ANGIOGENIC EFFECT OF CHEMICALS

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SABELL EMMI: ANGIOGENIC EFFECT OF CHEMICALS

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Verisuonitus on välttämätöntä kudosten hyvinvoinnille, koska veren kuljettamien ravinteiden ja hapen täytyy tavoittaa kehon jokainen solu. Uusien verisuonten muodostusta, angiogeneesia, tarvitaan sikiön kehityksen ja kasvun aikana, samoin haavan paranemiseen ja muihin fysiologisiin toimintoihin. Tapahtuessaan ilman tervettä fysiologista tarkoituksenmukaisuutta, se voi johtaa patologisiin prosesseihin, kuten tukemaan kasvaimen kasvua.

Lääkkeiden kehityksessä angiogeneesilla ja sitä inhiboivilla lääkkeillä on suuri rooli. Uusia lääkkeitä tutkitaan ja kehitetään jatkuvasti erityisesti syöpäkasvaimia vastaan. Lisäksi saadaan jatkuvasti uutta tietoa ympäristökemikaaleista ja niiden negatiivisista vaikutuksista angiogeneesiin. Eläinkokeita käytetään yhä laajalti, vaikka niiden tulokset eivät välttämättä vastaa vaikutuksia ihmisellä. Tämän vuoksi tarve validoiduille ihmissoluilla tehdyille *in vitro* -malleille on kasvanut ja kiinnostus niiden kehittämiseen lisääntyy jatkuvasti. Vaikka *in vitro* -verisuonimallit eivät välttämättä kata joka vaihetta angiogeneesissa, ne kuitenkin mallintavat hyvin tärkeimmät osa-alueet, ovat helposti toistettavissa ja tulokset vastaavat paremmin vaikutuksia ihmisellä.

Uusien verisuonten muodostamiseen tarvitaan endoteelisoluja, mutta putkimaisten rakenteiden muodostukseen tarvitaan myös ulkopuolista ärsykettä. Ihmisen napanuoran endoteelisolut (human umbilical vein endothelial cells, HUVECs) voivat muodostaa verisuonirakenteita, kun niitä viljellään maljalla oikeissa olosuhteissa. HUVEC-viljelmistä tehtyjen verisuonimallien avulla on mahdollista tutkia kemikaalien vaikutuksia angiogeneesiin.

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1 INTRODUCTION

1.1 Blood vessel formation

Vasculature is essential to tissue welfare because it is needed to bring oxygen and other necessary molecules to tissues and organs. Blood vessel formation is needed during development, growth and wound healing. (Carmeliet et al., 2011) New blood vessels are derived from two sources: they can extend from pre-extending vessels (angiogenesis) or they can be derived from progenitor cells (vasculogesis). (Auerbach, 2008)

1.1.1 Angiogenesis

The primary cells needed for new blood vessel formation from pre-existing vessels are endothelial cells. These cells arise from pre-existing vessels and are released by breaking down or passing through extracellular matrix. Then they migrate, proliferate and reorganize. (Auerbach, 2008) Vascular endothelial growth factor (VEGF) is the most important angiogenic factor and angiogenesis is initiated by binding of VEGF to tyrosine kinase receptors present in endothelial cells surfaces. Binding initiates number of intracellular signal pathways, which leads to new blood vessel formation. (Friis et al., 2013) Trough the whole process there are interactions between endothelial cells and surrounding tissues, such as pericytes and smooth muscle cells. Angiogenesis is highly regulated by systemic factors such as hormones and cytokines in addition to hypoxia and shear stress. (Auerbach, 2008) Angiogenesis can also be pathological when it takes place in inappropriate place or if it supports tumor formation and growth. (Carmeliet et al., 2011)

1.1.2 Types of angiogenesis

Sprouting angiogenesis means that endothelial cells basically grow towards angiogenic stimulus such as vascular endothelial growth factor (VEGF). Sprouting can add new blood vessels to tissues which did not have them earlier. The basic steps include endothelial cell proliferation, directed migration, tubulogenesis, vessel fusion, vessel pruning and pericyte stabilization. (Adair and Montani, 2010)

In intussusceptive angiogenesis vessel wall extends into the lumen and causes vessel to split in two. This process is faster than sprouting because it does not require endothelial cell proliferation or migration. Both ways of angiogenesis happens in growing fetus and in adults but intussusceptive angiogenesis happens when fast capillary formation is needed. Intussusceptive angiogenesis mainly forms capillaries where capillaries already exist. (Adair and Montani, 2010)

In cancer tumors tumor cells can hijack the existing vasculature or they can simply by themselves line blood vessels which is called vascular mimicry. Cancer stem-like cells can even generate tumor endothelium. (Adair and Montani, 2010) Angiogenesis is key process to tumor growth. Initially the growth of tumor is fed by nearby blood vessels but when tumor comes too big, lining blood vessels can not supply nutrients to whole tumor and angiogenesis occurs. That is why angiogenesis inhibition is desirable target when thinking new anti-cancer drugs. (Cook and Figg, 2010)

1.1.3 Vasculogenesis

De novo formation of blood vessels means that blood vessel tubule network is formed from stem cells. That happens mainly in extraembryonic and intraembryonic tissues of embryos. At the end of vasculogenesis, endothelial cells are lining the luminal surface of vessels and they are formed from angioblasts, which are derived from mesodermal stem cells. Vasculogenesis is dynamic process and it involves cell-cell and cell-extracellular matrix (ECM) interactions which include different growth factors. (Adair and Montani, 2010) Veins and arteries have both they own signal pathways (Carmeliet et al., 2011).

1.2 hASCs

Human adipose stromal cells (hASCs), isolated from adipose tissue as stromal-vascular fraction are pluripotent cells. In adipose tissue ASCs are localized in peri-endothelial layer of blood vessels and they are functionally and phenotypically equivalent to pericytes of microvessels. They secrete molecules which promotes endothelial cell survival and proliferation including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and granulocyte macrophage colonystimulating factor (GM-CSF). They also stabilize endothelial network in angiogenesis models. (Merfeld-Clauss et al., 2010)

1.3 Angiogenesis assays

Angiogenesis studies have become important area of interest because angiogenesis is involved in various physiological and pathological processes. (Donovan et al., 2001)

1.3.1 In vivo angiogenesis assays

In vivo angiogenesis assays give the most exact information of vascular events because they mimic angiogenesis as it occurs in normal environment. But there is still some disadvantages: they are usually expensive and more difficult to carry trough. They involve multiple cell types and potential metabolic processing of the studied agent, which complicates the analysis of the agent's mechanism of action. (Goodwin, 2007) *In vivo* tests are mainly animal tests, which do not necessarily predict effects in human but they still are used widely. (Sarkanen et al., 2011)

1.3.2 In vitro angiogenesis assays

In vitro angiogenesis assays are important tool of studying mechanisms of angiogenesis and also potential equipment to develop therapeutic strategies of vascularization. (Donovan et al., 2001) Because there are so many factors which regulate the angiogenesis (hormones, cytokines, hypoxia,

shear stress), *in vitro* assays never are entirely adequate. (Auerbach, 2008) Even though *in vitro* angiogenesis assay does not cover every step of angiogenesis process, it effectively mimics the key steps i.e. migration and differentiation of endothelial cells. (Sarkanen et al., 2011) It also allows identification of direct effects on studied cell function. There is no need for technical expertise in animal handling, which is required *in vivo* assays. (Goodwin, 2007) There is strong need for pre-validated human cell *in vitro* assays for reducing and replacing animal tests. (Sarkanen et al., 2011)

Before *in vitro* assays can be used to make regulatory decisions they must undergo a validation process which evaluates its reliability, relevance and fitness for purpose. Nowadays the validation means that the new test must provide equivalent or better way to do things than current procedures. Parameters which are evaluated are time, money and the process of making. (Judson et al., 2013)

Sarkanen et al. have studied angiogenesis assay, in which human foreskin fibroblast and human umbilical vein endothelial cell (HUVECs) co-cultivation was exposed to six reference chemicals. Chemicals were widely used pharmaceuticals that inhibit angiogenesis and their effect in human was known. The test results showed that a standardized *in vitro* angiogenesis assay mimics well the effects in man and it is reliable way to test angiogenesis modulators. (Sarkanen et al., 2011) Later Sarkanen et al. have also studied hASC+HUVEC co-culture angiogenesis assay in which cells are induced with natural growth factors and allowed to self-assemble into tubular network and vascular supporting structures. It provides completely human-based and simplified model of angiogenesis and it could be used for studying angiogenesis *in vitro* (Sarkanen et al., 2012).

1.4 Chemicals and blood vessels

1.4.1 Angiogenesis in cancer

Angiogenesis has become an attractive target for drug therapy because angiogenesis is a key process to tumor growth and many compounds are in pre-clinical development constantly. Physiologic angiogenesis is tightly regulated and organized by anti- and pro-angiogenic factors but in tumors new

blood vessels are irregular and leaky. The tumor endothelial cells divide more rapidly and express different markers. (Cook and Figg, 2010) Because endothelial cells line the vessels, they are better target to anti-cancer drugs than tumor cells themselves meaning that angiogenesis inhibitor therapy may prevent tumor growth instead of killing tumors. That is why angiogenesis inhibitors are most effective when combined with additional therapies. (Yoo and Kwon, 2013) When targeting tumor blood vessels with anti-cancer compounds, there are two strategies. First is anti-angiogenic therapy, where new blood vessel formation is inhibited. That has almost none effect on blood vessels that already exists. That is why effect on large tumors is very limited. The second strategy is vascular disrupting therapy (VDT), in which rapid and selective shutdown of already existing vasculature happens. It disrupts the tumor endothelium selectively, because it founds the difference between immature tumor vasculature and normal mature blood vessel. It has greater power in bigger tumors, but it has no effect on angiogenesis, which happens vigorously in tumor periphery. (Xuan et al., 2009)

One interesting finding is that many conventional chemotherapeutics actually possess previously unknown anti-angiogenesis activity. (Cook and Figg, 2010) Solid tumors often suffer from hypoxia, extracellular acidosis and nutrient deprivation which can lead to inflammatory response. That is why solid tumors often exhibit pro-inflammatory modules such as prostaglandins. Prostaglandins impact cancer cell behavior such as motility, invasion, vascularization and metastatic dissemination. Non-steroidal anti-inflammatory drugs (NSAIDs) can reduce inflammation by inhibiting COX activity and that is why NSAIDs are widely studied as anti-cancer agents. (Ackerstaff et al., 2007)

1.4.2 Other angiogenesis involving diseases

There are also other diseases than cancer where angiogenesis plays a central role. In a field of ophthalmology anti-angiogenic strategies are also used because VEGF have been implicated as a major factor in ocular angiogenesis including pathological processes such as proliferative diabetic retinopathy, neovascular AMD and neovascular glaucoma, which causes blindness. Pegaptanib was the first anti-VEGF agent specifically designed for intraocular use and after that several other intraocular anti-VEGFs have been approved. (SooHoo et al., 2014) In psoriasis, hypervascular skin

lesions overexpress angiogenic polypeptide IL8 and underexpress the angiogenic inhibitor thrombospondin 1. Other diseases where the therapeutic goal is inhibition of angiogenesis are hemangiomas, Kaposi's sarcoma, rheumatoid arthritis, endometriosis and atherosclerosis. (Yoo and Kwon, 2013)

