A Novel Imaging-based High-throughput Screening Approach to Anti-angiogenic Drug Discovery

Lasse Evensen, David R. Micklem, Wolfgang Link, James B. Lorens*

Abstract
The successful progression to the clinic of angiogenesis inhibitors for cancer treatment has spurred interest in developing new classes of anti-angiogenic compounds. The resulting surge in available candidate therapeutics highlights the need for robust, high-throughput angiogenesis screening systems that adequately capture the complexity of new vessel formation while providing quantitative evaluation of the potency of these agents. Available in vitro angiogenesis assays are either cumbersome, impeding adaptation to high-throughput screening formats, or inadequately model the complex multistep process of new vessel formation. We therefore developed an organotypic endothelial-mural cell co-culture assay system that reflects several facets of angiogenesis while remaining compatible with high-throughput/high-content image screening. Co-culture of primary human endothelial cells (EC) and vascular smooth muscle cells (vSMC) results in assembly of a network of tubular endothelial structures enveloped with vascular basement membrane proteins, thus, comprising the three main components of blood vessels. Initially, EC are dependent on vSMC-derived VEGF and sensitive to clinical anti-angiogenic therapeutics. A subsequent phenotypic VEGF-switch renders EC networks resistant to anti-VEGF therapeutics, demarcating a mature vascular phenotype. Conversely, mature EC networks remain sensitive to vascular disrupting agents. Therefore, candidate anti-angiogenic compounds can be interrogated for their relative potency on immature and mature networks and classified as either vascular normalizing or vascular disrupting agents. Here, we demonstrate that the EC-vSMC co-culture assay represents a robust high-content imaging high-throughput screening system for identification of novel anti-angiogenic agents. A pilot high-throughput screening campaign was used to define informative imaging parameters and develop a follow-up dose-response scheme for hit characterization. High-throughput screening using the EC-vSMC co-culture assay establishes a new platform to screen for novel anti-angiogenic compounds for cancer therapy.

Key terms
high-throughput screening; endothelial/mural cell co-culture; angiogenesis

INHIBITING angiogenesis is a validated therapeutic modality for cancer (1). Hence, new agents that potently inhibit pathological angiogenesis are a critical component of future combination cancer therapies (2). The identification of candidate therapeutics relies on current in vitro angiogenesis assays that measure effects on endothelial cell migration, proliferation, apoptosis and tube formation, and in vivo models such as the chick chorioallantoic membrane assay, corneal neovascularization assay, and Matrigel plug assays (3–5). Current in vitro angiogenesis assays are generally focused on specific behaviors of monocultured endothelial cells (EC) (e.g., migration, proliferation) and fail to model heterotypic perivascular interactions, whereas in vivo systems are not compatible with high-throughput screening. More complex endothelial tube formation in vitro assays, such as endothelial culture on Matrigel or in 3D collagen matrices, are cumbersome and not sufficiently robust for high-throughput screening. To address this, we have developed a microtiter format in vitro assay that
encompasses the three main components of blood vessels: EC, mural cells, and the basement membrane. Co-culture of primary human umbilical cord endothelial cells (HUVEC) and pulmonary artery-derivated vascular smooth muscle cells (PA-vSMC) results in formation of an organotypic capillary network recapitulating many facets of in vivo angiogenesis including morphology, pathfinding migration, loss of endothelial cell proliferation, basement membrane deposition, and quiescence. During network formation, EC are completely dependent on vSMC-derived VEGF. Inhibition of VEGF-signaling by treatment with anti-VEGF antibody (bevacizumab), VEGF receptor-targeting small molecule tyrosine kinase inhibitors, or RNA interference of VEGF expression in vSMC potentially blocks capillary-like network formation (6–9). However, once formed, EC networks acquire resistance to anti-VEGF therapeutics reflecting a phenotypic VEGF-switch characteristic of blood vessel maturation. This biphasic sensitivity profile can be utilized to classify potential anti-angiogenic therapeutics as normalizing agents (e.g. bevacizumab) or vascular disruptive agents (e.g. combretastatin) (8). Further, we have shown that image analysis of capillary-like network formation can be used to conduct quantitative structure-activity relationship analysis of tubulin-inhibitor natural compound derivatives (unpublished results). Here, we evaluate the biphasic co-culture assay as an automated high-throughput image-acquisition approach for anti-angiogenic compound discovery. Using a pilot HTS screening campaign, we evaluated image analysis parameters and assessed the robustness of the biphasic approach. We demonstrate that the HTS co-culture assay represents a novel organotypic assay approach suitable for large scale high-throughput screening.

