CPD100 as a hypoxia-activated therapy for ocular neovascular diseases is warranted. Future evaluation of CPD100 should include *in vivo* ocular safety, PK/PD, ADME and efficacy studies.

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Conflict of Interest

AAG is a member of the board of directors and stockholder of Cascade Prodrug Inc. GC and NP received consulting fees from Cascade Prodrug Inc. TC and CP are Nanolive's employees and stockholders.

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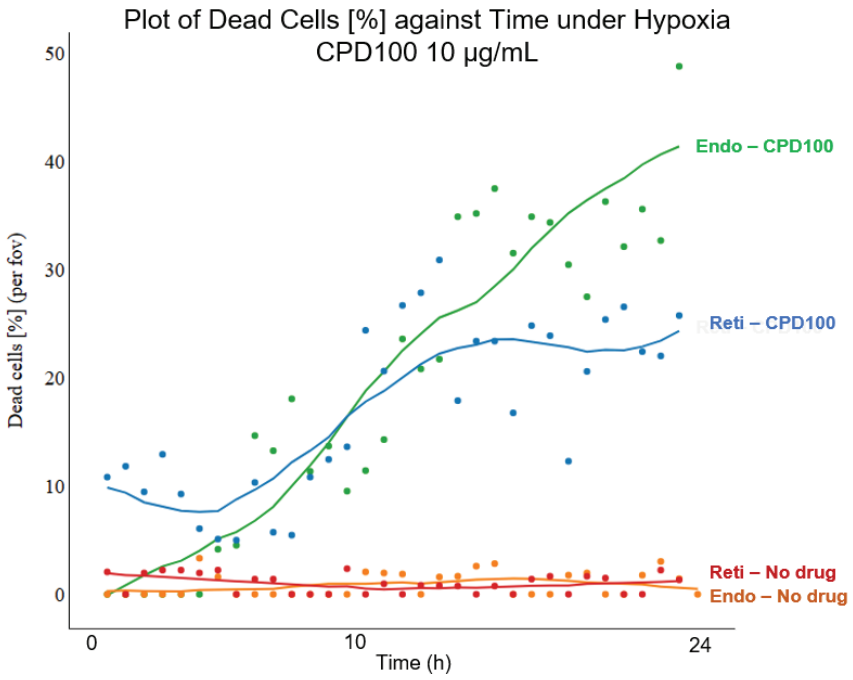

Figure 1. CPD100 induces time-dependent, preferential cytotoxicity in endothelial cells under hypoxia. Endothelial and retinal cells were exposed to CPD100 (10 µg/mL) under hypoxic conditions, and cell death was quantified over 24 hours. CPD100 produced a progressive increase in endothelial cell death, reaching approximately 40–45% by 24 hours, whereas retinal cell death increased more slowly and plateaued at ~25–30%. Untreated endothelial and retinal controls remained at baseline levels (≤5%), confirming that hypoxia alone did not contribute to cytotoxicity.