Stimulation of angiogenesis can also be a therapeutic goal. Such situation can be a need for collateral vessel formation for example in myocardial, peripheral or cerebral ischemia. In ischemia collateral vessels can improve blood supply to the area. Unfortunately currently there are no FDA-approved angiogenic drugs to treat ischemic cardiovascular disease. Stimulation of angiogenesis can also be needed in wound healing and reconstructive surgery. (Yoo and Kwon, 2013)

1.5 Chemicals as vascular disrupting compounds during embryo development

There is a large scale of chemicals which are known to be toxic for developing embryo. Some of them are medical drugs, such as thalidomide, estrogens, endothelins. For some time now also cigarette smoke is known to be toxic. (Knudsen and Kleinstreuer, 2011) Recently there have come out more knowledge concerning environmental chemicals acting also as vascular disrupting chemicals. Environmental chemicals are large and homogenous group of chemicals including food additives, plastic fillers and wide range of non-medical dugs. (Kleinstreuer et al., 2011)

The first organ system which begins to function in embryo during development is cardiovascular system. Cardiovascular system develops in first trimester, in which developing organ systems are most vulnerable against chemicals which enter mother body. There are also other factors than the onset of exposure which effects the chemical's capacity to disrupt embryogenesis such as dose, genetic susceptibility and chemical interactions with biological system. (Knudsen and Kleinstreuer, 2011) The two ways of blood vessel formation are described above. If blood vessel formation is somehow disrupted it likely leads to prenatal loss, malformations, placenta complications and problems in neuron development. Thalidomide is one commonly known vascular disrupting chemical. It was earlier used widely among pregnant women against nausea. (Kleinstreuer et al., 2011) Now

when there is more knowledge about negative sides of environmental chemicals there are also more studies. Heinonen et al. have shown that Octyl gallate, Triclosan, 1-hydroxypyrine, Disulfiram and Diethanolamine inhibit vascular tubule formation even lower concentrations than they are cytotoxic. (Heinonen et al.)

Typically chemical testing during pregnancy involves *in vivo* tests for pregnant animals such as rats and rabbits. These test mostly take time but are also not effective when only one compound can be tested at the time. Also zebrafish are used widely in *in vivo* tests but the results do not predict results in human embryo. Zebrafish embryo is so small that it can receive oxygen by passive diffusion even without blood vessels. But they cover better the field when blood vessel development is studied. (Knudsen and Kleinstreuer, 2011)

Nowadays there is need for more effective toxicity screening and *in vitro* assays are answer to that need. There are also high-throughput screening assays in which thousands of chemicals can be tested rapidly against huge amount of molecular targets. (Knudsen and Kleinstreuer, 2011)

1.6 Chemicals in this study

Ten chemicals were chosen in this study. Almost all of them are commonly known chemicals and their effects on angiogenesis are studied before: reference data in animal and man is available. Levamisole is alkaline phosphatase inhibitor, which is used as anti-helminthic agent but has also been used for adjuvant chemotherapy of colorectal cancer. The anti-angiogenic mechanisms of levamisole is not yet known, but it induces an endothelial cluster morphology and that way may involve the VEGF signaling pathway. (Friis et al., 2013) Erlotinib is epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which has anti-cancer activity. It is used as treatment of lung cancer. (Cook and Figg, 2010) Suramin is a polysulfonated naphtylurea and is reported to inhibit endothelial cell proliferation and migration by binding to VEGF. Also neutralizing antibodies to VEGF (anti-VEGF) is known to be an effective angiogenesis inhibitor. (Friis et al., 2013) 2-Methoxyestradiol is a human metabolite of estradiol, which inhibits tubulin polymerization, destabilizes the microtubules and causes cell cycle

arrest. It has anti-angiogenic and proapoptotic propertias and it also inhibits cell proliferation and migration. (Cook and Figg, 2010) NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide) belongs to sulphonamide class and is a COX-2-selective inhibitor. It binds uniquely to the cyclo-oxygenase channel. (Vecchia and Malkowski, 2011) Acetylsalicylic acid (ASA, aspirin) in a non-selective non-steroid anti-inflammatory drug (NSAID), which is used for treating pain and fever and also as anti-thrombotic agent. Aspirin use is associated with decreased risk for several cancers e.g. colon cancer. (Pathi et al., 2012) Indomethacin belongs to group of drugs called indoleacetic acids and it is also a non-selective NSAID. (Ackrerstaff et al., 2007) Triclosan (2.2.4'-thrichloro-2'hydroxydiphenyl ether) in an antimicrobian combound used as a bacteriostat, fungisatat and deodorizer. It is used in several custom products for example sanitizing products like soaps, toothpaste and hairproducts. There is a lot of worries in overuse of triclosan both in health care and in environmental studies. (Stoker et al., 2010) Pyridaben is a pyridazinone derivate and it is an acaricide and insecticide for control of mites and some insects. (Hajime et al., 1994)

2 MATHERIALS AND METHODS

The study was carried out in the spirit of GLP and according to FICAM's SOPs when applicable.

2.1 Test systems/cells used

hASCs (several different Masterbanks, e.g. K73-hASC014 for constructing the angiogenesis model) were used. Cells have been isolated from stromal vascular fraction of adipose tissue (enzymatically with 0.1% collagenase I). Adipose tissue is received as a leftover material from surgeries. Utilization of human adipose stem cells used in this study has received an approved statement of Joint Municipal Authority of the Pirkanmaa Hospital District with ETL-code R003058. Cells were used in the angiogenesis model at passages 2-3.

HUVECs (several different Masterbanks, e.g. HUVEC012 for constructing the angiogenesis model) were used. HUVEC have been isolated from human umbilical cord veins (enzymatically with 0.05% collagenase), which are obtained from scheduled cesarean sections. Utilization of endothelial cells used in this study has received an approved statement of Joint Municipal Authority of the Pirkanmaa Hospital District with ETL-code R08028. Cells were used in the angiogenesis model passage 4.

2.2 Tested chemicals

Ten chemicals were tested in this study: Erlotinib (Roche), Acetylic salicylic acid (ASA) (Sigma), Levamisole (Sigma), 2-methylestradiol (Sigma), Anti-VEGF (Sigma), Suramin sodium salt (Sigma), Triclosan (Sigma), NS398 (Sigma), Indomethacin (Sigma), Pyridaben (Sigma).

2.3 Experimental procedure

2.3.1 Cell cultivation

Both hASCs and HUVECs were thawed and cultivated separately in 75 cm^2 culture bottles until they were confluent. Cells were counted using Bürger's chamber and living/dead cell ratio was determined by using Trypan Blue. Angiogenesis assay was built in the following manner. First hASCs were plated on the bottom of cultivation well. After they had attached properly HUVECs were seeded on top of them. The co-culture was allowed to grow for one day before any experiments.

2.3.2 Exposing co-cultivation to chemicals

Co-cultures were exposed to ten different chemicals and every concentration of each chemical was tested in at least two wells of co-culture. Chemicals were dissolved according to manufacturer's instruction and then diluted in CSFM based stimulation medium. If chemical did not dissolve to medium it was first diluted to DMSO or ethanol. Dilution agents for each chemical are seen on table 1. Concentration of DMSO never exceeded 0.3%. Each well was exposed two times to same

concentration, both cultivation periods were three days. Cultivation medium/chemical was removed before new exposure. Every plate had also positive and negative control and also 0.3% diluent control if chemical did not dissolve to CSFM based stimulation medium.

Table 1 | Dilution agents for each chemical. If chemical did not dissolve straight to medium it was first diluted to DMSO or ethanol. Concentration of DMSO never exceeded 0.3%.

Chemical name	**Erlotinib**	**Suramin**	**NS-398**	**ASA**	**2-Methoxy-estradiol**
Dilutive agent	DMSO	Stimulation medium	DMSO	Stimulation medium	DMSO
Chemical name	**Pyridaben**	**Indomethacin**	**Anti-VEGF**	**Levamisole**	**Triclosan**
Dilutive agent	DMSO	DMSO	Stimulation medium	Stimulation medium	Ethanol

2.3.3 Immunocytochemical staining

Immunocytychemical staining was performed on sixth day after first exposure. Cells were fixed with ice-cold 70% ethanol and after that treated with 0.5 % Triton X-100 and blocked for unspecific staining with 10% BSA. Then cells were incubated with 120 µl of primary antibody, anti-von Willebrandt (produced in rabbit) and anti- Collagen IV (produced in mouse diluted in 1% BSA in PBS, at 4°C overnight or 1 hour at room temperature. Cells were then incubated with secondary antibody (Anti-Rabbit IgG TRITC or anti-mouse IgG FITC, diluted with appropriate dilution ratio) for 30-45 min at room temperature in dark.

2.3.4 Toxicity test

The purpose of the test was to find out highest non-toxic concentration for each chemical. Different concentrations of each chemical were tested and WST-1 reagent was used to determine which

concentration gives the viability of 80 % or higher. WST-1 was performed according to manufacturer's instructions and the chemical exposure time was 6 days. Absorbances were measured at 440 nm with plate reader after 1,5h of incubation in 37°C. WST-1 was performed on the same plate as immunostaining but before it.

2.3.5 Result analysis

After staining formed vessel tubes were graded in scale 0-10 and photographed with Cell-IQ with 10x enlargement. Examples of grades are seen in figure 1. Good positive and negative control are seen in figure 2. Results were analyzed and figures done from semi-quantitative and quantitative results. Resulting absorbances from toxicity test were measured with plate reader (VarioSkan, Thermo Scientific) and the values were processed with Microsoft Office Excel. Data handling was done with GraphPad Prism and photographs were processed with Adobe Photoshop CS6.