**Material and Methods**

**Compounds**

PTK787/ZK (10) and CHIR258 (11) were kindly provided by Novartis (Oncology Research, Novartis Institutes for BioMedical Research). CK246 was kindly provided by Professor Karl-Heinz Altmann (Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Zurich Switzerland) (12). Velcade was kindly provided by Prof. Bjorn Tore Gjertsen, Haukeland University Hospital, Norway (13). Flavopiridol was kindly provided by the NCI, National Institutes of Health. All other chemicals were purchased from commercial sources. 5-Aza-2’-Deoxycytidine, Bapta-AM, Doxorubicin, JAK3 Inhibitor VI, JNK Inhibitor VIII, Kenpaullone, KN-62, Resveratrol, W-13 HCl, and W-7 HCl were purchased from Calbiochem (San Diego, CA). Genistein, Rapamycin, Thapsigargin (THAPS), and U0126 were purchased from LC Laboratories. (Woburn, MA); Caffeine, Hydrocortisone, Nicotinamide, Paclitaxel, STO-609, 12-O-tetradecanoylphorbol-13-acetate (TPA), Trichostatin A (TSA), and Vinblastine were purchased from Sigma-Aldrich (St. Louis). D000 was purchased from Labotec (Niederschoena, Germany); Stock solutions of the test compounds were deposited onto 96-well mother plates, transferred to multiple replica plates, and frozen at −80°C.

**Cell Culture**

HUVEC and PA-vSMC were purchased from Lonza (C2517A, CC2581). To simplify imaging, early passage HUVEC cells were infected with retrovirus carrying a fluorescent-expressing construct. HUVEC cells used in screening experiments were infected with a dsTomato construct, whereas a GFP construct was used for the experiments testing the use of propidium iodide staining. Cells were maintained in culture in the supplier’s recommended complete medium (EGM-2, SmGM-2) at 37°C, 5% CO2. The growth medium was changed every third day, and cells were passaged before reaching confluence. The maximum passage number used for experiments was eight (HUVEC) and 10 (PA-vSMC).

**EC-mural Cell Co-culture Assay**

Separated cultures of PA-vSMC and HUVEC were trypsinated, counted and mixed in a falcon-tube, and centrifuged at 200g for 5 min. To seed the co-cultures, the cell pellet was resuspended in the desired amount of EGM-2 before distributing to the wells of a 96-well plate. The plate was then centrifuged briefly at 200g to achieve an even distribution of cells and cultured for 72 h at 37°C, 5% CO2 to allow network formation. Cell numbers and culture volume were as follows (per well): 96-well plates: 5 × 10^4 PA-vSMC, 10–15 × 10^3 HUVEC, 200 µl EGM-2.

**Compound Treatment**

Addition of compounds was performed as follows: Concentrated stock solutions were diluted to 2× concentrations in a 96-well plate containing 100 µl EGM-2 per well and mixed thoroughly; 100 µl medium was removed from seeded co-cultures and substituted with 100 µl EGM-2 containing 2× compound concentrations. DMSO concentrations were kept at 0.25%.