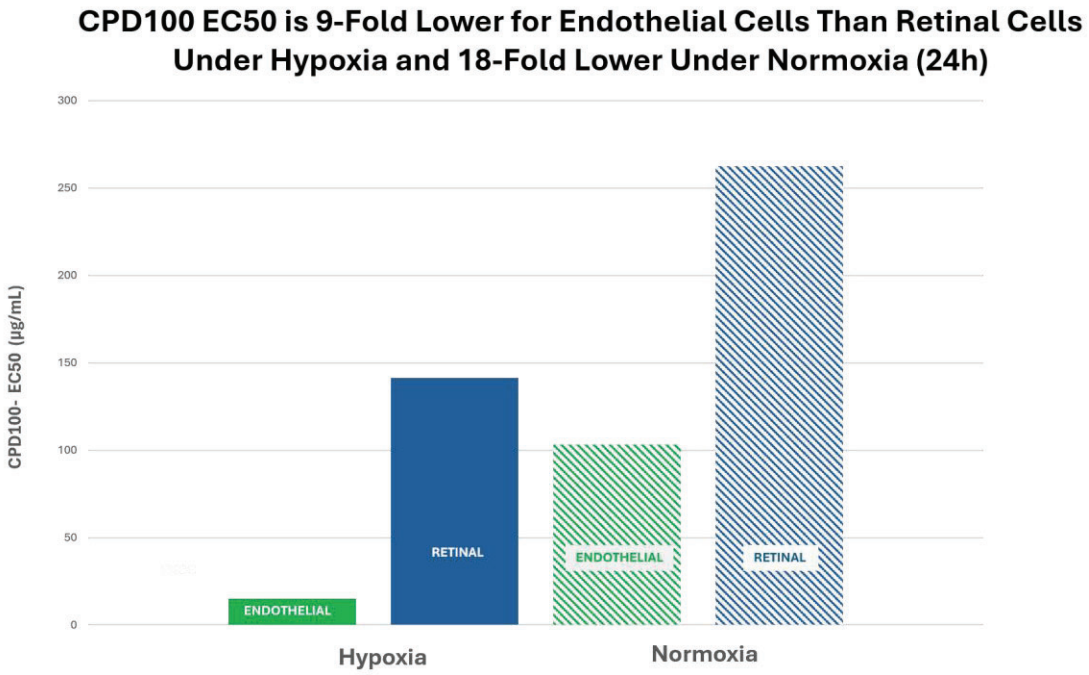

Figure 2. CPD100 demonstrates hypoxia-dependent enhancement of cytotoxic potency in endothelial and retinal cells. Concentration–response curves were generated for CPD100 under hypoxic and normoxic culture conditions, and EC50 values were calculated as the concentration required to achieve 50% maximal cytotoxicity. Under hypoxia, EC50 values were reduced approximately 9-fold in endothelial cells and 18-fold in retinal cells relative to normoxic controls, indicating a substantial increase in drug potency in low-oxygen environments. Normoxic cultures exhibited markedly higher EC50 values, consistent with attenuated activation or reduced susceptibility in oxygen-replete conditions. These findings support a hypoxia-activated mechanism of action and demonstrate preferential cytotoxic activity of CPD100 in hypoxic cell populations.