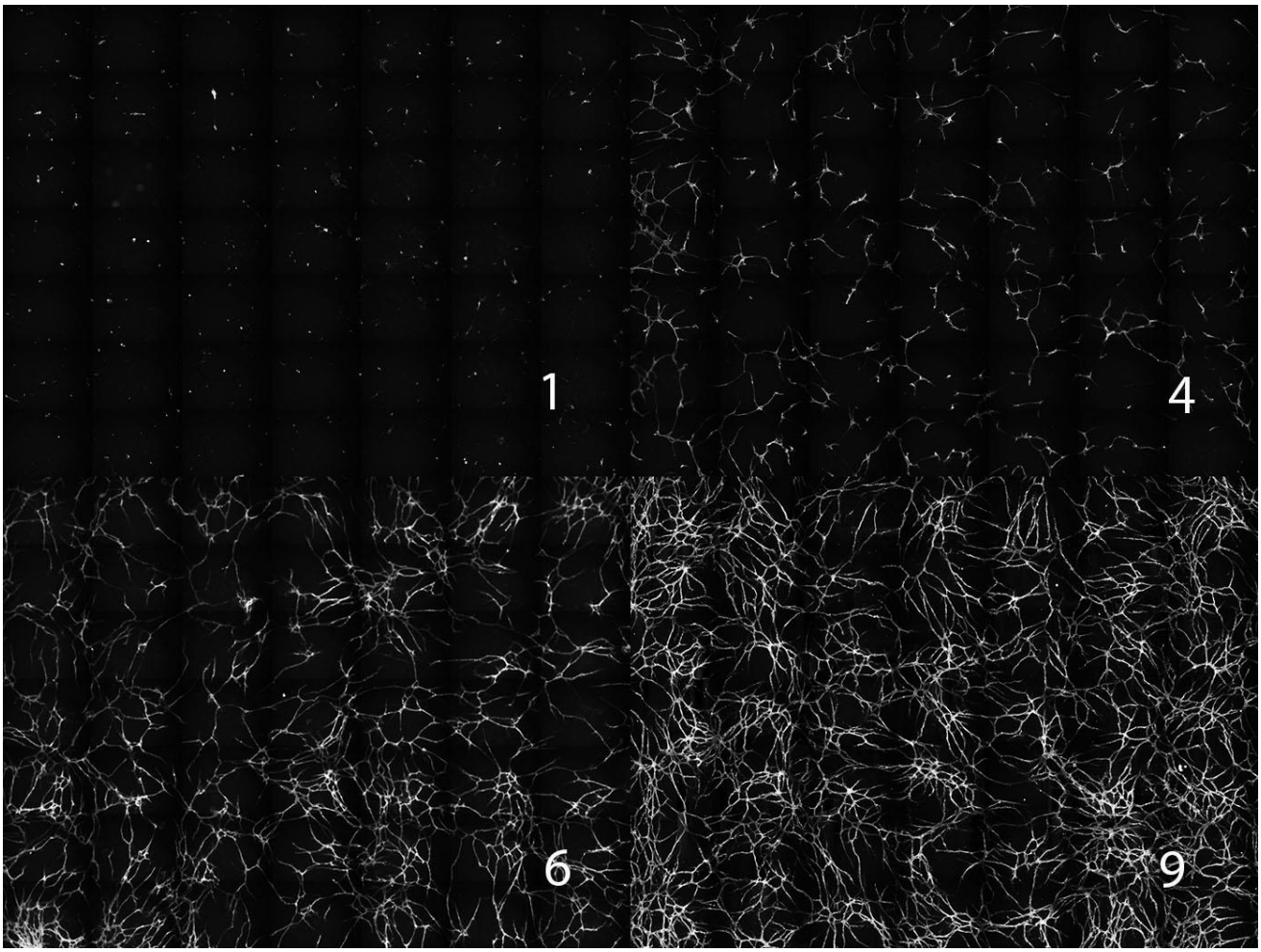


Figure 1 | Blood vessel tubes were graded in scale 0-10. 0 = no tubule formation and 10 = good tubule formation, tubules connect each other and tubule network is constant.

3 RESULTS

Each chemical was tested as separate experiment and the data was collected each step of the process. Cell cultures were exposure to different concentrations of chemicals and cell toxicity test was done afterwards. EC_{80} value represents the concentration that gives the cell viability of 80 % or higher. Same concentrations were also used for determine each chemical's potential to inhibit angiogenesis. IC_{50} value represents the concentration in which half of reactions are inhibited. All calculated data is collected in table 2.

Table 2 | IC_{50} and EC_{80} values for each chemical. * = Value can not be calculated. ** = reference value not found.

Chemical	**Erlotinib**	**Suramin**	**NS-398**	**ASA**	**2-Methoxy-estradiol**
IC_{50}	6.316 µM	135.0 µM	323.6 µM	3.324 mM	720.6 µM
IC_{50} in literature	Moderate 10 µM (Sarkanen et al., 2012)	100 µM (Friis et al., 2013)	**	500µM (Borthwick et al.,2006) 250 µM (Friis et al., 2013)	Moderate 50 µM (Sarkanen et al., 2012)
EC_{80}	~0.7755 µM	*	*	*	70.64 µM
Chemical	**Pyridaben**	**Indomethacin**	**Anti-VEGF**	**Levamisole**	**Triclosan**
IC_{50}	0.02395 µM	158.4 µM	6.382 µM	494.3 µM	31.66 µM
IC_{50} in literature	**	2.5 mM (Friis et al., 2013)	Strong 5 µM (Sarkanenet al., 2012)	Moderate 750-1000 µM (Sarkanen et al., 2012)	**
EC_{80}	*	*	0.2047 µM	~1272 µM	18.04 µM

EC_{80} value was not possible to calculate for every chemical meaning that those chemicals did not effect on cell viability with tested concentrations. In future further tests can be carried out with higher concentration. Even though they are not cell toxic they still have effect on angiogenesis. Only three quite reliable values were able to be calculated and those are with 2-methoxyestradiol, Anti-VEGF and Triclosan. For Levamisole and Erlotonib the calculated values are just estimates.

IC_{50} value was possible to calculate for every chemical but there were situation where an extra concentration (10x compared to highest tested chemical concentration) had to be added for to have a good negative slope. In statistics, the same tubule value as in the highest tested concentration was added to the extra concentration. In those cases the results are not as reliable as other values and even higher concentrations should be tested in future. This was the case with Suramin, NS-398 and Indomethacin. Results for each chemical will be described below.

3.1 Erlotinib

EC_{80} value is only estimate and as seen in figure 2, the absorbance slope is not linear. Tubule formation gives good slope although positive control in this plate got only grade 5.

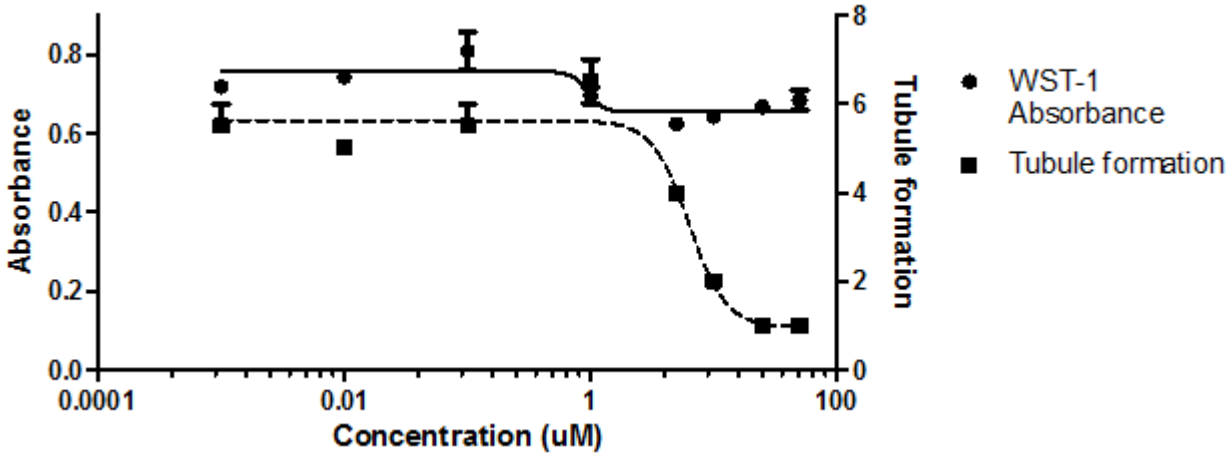


Figure 2 | Erlotinib tubule formation and WST-1 absorbance.

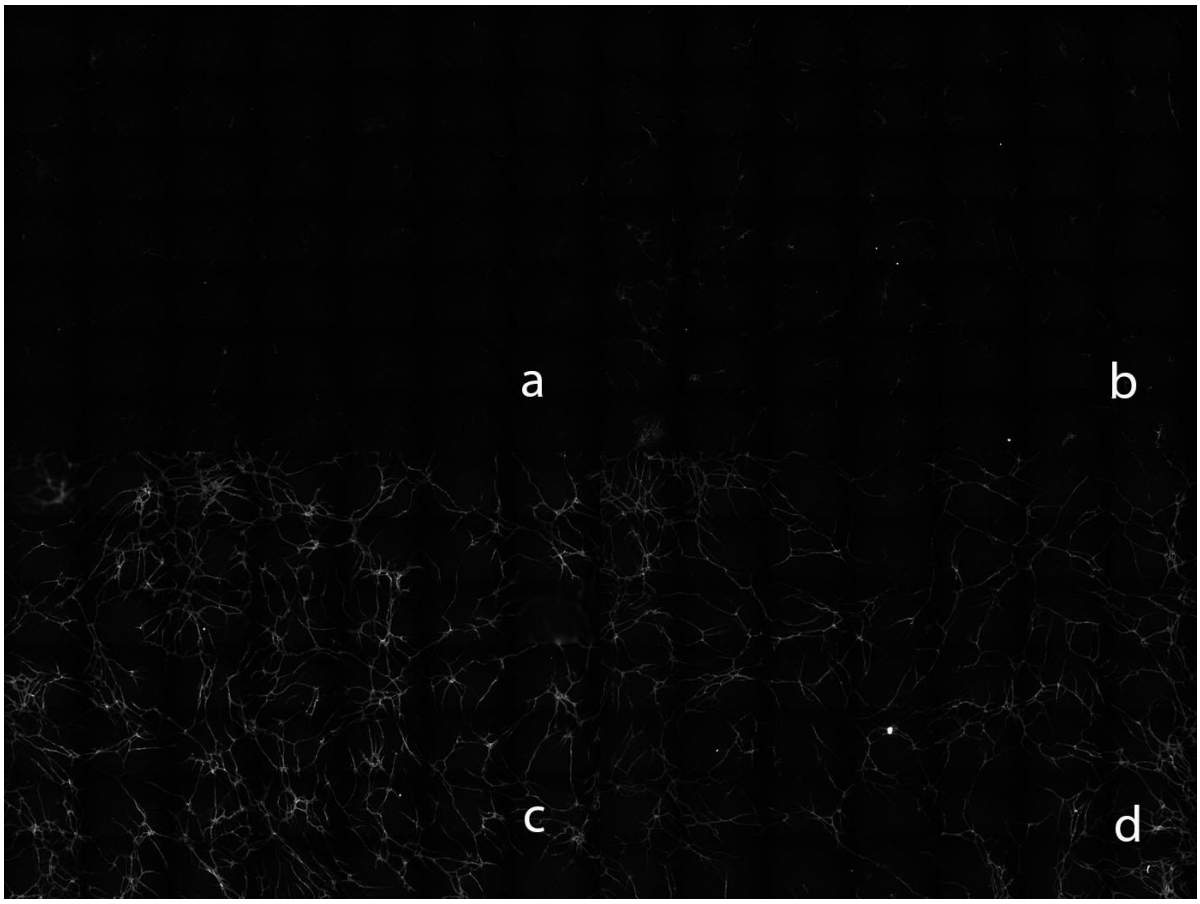


Figure 3 | Erlotinib tubule formation. a = highest tested concentration 50 µM, b = 10 µM, c = 1 µM, d = smallest tested concentration 0.01 µM. In c tubule formation is better than on d.