**Microscopy and High-content Imaging**

For quantitative analysis of the co-cultures, a BD Pathway 855 bioimaging system (BD Biosciences, San Jose, CA) was used for automated high-throughput-imaging. Statistical analysis of acquired images was done with BD Image Data Explorer software. Images demonstrating network formation were acquired as 5 × 5 montages using a 10× objective (Fig. 1). To decrease disk usage and acquisition times, images from screening experiments and dose-response experiments were acquired as 2 × 2 montages using a 10× objective. Filters used: Propidium Iodide: excitation filter 555/28 and emission filter 655/75, Hoechst: excitation filter 380/10 and emission filter 435LP, dsTomato HUVEC cells: excitation filter 548/20 and emission filter 570LP, GFP HUVEC cells: excitation filter 488/10 and emission filter 520/35. Background subtraction, noise reduction (rolling ball 25 × 25), and image thresholding were performed using the AttoVision v1.6.1 software supplied by BD Biosciences. Statistics on tube branch lengths per region of interest were obtained using the “Tube Formation” image analysis module of AttoVision v1.6.1.
dsTomato/GFP-Expression

Expression of proteins in cells was performed by transfection of a 293 T packaging cell line with a retroviral expression vector containing the DNA of interest (14). Virus was harvested 24–48 h after transfection in medium suited for the target cells. Infection was performed by filtering the virus containing medium, addition of protamine sulfate, and transferring of the virus containing medium to the target cells that were incubated further for 24 h. Infection was ended by changing the medium. Protocol will be given out by request. GFP was expressed from pCGFP (15). tdTomato was expressed from pCtdTomato, a derivative of pCGFP with the GFP replaced by tdTomato (16).

Hoechst and Propidium Iodide Staining

Co-cultures were stained with 1 µg/ml Propidium Iodide (dead cells) and/or 10 µg/ml Hoechst 33,342 (nuclei) in EGM-2 for 30 min at 37°C, 5% CO2, and imaged as described. To avoid difficulties with separating propidium iodide fluorescence from tdTomato fluorescence, these experiments were conducted with GFP-expressing HUVEC cells.

IC50 Calculations

IC50 values were calculated from a nine-point dose-response curve drawn from two replicates using the log[inhibitor] versus response function in Prism Graphpad 5.

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Figure 1. Parameter evaluation. (a) Twelve EC-vSMC co-cultures were seeded in a 96-well plate and networks were allowed to develop without any interference. Images were acquired for every third day as 5×5 montages with a ×10 objective and quantified with the parameter tube total length (BD Attovision software). Once the network is formed at day 3, this parameter reflects the stability of the network (day 3—12), and inhibition of network formation due to drug treatment can be measured. (b) Images from a developing network acquired every third day during a 12-day period showing fluorescent EC (gray). Individual seeded EC (day 0), the EC gradually interact to generate a capillary-like network (day 3). The generated network is remodeled and matures over time as the branches become more even and defined (day 9). The final network is stable and can be kept alive over long periods of time (Day 12). vSMC are not visible in the images but generate a confluent layer beneath the fluorescent EC.
Statistics

Nine DMSO-treated co-cultures were included on each of the replicate plates. Threshold values corresponding to three standard deviations were calculated based on the mean tube total length from these DMSO-controls. In the screen, wells producing tube total length values outside the threshold values were defined as a hit. In order for a compound to be included in follow-up dose-response experiments two out of three replicates had to be scored as a hit.

Standardization

Values from replicate plates with treated co-cultures were standardized for direct comparison in the same plot using the formula:

\[
\frac{(\text{Tube total length single well} - \text{Mean whole plate})}{\text{St Dev Whole plate}}
\]

Z-factor Calculation

A 96-well plate containing 84 DMSO-treated (0.25 %) and 12 CHIR258 (30 nM) co-cultures was incubated for 3 days and imaged as 2 × 2 montages. Average total tube lengths and standard deviations were quantified for the two treatment groups, and noise/signal ratio calculations were performed using the formula:

\[
1 - \frac{3 \times (\text{SD}_\text{NegCtrls} + \text{SD}_\text{PosCtrls})}{\text{Avg}_\text{NegCtrls} - \text{Avg}_\text{PosCtrls}}
\]

RESULTS

Measuring Cellular Networks

The co-culture assay entails live image analysis of fluorescent protein-expressing primary human EC that form tubular networks within 72 h postseeding (6). Unless otherwise specified, the EC used in this study were transduced with a tdTomato retroviral expression construct to ensure stable expression of the fluorescent marker.