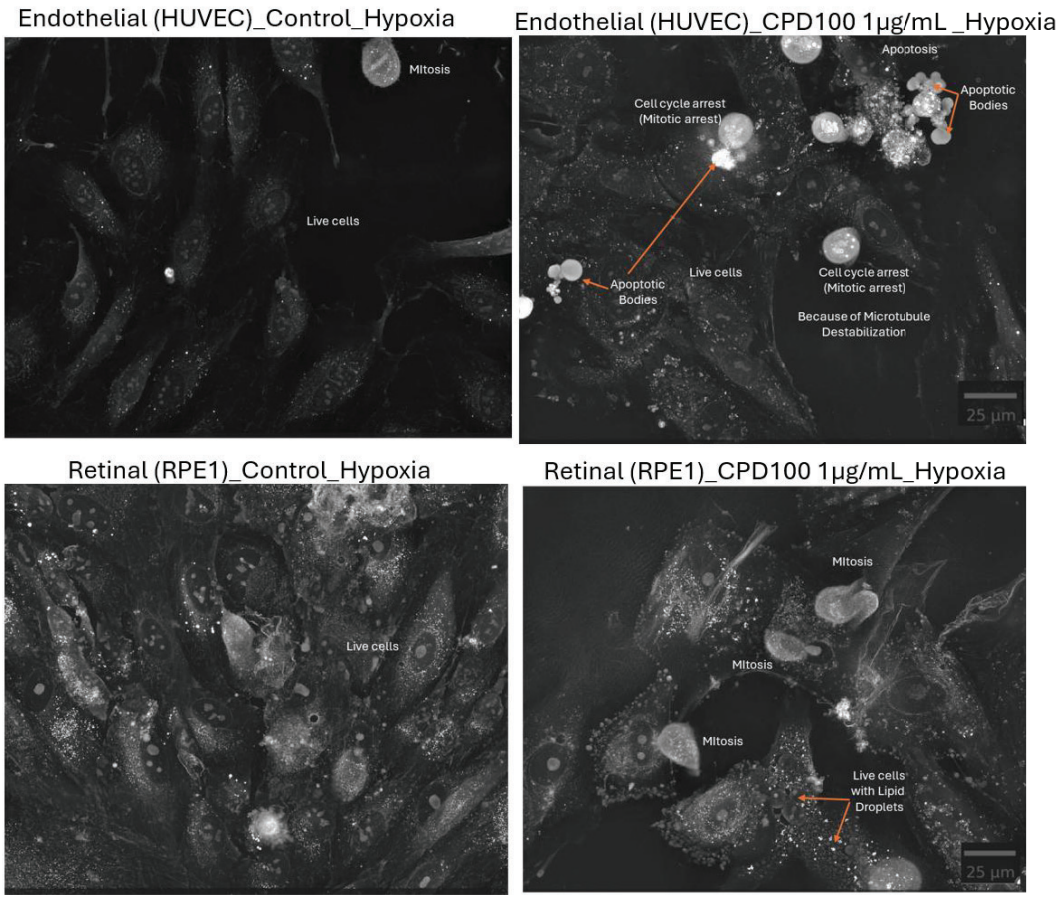

Figure 3. Representative microscopy images demonstrating progression from normal proliferation to mitotic arrest and apoptosis in endothelial cells, and to lipid-laden stress in retinal cells following microtubule destabilization with CPD100. Images depict untreated cells with normal morphology and active mitosis, contrasted with cells exposed to a microtubule-destabilizing insult with CPD100. Treated endothelial cell cultures show prominent mitotic arrest, characterized by rounded, condensed cells with disrupted spindle progression, followed by the appearance of apoptotic bodies and fragmented apoptotic cells. In contrast, retinal cells cultured under the same conditions only reveal lipid-accumulating cells but no cell death, consistent with metabolic stress associated with CPD100 exposure.

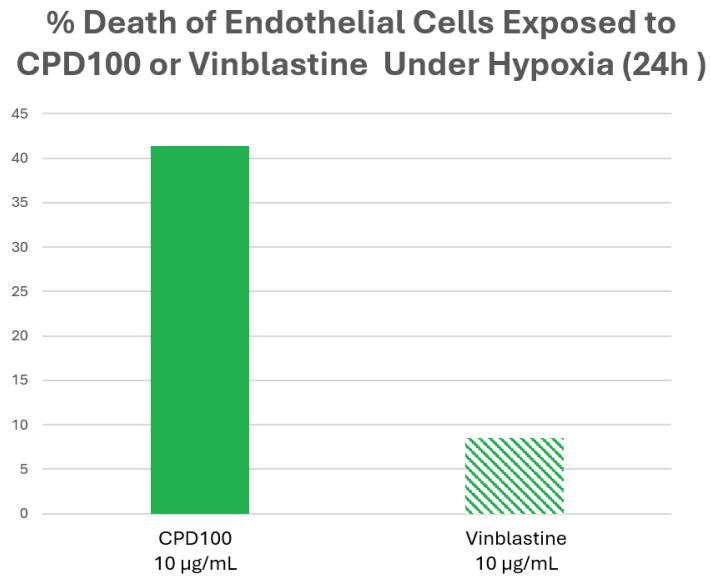

Figure 4. CPD100 induces substantially greater endothelial cell death under hypoxia than vinblastine at an equivalent concentration. Endothelial cells were exposed to CPD100 (10 µg/mL) or vinblastine (10 µg/mL) for 24 hours under hypoxic conditions, and cytotoxicity was quantified as percent dead cells per field of view. CPD100 produced robust endothelial cell death (~40%), whereas vinblastine induced only minimal cytotoxicity (~8%) despite identical dosing and environmental conditions. These results demonstrate that vinblastine does not replicate the hypoxia-dependent cytotoxic profile of CPD100, supporting a mechanism of action distinct from classical microtubule-targeting agents.