3.2 Suramin

There was some challenges when evaluating tubule formation in experiment with Suramin. Cells should not grow in lumps and the tubule network should be constant. In some wells cells have made big lumps in the middle of well but on the sides there might have been good tubule formation. That made the evaluation challenging because that lowered the grade even when tubule formation was very good in sides of the well. Still the results are reliable when calculating the effects on angiogenesis, IC_{50} value is 135.0. Any results from toxicity test can't be calculated because the absorbance actually rose with highest concentrations.

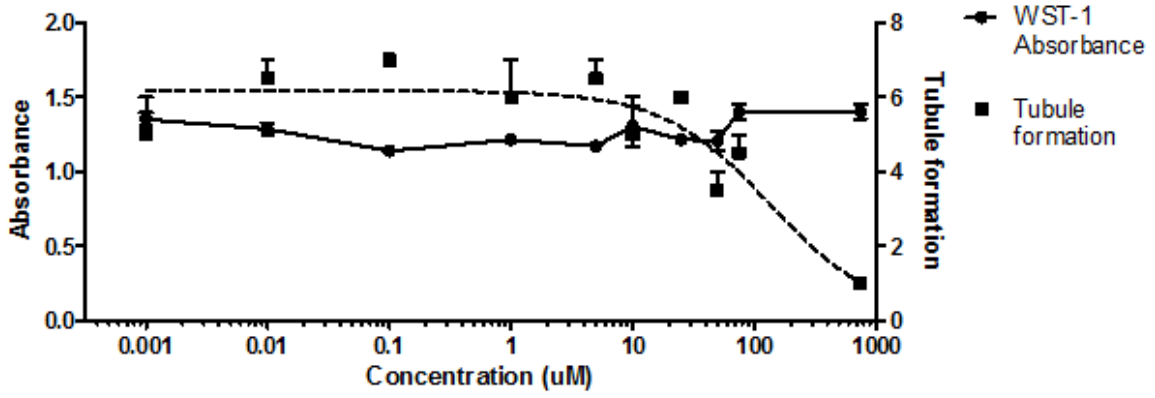


Figure 4 | Suramin tubule formation and WST-1 absorbance.

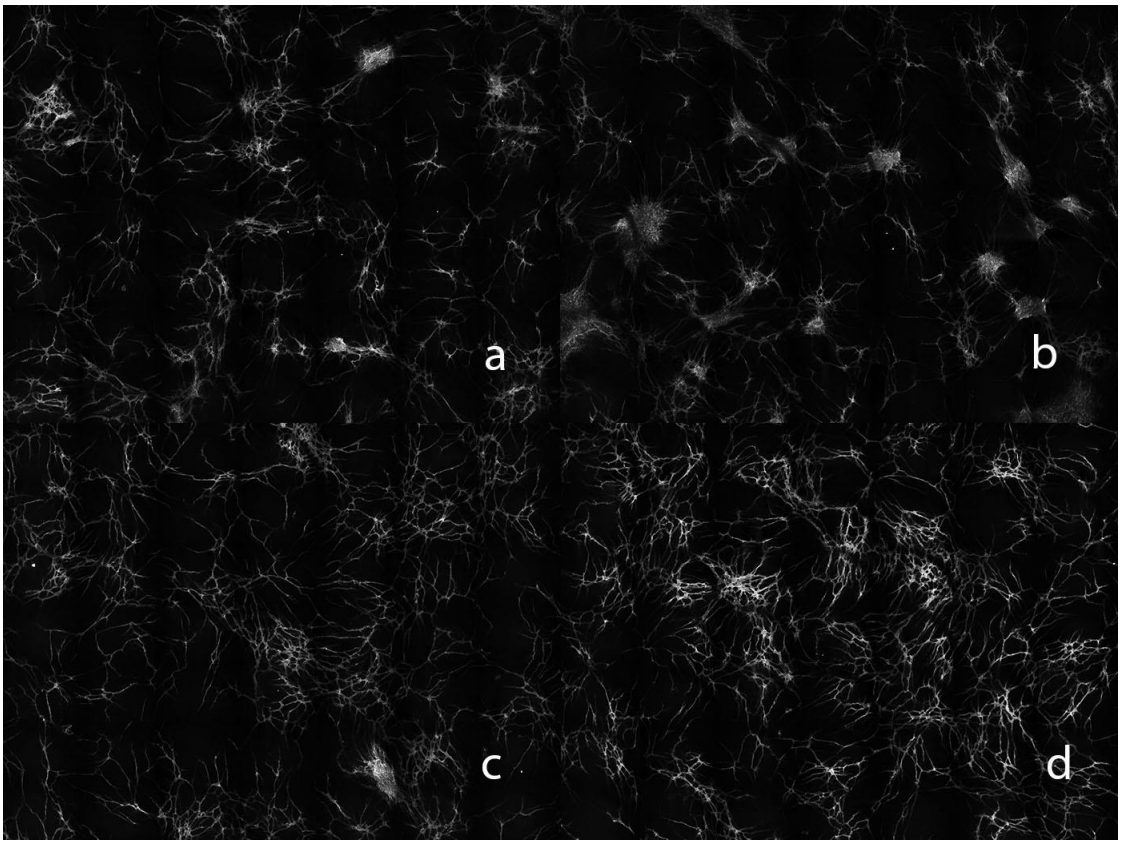


Figure 5 | Suramin tubule formation. a = highest tested concentration 75 µM, b = 50 µM, c = 5 µM, d = smallest tested concentration 0.01 µM. B demonstrates the cell lumps which were seen in many wells.

3.3 NS-398

NS-398 gives very disturbing slope from toxicity test and it is not reliable. It would be necessary to repeat the experiment for to get reliable results about toxicity. Results from angiogenesis test are more adequate giving IC_{50} value 323.6 and the slope is demonstrative but the results are not unambiguous as seen in figure 6. Even though the slope goes naturally downwards.

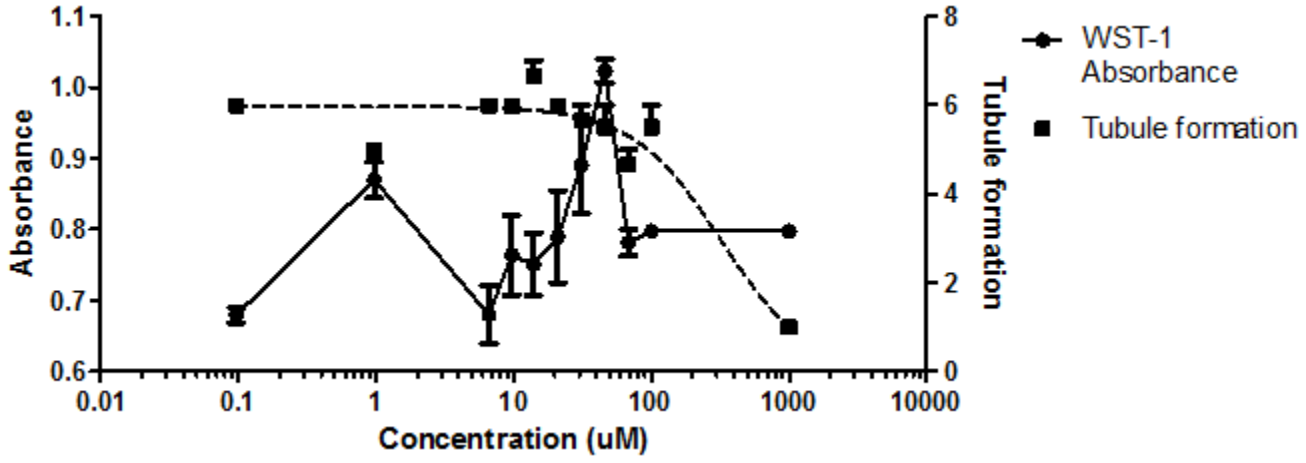


Figure 6 | NS-398 tubule formation and WST-1 absorbance.

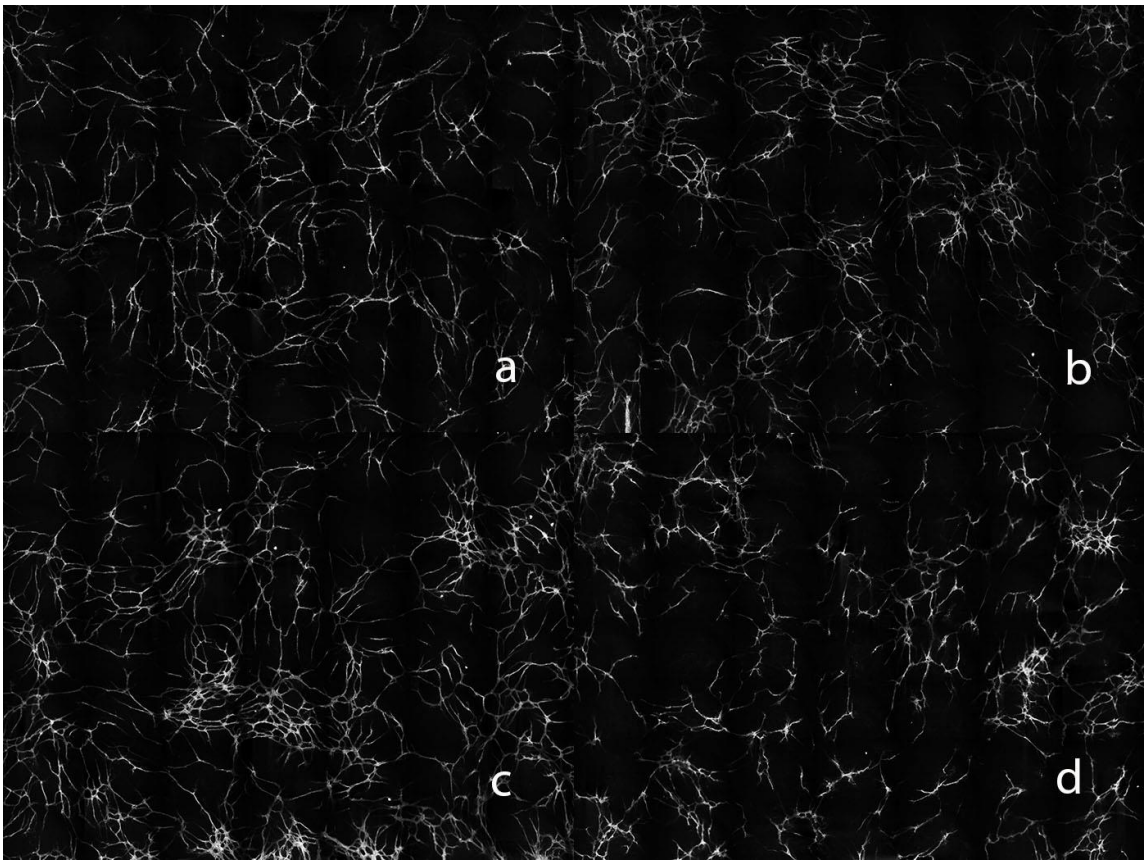


Figure 7 | NS-398 tubule formation. a = highest tested concentration 100 µM, b = 46 µM, c = 9.7 µM, d = smallest tested concentration 0.98 µM. The picture is not very informative because results were very simiral troughout the whole experiment.