The EC networks form over a period of 3 days. At day 0 and day 1, the EC are elongated and display extensive migratory activity (data not shown). As they begin to integrate into a proper network (Day 3, Fig. 1b), the EC interdigitate to generate tubes with uniform diameter (6). The EC do not proliferate when co-cultured with vSMC, so the networks do not grow in size. However, the connectivity increases until the network stabilizes, and once formed, the network can be kept alive for long periods (Figs. 1a and 1b). Although early networks are susceptible to anti-VEGF therapeutics, stable networks become resistant to anti-VEGF therapeutics (Evensen et al., submitted).

Ideally, the parameters used to quantify EC networks should reflect the stability of a generated network and also be able to identify any inhibited phenotype. To investigate the stability of the parameters available in the BD Attovision v1.6.1 software, 12 untreated co-cultures were seeded in a 96-well plate and monitored over a period of 12 days. The most stable imaging parameter was found to be tube total length (Fig. 1a), which estimates the total length of cellular tubes within the field of view. Developed networks, with their extensive interconnections, have longer total tube lengths than inhibited phenotypes (Figs. 2c, 2f, and 2g).

The tube total length parameter is calculated on the basis of a segmentation mask-defined preanalysis that divides the image into regions that are part of the cellular network and regions that are considered to be background (Figs. 2b and 2e). The segmentation mask can be used to define both the tube bodies (Figs 2b and 2e) and to generate a one-pixel-wide skeleton representation of the network structure (Figs. 2c and 2f). The tube total length parameter corresponds to the total number of pixels in this skeletonised image. To optimize images during analysis, noise was reduced by background subtraction and a 25×25 pixel sized rolling ball filter. A further size-based filter was included to exclude any objects under 8000 pixels in size from the analysis.

The Z-factor for tube total length was calculated from 84 individual DMSO treated co-cultures (negative control) and 12 co-cultures treated with the VEGFR2 tyrosine kinase inhibitor CHIR258 (positive control) in a 96-well plate and imaged as 2 × 2 montages with a 10× objective at day 3 (Fig. 2). CHIR258 generates rounded-up, single viable EC morphology with a disrupted plasma membrane) could be used to identify co-cultures with extensive cytotoxicity and exclude them from follow-up dose-response experiments. Both positive control by bortezomib (Velcade) at 200nM and anti-VEGF treatment with PTK787/ZK (100 nM) result in inhibition of network formation. However, the Velcade causes...
significant PI uptake indicating that it has cytotoxic effects. In contrast, the inhibitory effect of PTK787/ZK is not due to cytotoxicity. Because PI-staining overlaps with tdTomato (RFP) emission, these experiments were performed in combination with GFP-expressing EC.

**Proof of Principle: High-throughput Screening**

To evaluate consistency in screening hit identification, a double-blinded pilot screen was conducted using a prearrayed compound plate comprising randomly distributed small molecule VEGFR2 tyrosine kinase inhibitors, PTK787/ZK (200 nM and 50 nM), and CHIR258 (200 nM and 50 nM), and two vascular disruptive agents, combretastatin CA-4 (100 nM and 20 nM) (17–19), an inhibitor of microtubule polymerization, and CK246 (100 nM and 20 nM), an epothilone analog functioning as a microtubule stabilizer (12), among DMSO control wells. Two 96-well plates seeded with EC-vSMC co-cultures were treated with the compound plate that also contained nine DMSO controls for calculation of a threshold value (Fig. 4a, red lines). After a 72 h incubation, the two screening plates were analyzed to identify active compounds. Plate 1 identified 26 wells and plate 2 identified 28 wells with tube total lengths below the threshold value. Unblinding the compound plate layout showed that 29 wells contained an active compound corresponding to a correct hit identification of over 90%; one false positive well was identified (0.05%) (Fig. 4b). Standardization of the data for direct comparison of the two plates show that the negative value distributons overlap, indicating that the co-culture assay consistently identifies inhibited phenotypes (Fig. 4c). Images from treated co-cultures show severely inhibited phenotypes that is clearly distinguishable from developing networks (Fig. 4d).

**High-throughput Screening of a Small Molecule Compound Library**

A blinded image-based co-culture HTS screen was performed using a subcollection of commercially available small molecules.
molecule compounds previously used for chemical genetic studies (20). Compound screening plates comprised prear-rayed DMSO-dissolved agents that were added directly to seeded co-cultures. Both the identity and location of the active agents versus multiple DMSO vehicles were blinded. Three identical co-culture HTS assays were conducted such that each compound screening plate well was assayed in triplicate. After incubation for 72 h to allow EC network formation, wells were imaged automatically as 2×2 montages with a 10× objective and tube total length was quantified for each image (Fig. 5a). At least two of three replicates had to show effects on tube total length greater than three standard deviations from the mean of DMSO control wells to qualify as a primary screening hit. Figure 5b shows the overlaid standardized screening data from all three plates to illustrate the consistency of the assay screening hits. Overall, the replicates showed very good consistency with the identical 27 wells being indentified as screening hits in each assay plate.

**Dose-response Experiments with Screening Hits**

The primary pilot screening campaign was performed double-blinded and revealing compound identity showed the most potent agents comprised three major classes. The three compound classes included the microtubule-interfering agents vinblastine and paclitaxol, the histone deacetylase- (HDAC) inhibitor trichostatin A (TSA), and finally the Ca^{2+}-homeostasis interfering agent THAPS. Two other agents associated with Ca^{2+}-signaling, BAPTA and W13, were included to further investigate the importance of this process as they have opposite effects to THAPS. Thus, in total six compounds were further investigated in dose-response experiments to confirm their activity.
Microtubule-inhibitor compounds: Vinblastine and paclitaxel. Vinblastine, a member of the vinca alkaloids compound class, is an antimitotic drug with high affinity for tubulin (21). Upon binding tubulin, vinblastine inhibits microtubule formation, thereby arresting cells in the M phase of the cell cycle and inhibiting cell division. Vinblastine blocks formation of the endothelial tubes at picomolar concentrations and is a potent anti-angiogenic agent in the in vivo chick embryo chorioallantoic membrane (CAM) assay (22).

Figure 4. Proof-of-concept: High-throughput screening. Four compounds, two anti-VEGF therapeutics and two vascular disruptive agents were distributed (double-blind) on a 96-well compound plate and two replicate plates with freshly seeded co-cultures were treated with nontoxic concentrations. (a) Nine co-cultures known to be treated with 0.25% DMSO were the basis for calculation of the threshold value of three standard deviations away from the DMSO-mean (red line). (a, b) Plate 1 and plate 2 identified 26 and 28 wells producing tube total lengths below the threshold value, respectively. On the two replicate plates only one false positive was produced. (c) Standardization of the screening data for direct comparison show overlapping results in the negative area emphasizing the consistency in hit identification. (d) The inhibited phenotypes produced by the individual compounds are easily distinguished from the developing DMSO-treated networks and score as significantly shorter networks when measured by the parameter tube total length. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
bundles (23). Paclitaxel exhibits similar inhibitory effects (IC50 = 1.3 nM) on network formation as vinblastine (Fig. 6b).

**HDAC inhibitor TSA.** The HDAC-inhibitor TSA blocks class I and II HDACs leading to alterations in chromatin structure that modulate gene expression (24). TSA has been shown to inhibit hypoxia- and VEGF induced angiogenesis (25–27). In vivo, it is a potent inhibitor of angiogenesis as it reduces neo-vessel formation in the CAM-assay and inhibits bFGF-induced angiogenesis in the Matrigel-plug assay in mice. In the primary screen performed with the EC-vSMC co-culture, TSA supported the inhibiting effects reported, although at very high concentrations (20 μM). Dose-response experiments showed that the IC50 value was 1.5 μM and maximum blocking effect was first observed at 5 μM (Fig. 6c).