3.4 ASA

When viewing the cells under the microscope after the first three days from chemical exposure there was some crystals seen in highest concentration. We can not be sure if it has affected on tubule formation because the grade was zero but it seems that crystals are not the only reason for bad grade because tubule formation was poor also with lower concentrations. Nevertheless, the slope is highly demonstrative. Based on WST-1 test absorbances it can be said that ASA is not toxic with tested concentrations.

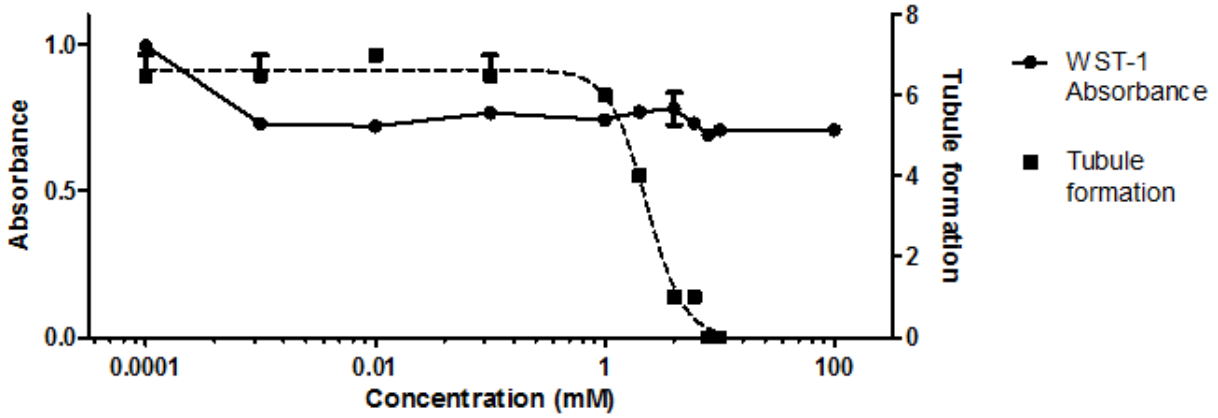


Figure 8 | ASA tubule formation and WST-1 absorbance.

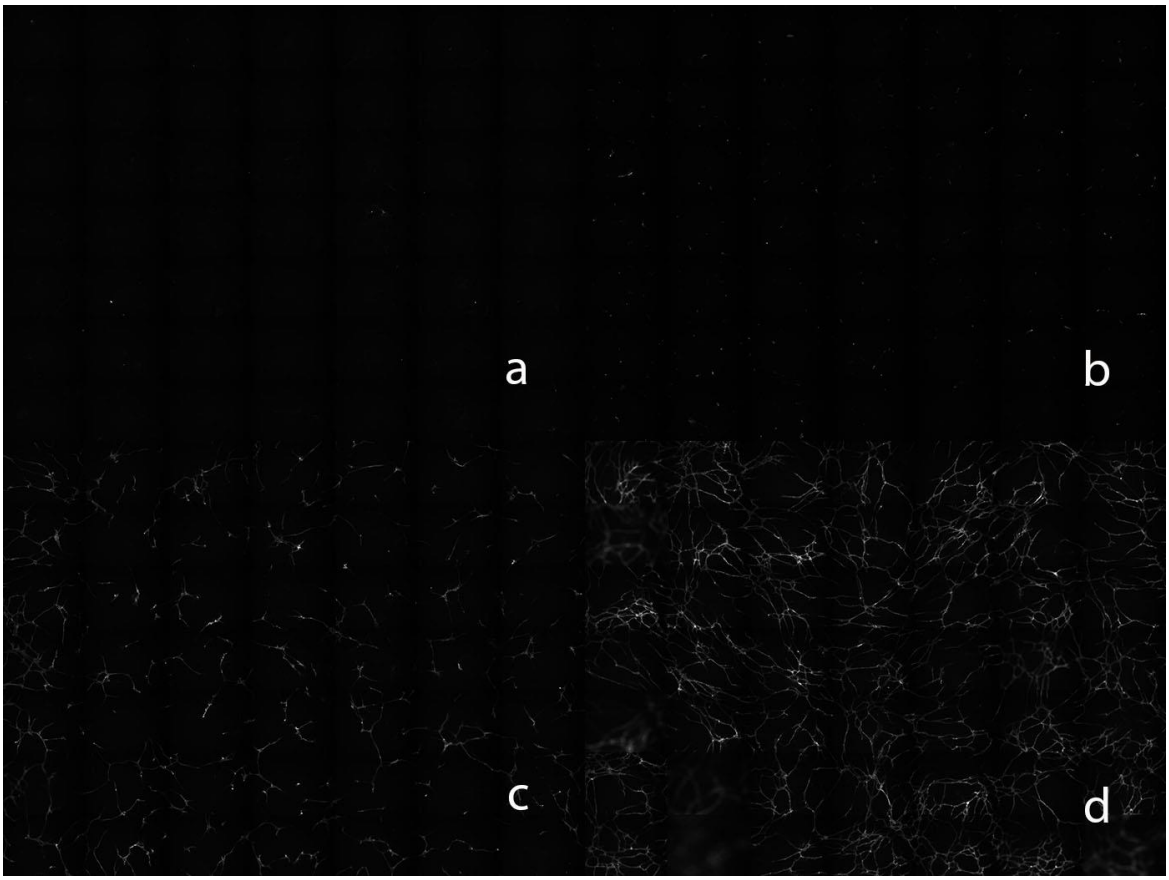


Figure 9 | ASA tubule formation. a = highest tested concentration 10 mM, b = 4 mM, c = 2 mM, d = smallest tested concentration 0.001 mM. Tubule formation was very poor when concentration was more than 2 mM.

3.5 2-Methoxyestradiol

Tubule formation was good with small concentrations and grades did not differ from each other. With higher concentrations there was more variation between the wells of same concentrations. One explanation is that there was some lump formation in wells which lowered the grade. In toxicity the slope is highly informative.

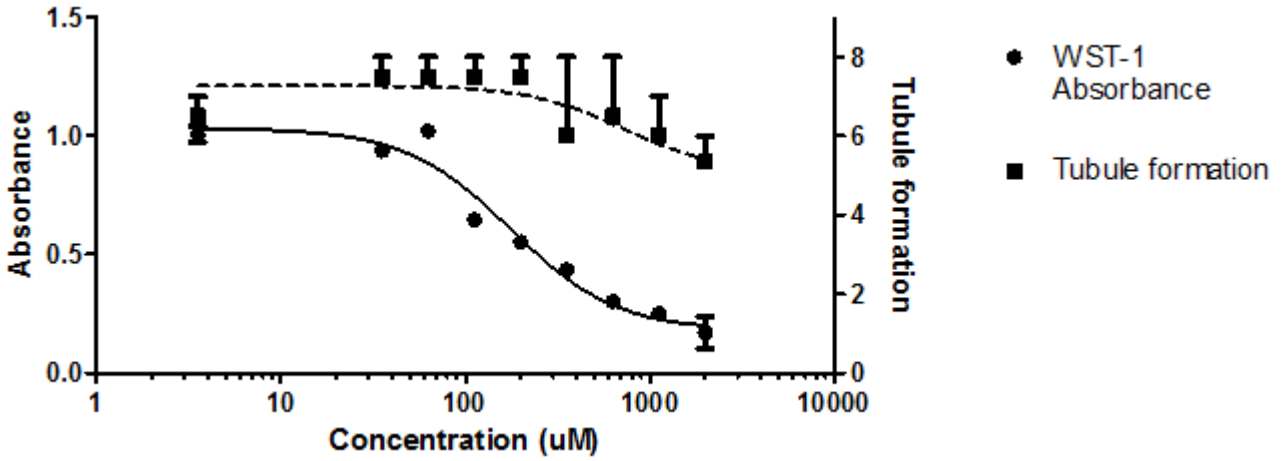


Figure 10 | 2-Methoxyestradiol tubule formation and WST-1 absorbance.

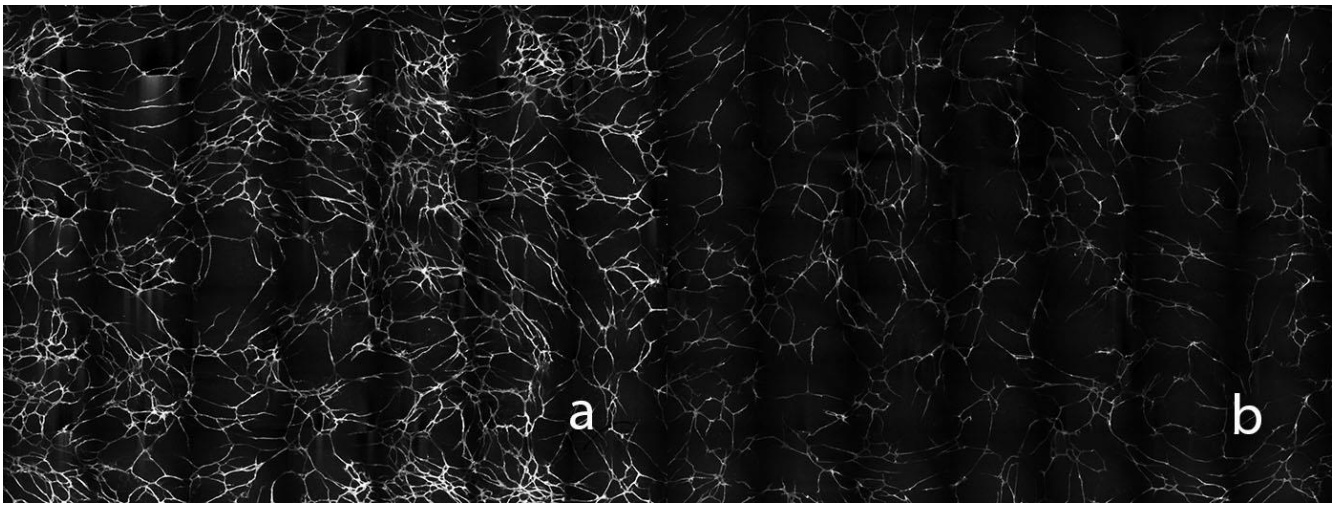


Figure 11 | 2-Methoxyestradiol tubule formation. a = highest tested concentration 2000 nM, b = smallest tested concentration 35.33 nM.

3.6 Pyridaben

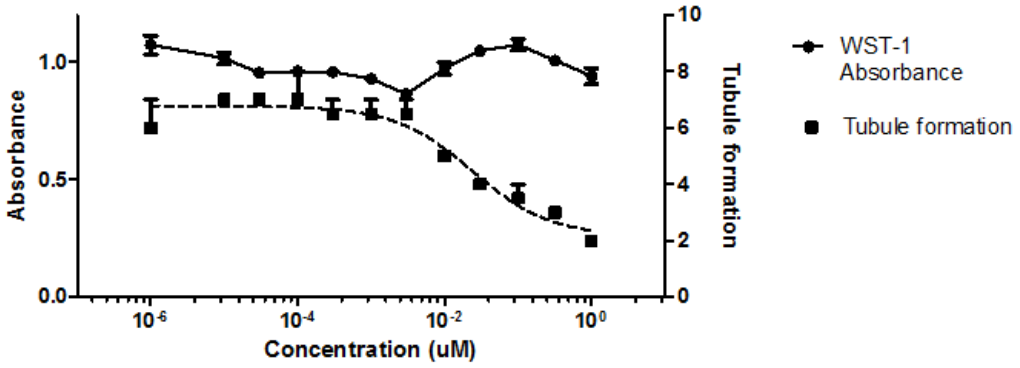


Figure 12 | Pyridaben tubule formation and WST-1 absorbance.

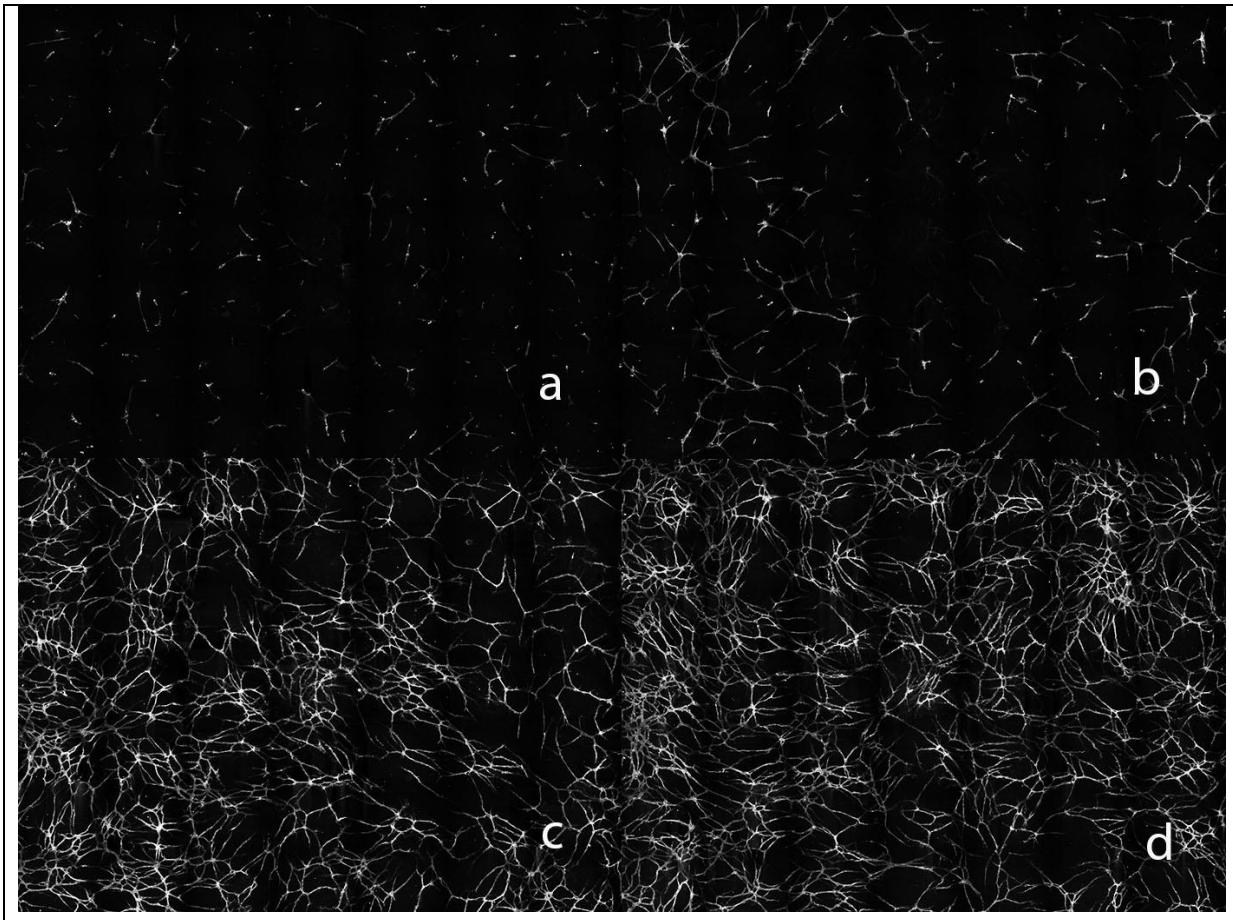
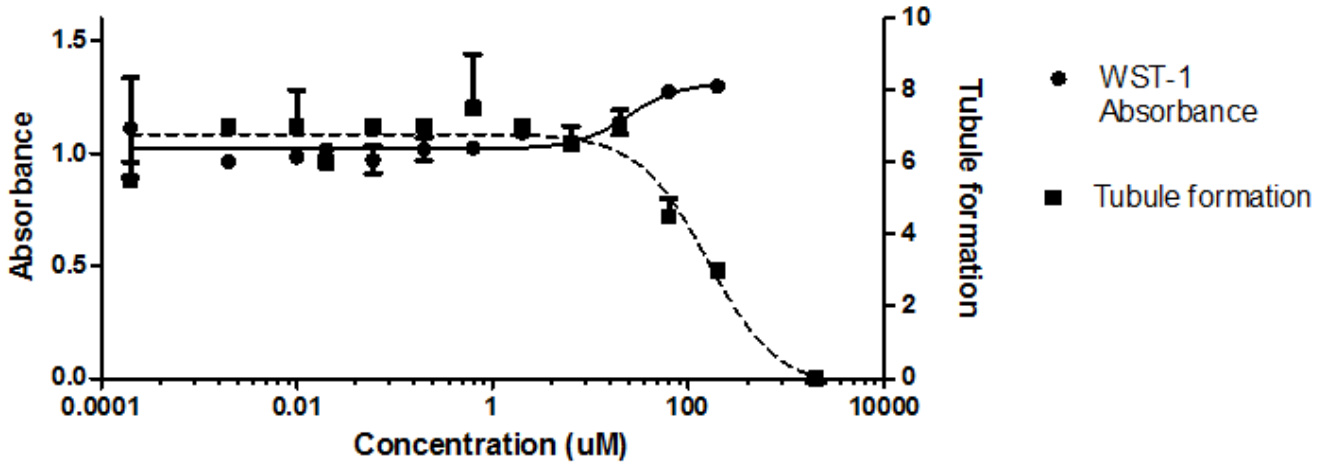


Figure 13 | Pyridaben tubule formation. a = highest tested concentration 1.00 µM, b = 0.03 µM, c = 0.001 µM, d = smallest tested concentration 0.00001 µM.

3.7 Indomethacin

Toxicity test gives no reliable results because cell viability rose with high concentration. Results from angiogenesis test gave more adequate data. Only down side is that positive control got lower grading than smallest tested indomethacin concentration.



Picture 14 | Indomethacin tubule formation and WST-1 absorbance.

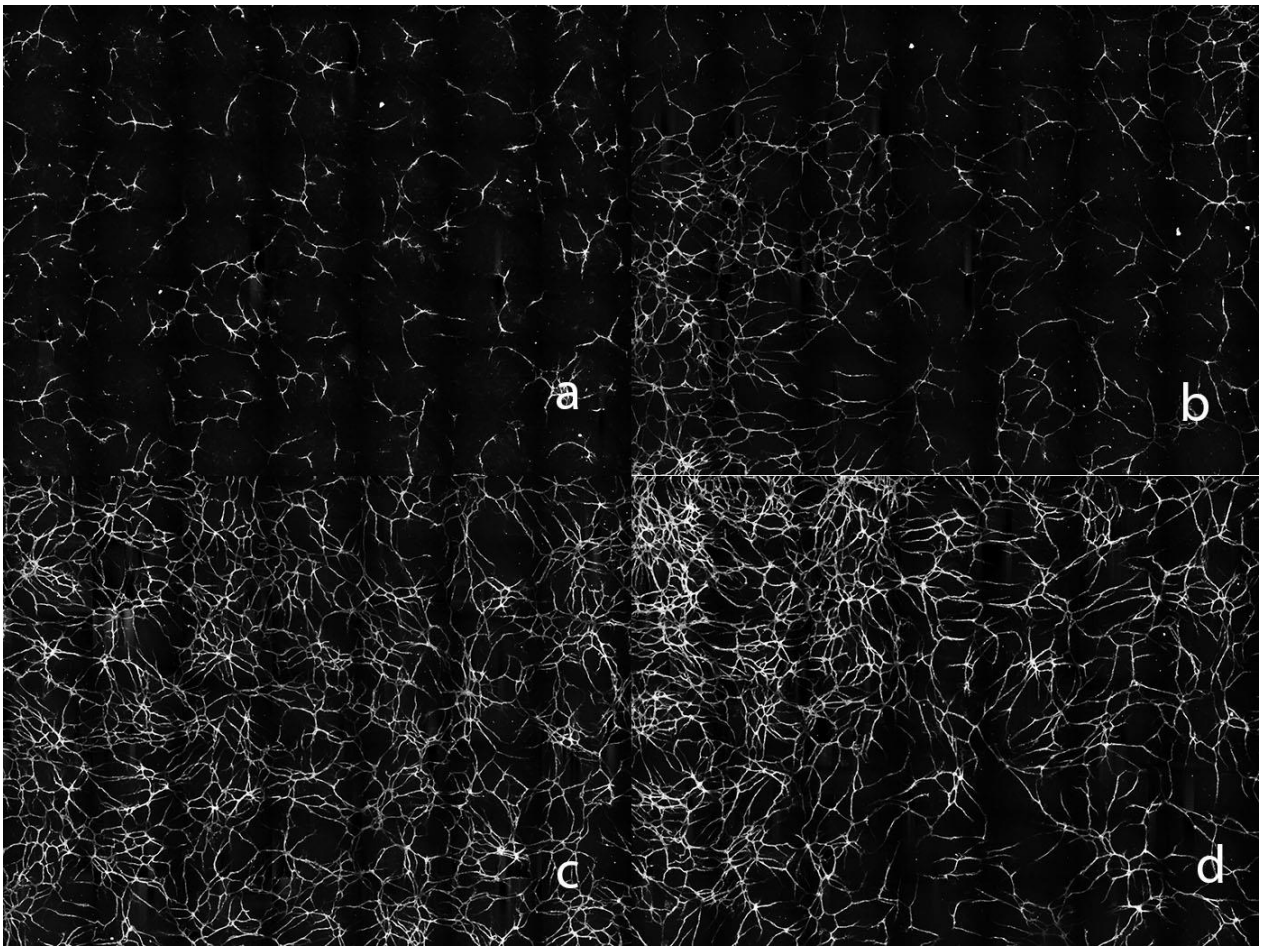


Figure 15 | Indomethacin tubule formation. a = highest tested concentration 200.00 µM, b = 63.29 µM, c = 20.30 µM, d = smallest tested concentration 0.002 µM.