Ca²⁺-homeostasis interfering agents. Ca²⁺-homeostasis interfering agents were identified in the primary screen. THAPS is an inhibitor of the Ca²⁺-dependent ATPase pump that concentrates calcium in the sarcoplasmic and endoplasmic reticulum. By blocking this ATPase pump, THAPS treatment raises cytosolic Ca²⁺ concentrations (28). In the EC-vSMC co-culture assay, THAPS in the concentration range tested showed an inhibitory effect (IC50 5.7 μM, Fig. 6e). In contrast, BAPTA, a Ca²⁺-chelator, only showed inhibitory effects above 10 μM suggesting that depleting Ca²⁺ in the medium does not have a substantial effect at subtoxic concentrations (Fig. 6f). Cytoplasmic Ca²⁺ is sequestered by Ca²⁺-binding proteins, including calmodulin, that alter conformation and interact with specific target proteins, such as Ca²⁺/calmodulin-dependent protein kinases (29). The calmodulin inhibitor W13 did not affect network formation indicating that calmodulin-dependent signaling is not required for endothelial network formation (Fig. 6d).

Together, these results confirm the inhibitory effects of vinblastine, paclitaxel, TSA, and THAPS found in the primary pilot high-throughput screening campaign. This demonstrates that the EC-vSMC in vitro angiogenesis co-culture assay is a reliable high-throughput screening platform to screen for novel anti-angiogenic agents.

**DISCUSSION**

**Image Analysis Parameters for a High-throughput Screen**

The live cell imaging-based high-throughput screening approach using the EC-vSMC co-culture assay robustly generates automated universal format (TIFF) images of endothelial cellular networks suitable for processing by any image analysis software. To obtain reliable, quantitative data, a primary imaging parameter must be used that accurately reflects the temporal acquisition of a stable endothelial network (8). We evaluated several imaging algorithms available in the Attovision v1.6.1 software package (Becton-Dickinson) that were designed to measure features of a branching network. The most reliable parameter was tube total length (field summary), which quantifies (in pixels) the total length of all tubes in the image field. This parameter is suitable as inhibited or disrupted networks comprise unconnected, rounded-up EC and thus, will score as “shorter” than fully developed networks (Fig. 6). Several other parameters were evaluated including tube average length, number of branch points, and tube max length. However, although the number of branch points is a highly suitable parameter to quantify networks, it did not reflect true endothelial network branch points. Figure 2 illustrates two stages in the image analysis: initial thresholding produces a segmented image where the fluorescent protein-expressing EC are distinguished from the background. The segmented image is then trimmed to a single pixel wide skeleton that represents all of the branches of the
As shown in 2c, the algorithm used in the Attovision software package produces spurious circular structures within the skeletonized network that interfere with calculation of the correct number of branch points.

The Z-factor for the parameter tube total length is 0.68 and thus meets the standards for high-throughput screening (30). The value of the Z-factor is dependent on the number of EC seeded in each well. For calculation of the Z-factor, 15,000

Figure 6. Dose-response experiments with identified hits. Six compounds were followed up in dose-response experiments based on the hits in the screen. (a) Vinblastine and paclitaxol are well known vascular disruptive agents and display very potent dose-dependent inhibitory effects in the nanomolar range verifying their anti-angiogenic effect in this assay. (b) The HDAC-inhibitor TSA was tolerated up to 1 µM but higher doses showed a sharp inhibitory effect. (c) The initial screen identified three Ca²⁺-homoeostasis regulating compounds: THAPS, a compound that leads to raised cytoplasmic Ca²⁺, showed strong inhibitory effects at all doses tested whereas BAPTA, a Ca²⁺-chelating agent had little effect except at very high doses. W13, a calmodulin inhibitor, did not exert any effect on network formation.
Dose-response Experiments