3.8 Triclosan

In experiment with Triclosan positive control got only grade 6 but so did the smallest concentration of chemical. Slopes give good information in figure 16.

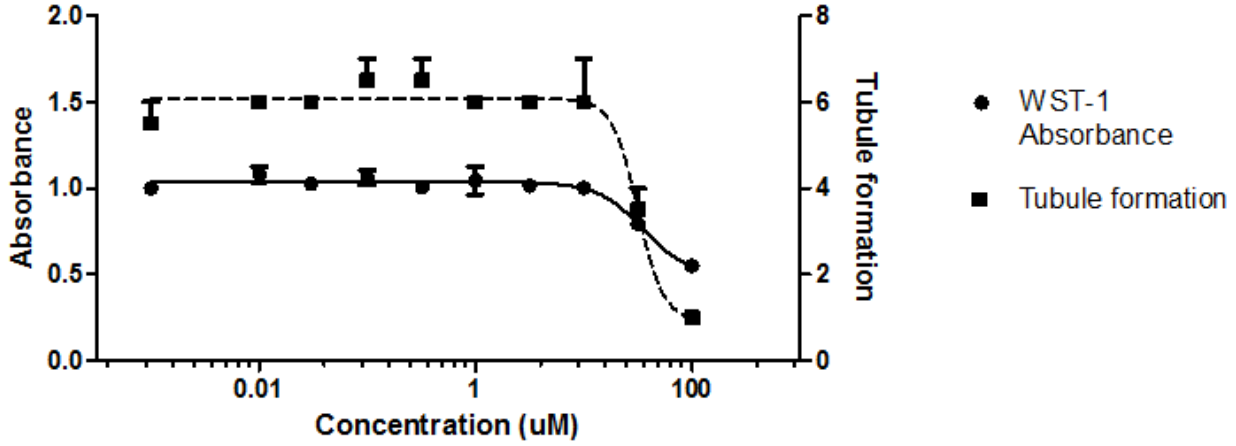


Figure 16 | Triclosan tubule formation and WST-1 absorbance.

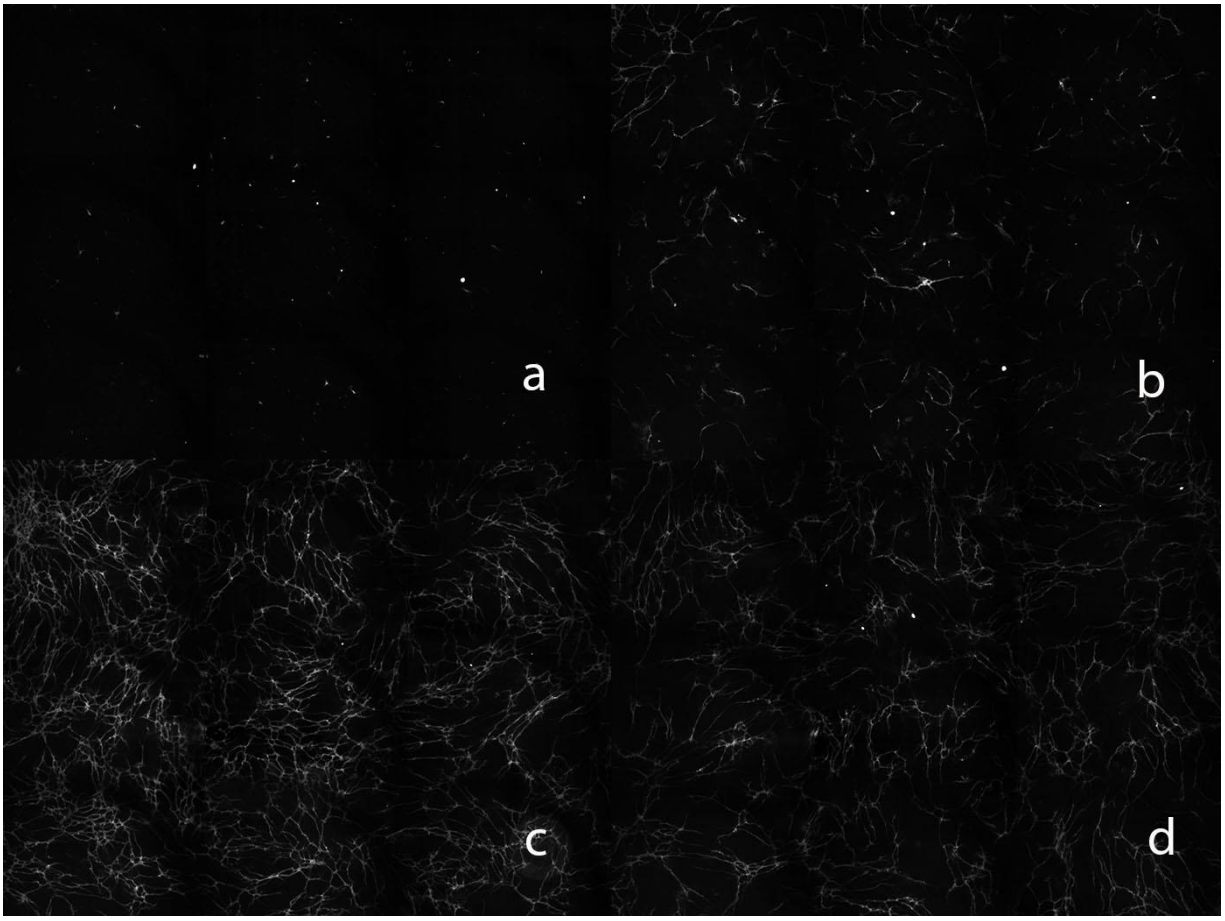
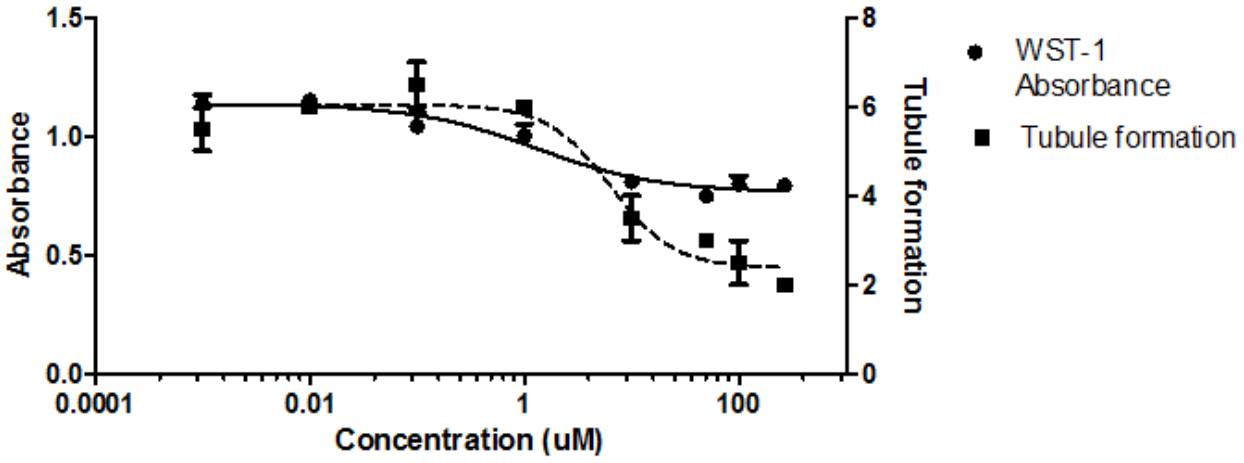


Figure 17 | Triclosan tubule formation. a = highest tested concentration 100 µM, b = 31.65 µM, c = 0.32 µM, d = smallest tested concentration 0.01 µM. Tubule formation were better in other 0.32 µM well than with smallest concentration. Immunostaining is not so bright than in other plates.

3.9 Anti-VEGF



Ficure 18 | Anti-VEGF tubule formation and WST-1 absorbance.

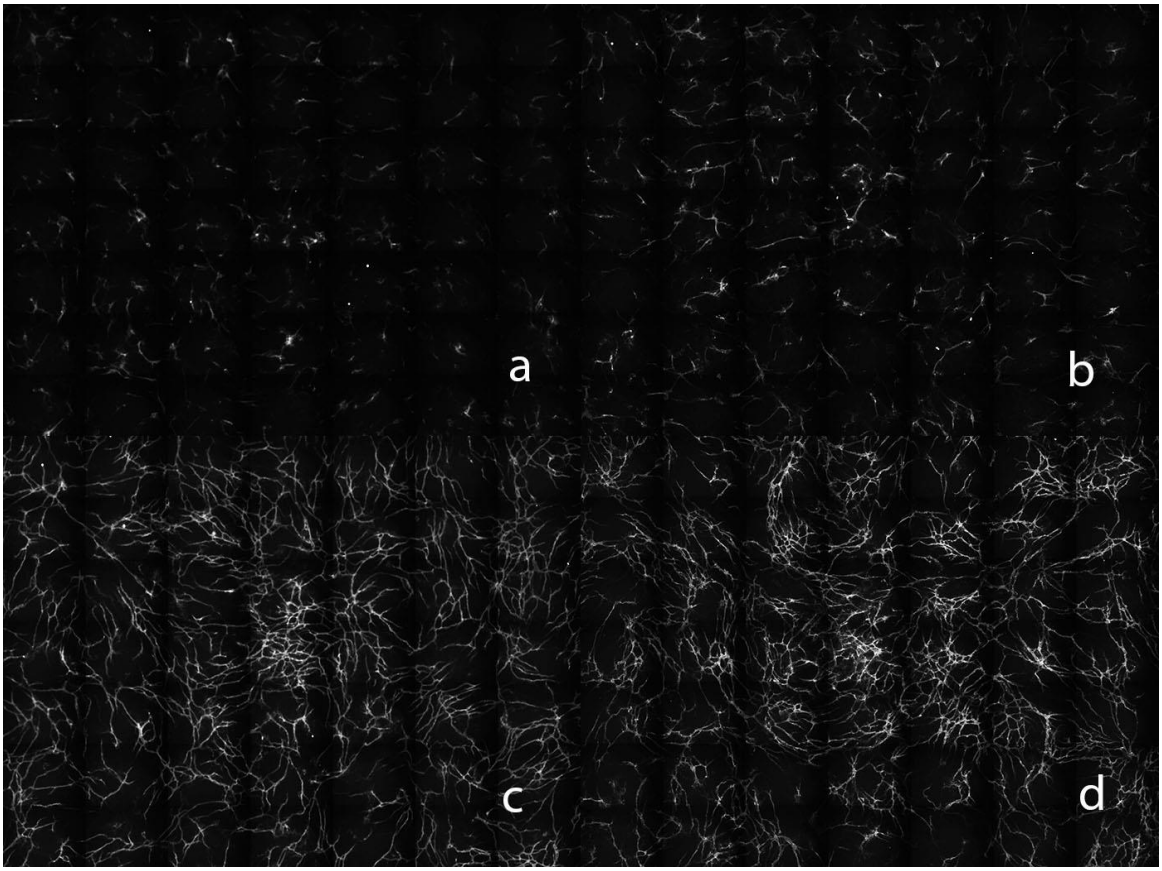


Figure 19 | Anti-VEGF tubule formation. a = highest tested concentration 267.00 µM, b = 50.00 µM, c = 1.00 µM, d = smallest tested concentration 0.01 µM

3.10 Levamisole

When evaluating the tubule formation in experiment with Levamisole, it turned out that there was plenty of cells and cell lumps growing in the middle of wells. There were also many wells where the cells were detached from the bottom of the plate on the left side of the well. One explanation for lumps can be inappropriate stirring when passaging the cells while doing angiogenesis assay. Too rough pipetting can explain the detachment of the cells from the left side. Both reasons clearly depends on person who did the experiment and that problem can be solved if further experiments are carried out. Nevertheless there was tubule formation outside the middle and the grading was done systematically from there.

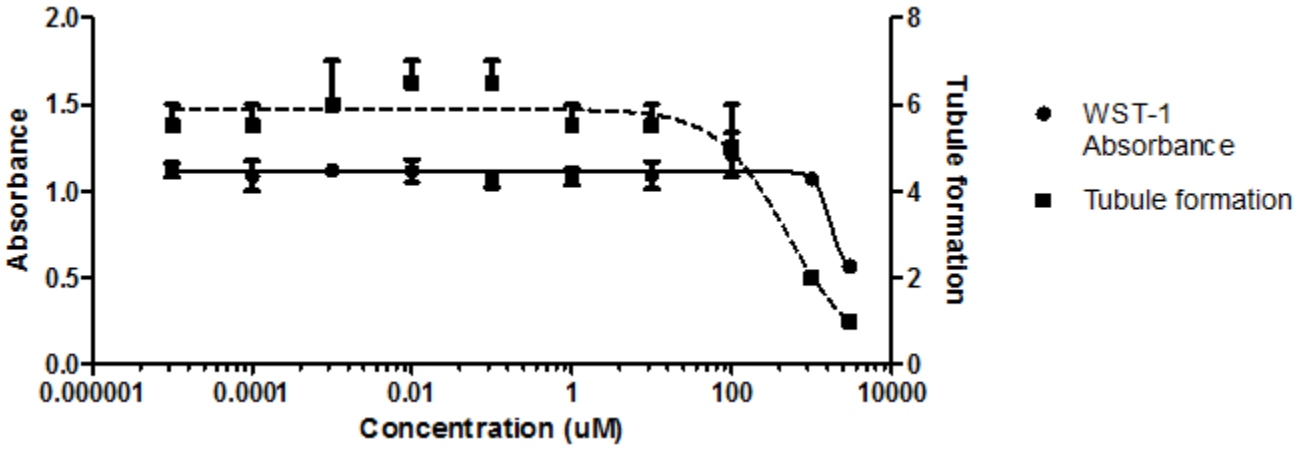


Figure 20 | Levamisole tubule formation and WST-1 absorbance.

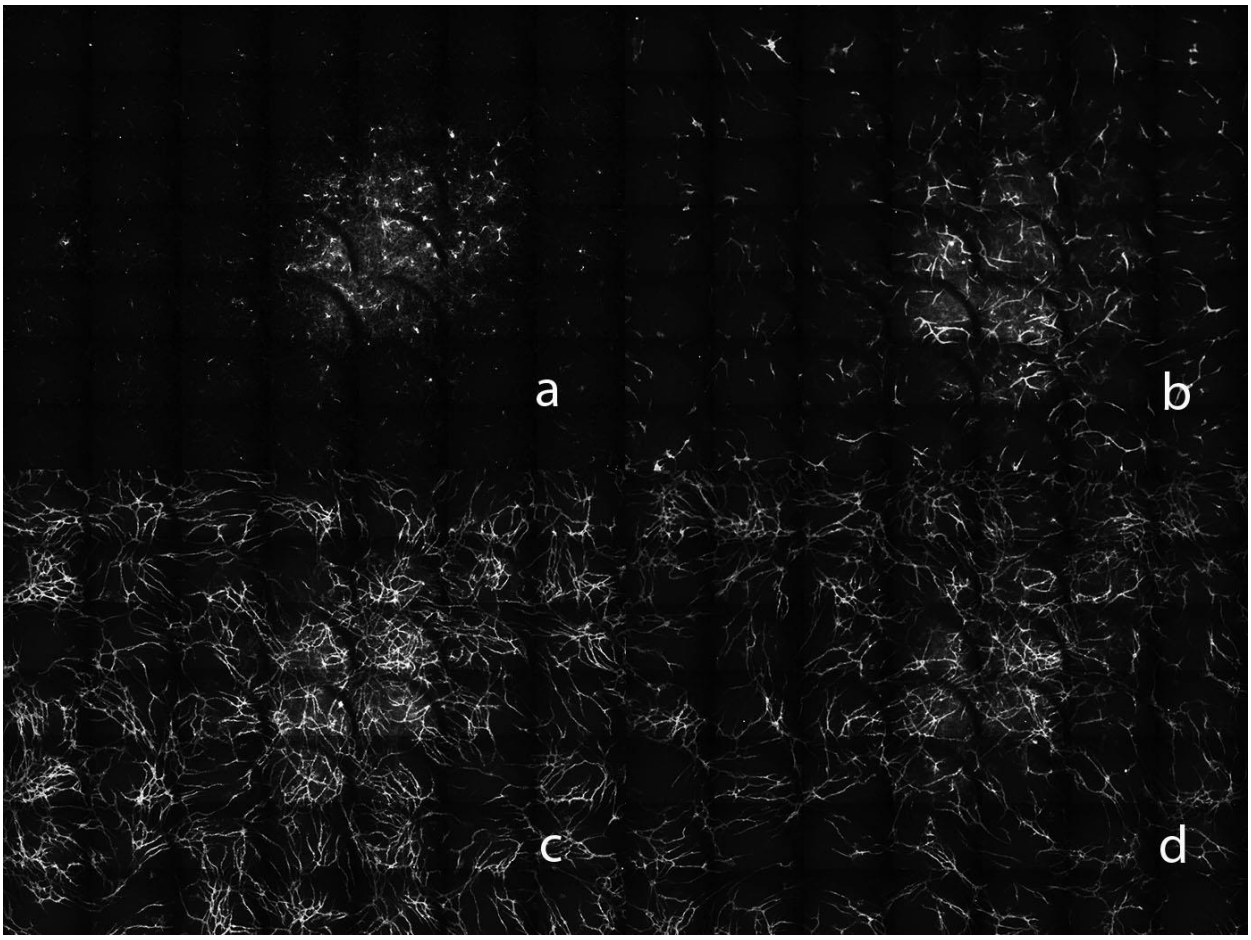


Figure 21 | Levamisole tubule formation. a = highest tested concentration 3000 µM, b = 1000 µM, c = 0.01 µM, d = smallest tested concentration 0.0001 µM. Bright areas seen in the middle of wells.

4 CONCLUSIONS

There is strong need for pre-validated human cell *in vitro* assays for reducing and replacing animal tests. Animal tests do not necessarily predict effects in human but they still are used widely. (Sarkanen et al., 2011) *In vitro* angiogenesis assays are important tool of studying mechanisms of angiogenesis and also potential equipment to develop therapeutic strategies of vascularization. (Donovan et al., 2001) Even though they do not cover every step of angiogenesis process, they effectively mimic the key steps i.e. migration and differentiation of endothelial cells. (Sarkanen et al., 2011) Nowadays there is high standards for in vitro assays and they must undergo validation process

before they can be taken in proper use. Reliability, relevance and fitness for purpose must be evaluated and the new test system should provide equivalent or better results than current procedures to even consider to become the new standard, time and money play central role. (Judson et al., 2013)

Sarkanen et al. have studied hASC+HUVEC co-culture angiogenesis assay in which cells are induced with natural growth factors and allowed to self-assemble into tubular network and vascular supporting structures. It provides completely human-based and simplified model of angiogenesis. (Sarkanen et al., 2012) The aim of this study was to validate the hASC+HUVEC co-culture by exposing it to ten commonly known chemicals, which effects on angiogenesis have been studied before. We evaluated reliability, relevance and fitness for purpose by comparing the test results with results from literature and earlier studies. Also other side of validation was put under examination; is the test system repeatable and is the process of making worth of money and time spent.

We tested both cell toxicity and effects on angiogenesis of each chemical to see if the effects are caused by specific anti-angiogenic property of the substance or if it is just common cell toxicity. Several different concentrations were tested and at least in two wells simultaneously. EC_{80} value was not possible to calculate for every chemical meaning that those chemicals did not affect on cell viability with tested concentrations. These chemicals found to have an effect specifically on angiogenesis. In future higher concentrations can be tested if there is a need to determine concentration which causes cell toxicity. IC_{50} value was calculated for every chemical but there were three chemicals for which extrapolation had to be used to be able to calculate the IC_{50} value. With Suramin, NS-398 and Indomethacin the results may not be accurate and even higher concentrations should be tested in future for to have even more reliable results.

When comparing toxicity test results with previously known values there can be seen some difference. Some reference values could not be found (see table 2) and some are quite far from results from this study. However, test gave some IC_{50} values for each chemical. These things (result

differences) should be considered in the future and still more test need to be done to complete validation process of this *in vitro* angiogenesis assay.

There were also some challenges to evaluate tubule formation completely adequate. The best scene should have been that cells did not grow in lumps and the tubule network should have been constant. For some chemicals lumps existed in several wells and with Levamisole there was also detachment of the cells from the left side of the well. Some reasons may depend on person who executed the experiments; not enough stirring while passaging the cells or too rough cell cultivation technique. If so, in the future these things should be considered. One explanation for lumps may just be chemical's effect on cells; the chemical might be inhibiting the endothelial migration and for this reason proper tubule formation is not possible when exposed to chemical.

When thinking overall the test process, there is one main point which comes out; it is mainly executed by human. The success of test results highly depend on person who executes the cell cultivation and chemical exposition. There are several steps when testing each chemical and also different cell cultivations must be done to every different chemical. Even though the test procedure is easy to learn, the executor must be trained to do cell cultivation. That is one way how different cell cultivations can be as alike as possible. It is also important that the same person does most of the work. Also the final evaluating of tubule formation is done by human, but the variation there is not so apparent because standards are already done for each grade and same person is executing the evaluation. The grading can be even more reliable if other person also does it.

The final conclusion is, that this hASC+HUVEC co-culture angiogenesis assay has a lot of potential to become standard procedure when testing chemical effects on angiogenesis. Some more test need to be done in future to get more reliable results on toxicity tests to complete the validation process.

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