Previous findings demonstrate that EC in co-culture with vSMC do not proliferate (8,31). It is therefore somewhat unexpected that vinblastine and paclitaxel have such potent inhibitory effects as they are primarily used as antimitotic drugs in cancer treatment. However, their modes of action might explain their strong inhibitory effect: vinblastine inhibits microtubule assembly by binding to tubulin subunits, whereas paclitaxel hyperstabilizes the structure of microtubules and prevents them from disintegration (23,32). As a result both drugs, in addition to being anti-proliferative, affect cell motility and morphological changes that are dependent on microtubule dynamics (33). Hence, the inhibitory effect on network formation is likely due to impaired elongation and migration characteristic of pathfinding EC during angiogenesis. Our findings confirm the reported anti-angiogenic effects of vinblastine and paclitaxel observed in other in vitro and in vivo angiogenesis assays (22,34).

TSA is a selective inhibitor of histone deacetylase classes I and II but not class III. TSA alters gene expression by inhibiting the removal of acetyl groups from histones, hence changing the ability of DNA transcription factors to interact with DNA and altering DNA transcription (24). Gene transcription profiles have been shown to change in both EC and vSMC when co-cultured (35). Thus, identification of this compound in the primary screen likely reflects alteration of the EC gene expression changes requisite for network formation, albeit only at relatively high concentrations. In addition, it has been shown that TSA reduces hypoxia-induced migration and adherence of bovine aortic endothelial cells to diverse extracellular matrix proteins, among them collagen Type IV and fibronectin (26). Therefore, TSA might also modulate the interaction between EC and basement membrane proteins (e.g. collagen IV, XVIII, fibronectin), (8) deposited during network formation and inhibit haptotactic endothelial cell migration (Fig. 6c).

\[ \text{Ca}^{2+} \] -homeostasis is important for numerous EC functions including proliferation, invasion, and differentiation (36), and perturbations of the fine-tuning of \[ \text{Ca}^{2+} \]-levels results in altered cell signaling. For example, THAPS potently inhibited angiogenesis in the rat aorta assay in a dose-dependent manner via inhibition of proliferation and migration of EC (28). THAPS identified in the primary screen, as shown in the dose-response experiments, exhibits effects at the lowest dose tested (1.5 \( \mu \)M) and ideally, it should be tested in much lower concentrations. In fact, it has been shown that doses lower than 10 nM were able to potently inhibit migration and proliferation of vSMC in vitro monocultures (37,38), suggesting that the observed effects on EC networks could be attributed to effects on vSMC functions. Nevertheless, the result shows that increasing calcium in the cytosol efficiently inhibits network formation. The opposite effect, \[ \text{Ca}^{2+} \]-depletion, was also tested by addition of BAPTA that chelates exogenous \[ \text{Ca}^{2+} \]. However, an IC50 value at 45 \( \mu \)M indicates that \[ \text{Ca}^{2+} \] in the medium is not important for network formation. The calmodulin inhibitor W13 also did not have any effect on network formation. Together, these results suggest that intra-cellular \[ \text{Ca}^{2+} \]-levels must be kept below a threshold level to maintain the ability of network formation.

In conclusion, we show here that the organotypic endothelial-mural co-culture capillary-like network approach represents a robust new platform for live cell high-content imaging high-throughput screening for novel anti-angiogenic agents. Via a pilot HTS small molecule compound screening campaign, we demonstrate that informative imaging parameters facilitate the identification of known and novel angiogenic modulators. The screening system interrogates endothelial network formation in a native context comprising the three main components of blood vessels: EC, mural cells, and the basement membrane deposited as a result of the heterotypic cell-cell interaction. The screening system affords the opportunity to conduct HTS campaign on networks modeling both immature and mature vasculature.

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LITERATURE CITED


