Tampereen yliopisto

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CYTOTOXICITY OF PHTHALIC ANHYDRIDE AND METHOTREXATE EXPOSED HUVEC CELLS

Bachelor's thesis
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ABSTRACT

Anni Mäkinen: Cytotoxicity of methotrexate and phthalic anhydride exposed HUVEC cells
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Pulmonary fibrosis is a progressive fibrotic disease that occurs in the lung tissue. In the fibrotic lung tissue, excess number of fibroblasts and immune cells accumulate to the interstitial lung tissue. This disease does not have a working medication and thus will lead to the death of the patient without lung transplant. Due to these factors, finding a medication for pulmonary fibrosis would be an important step. Creating a medication for disease requires understanding of its mechanisms. In order to find the mechanisms of pulmonary fibrosis in molecular level, expressed genes specific of a fibrotic cell need to be determined. For this purpose, genes expressed in fibrotic cells need to be compared with expressed genes of a cell that has been exposed to poisonous substance that does not cause fibrosis. By this comparison, it is possible to separate the effect of poisonous chemical from specifically pulmonary fibrosis inducing chemical.

The aim of this bachelor's thesis was to contribute to the creation of pulmonary fibrosis *in vitro* model to be able to create a medication for this disease. The goal of this experiment was to determine whether methotrexate and phthalic anhydride (PAH) could be used as reference chemicals in the *in vitro* model of pulmonary fibrosis. This was done by measuring the cytotoxicity of these substances and calculating their IC50 and IC20 values.

The experiment was conducted with human umbilical vein endothelial cells (HUVEC). These cells model the structure of endothelial cells in the walls of the pulmonary veins. HUVEC cells were cultivated in cell culture bottles for a week to ensure sufficient number of cells. After cultivation, cells were plated to 96-Well Microplates. Both potential reference substances had their own plate and then third plate was seeded to positive control substance sodium dodecyl sulfate. Exposure chemicals were added to their plates in 8 different concentrations with tracer compounds. After exposure, cell viability was measured from the plates with luminescence reader after 24,48 and 72 hours. One measurement round took 2 weeks to complete, and 3 rounds were performed to guarantee repeatability.

The results of this experiment were not according to the set objective. Within the used concentrations methotrexate did not decrease the viability of cells. Thus, it was not possible to determine the IC values for this substance. Phthalic anhydride showed inconsistent behavior through the experiment, and it was not possible to determine the IC values for this substance either. Based on the results of this study, higher concentrations for both substances could be used in future studies. Higher concentrations might be able to reduce the cell viability in the expected manner and then be suitable as reference chemicals for the *in vitro* model.

Keywords: Methotrexate, phthalic anhydride, endothelial cells, *in vitro* model, cytotoxicity, pulmonary fibrosis

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TIIVISTELMÄ

Anni Mäkinen: Metotreksaatilla ja ftaalianhydridilla altistettujen HUVEC solujen sytotoksisuus
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Keuhkofibroosi on krooninen ja etenevä keuhkojen kudoksen tulehdustila. Fibroottisessa kudoksessa fibroblasteja ja immuunisoluja kertyy keuhkojen välikudokseen ylimäärin. Keuhkofibroosiin ei ole olemassa olevaa lääkitystä ja se johtaa potilaan kuolemaan ilman keuhkosiirrettä. Koska tauti johtaa lähes aina potilaan kuolemaan, parannuskeinon löytäminen olisi tärkeä askel lääketieteelle. Tämä vaatii keuhkofibroosin tautimekanismien ymmärtämistä. Jotta voitaisiin ymmärtää keuhkofibroosin tautimekanismit molekyylitasolla, on määritettävä fibroottisessa solussa ominaisesti ilmenevät geenit. Fibroottiselle solulle ominaiset ilmenevät geenit löydetään, kun fibroottisessa solussa ilmeneviä geenejä verrataan myrkylle altistetun mutta ei fibroottisen solun ilmeneviin geeneihin.

Tämän tutkimuksen tarkoitus oli osallistua keuhkofibroosin *in vitro* mallin luomiseen, jonka avulla voitaisiin mahdollisesti kehittää lääke tähän sairauteen. Tässä tutkimuksessa pyrittiin selvittämään, sopisivatko metotreksaatti ja Ftaalianhydridi *in vitro* mallin referenssi kemikaaleiksi aiheuttaen soluissa vasteen myrkylliselle aineelle mutta eivät aiheuttaisi fibroosia. Aineiden sopivuutta pyrittiin arvioimaan mittaamalla niiden IC50 ja IC20 arvot.

Kokeet toteutettiin ihmisen napanuoran endoteelisoluilla eli HUVEC soluilla. Nämä solut mallintavat melko hyvin verisuonten seinämien endoteelisoluja. HUVEC soluja kasvatettiin solukasvatus pulloissa viikon ajan, jotta soluja on tarpeeksi kokeen tarkoitukseen. Kasvatuksen jälkeen solut istutettiin 96- mikrokaivo levylle. Molemmille tutkittaville aineille valmisteltiin levy ja lisäksi kontrollikemikaali natriumlauryylisulfaatille myös oma levy. Altistuksessa aineita lisättiin levylle 8 eri konsentraatiossa ja mittauksia varten levylle lisättiin myös merkkiaineita, jonka avulla lumisenssi mittaukset tehdään. Altistuksen jälkeen solujen viabiliteettiä mitattiin lumisenssi lukijan avulla 24, 48 ja 72 tunnin jälkeen. Yksi mittaussarja kesti viikon ja sama mittaus toistettiin kolme kertaa toistettavuuden varmistamiseksi.

Tutkimuksen tulokset eivät olleet asetetun tavoitteen mukaisia. Metotreksaatista käytetyt konsentraatiot eivät vähentäneet solujen viabiliteettia. Tästä johtuen ei ollut mahdollista määrittää IC arvoja tälle aineelle. Ftaalianhydridi käyttäytyi epäjohdonmukaisesti eikä tästäkään aineesta ollut mahdollista määrittää haluttuja IC arvoja. Saatujen tulosten mukaan mahdollisissa jatkotutkimuksissa voitaisiin käyttää molemmista aineista korkeampia konsentraatioita. Korkeammat konsentraatiot saattaisivat pystyä vähentämään solujen viabiliteettia halutulla tavalla ja siten soveltua *in vitro* mallin verrokeiksi.

Avainsanat: Metotreksaatti, ftaalianhydridi, endoteelisolut, solumallit, sytotoksisuus, keuhkofibroosi

The AI tools used in my thesis and the purpose of their use have been described below:

Name of the tool: Scopus AI 20
Purpose of use and the part in which it was used: AI was used to find articles about molecular pathways of phthalic anhydride in the Background chapter.

I am aware that I am responsible for the entire content of the thesis, including the parts generated by AI, and accept the responsibility for any violations of the ethical standards of publications.

TABLE OF CONTENTS

1. INTRODUCTION

Idiopatic pulmonary fibrosis is a disease that occurs in the interstitial of lung tissue and mostly occurs in older people, mean age of patients being around 65 years (1). The characteristics of this illness are chronic and progressive fibrosis in the tissue, unknown cause and poor prognosis. (2) Due to unknow factors behind the disease and poor prognosis, the creation of a disease model is an important step towards discovering a treatment method. This study is part of the effort towards the creation of the model. In vitro models that mimic the structure of lung tissue have been developed and pulmonary fibrosis causing substances have been investigated with them (3,4). In this study, the aim is to find reference chemicals that are poisonous but don't cause pulmonary fibrosis. Comparing the effects of pulmonary fibrosis causing chemical and otherwise poisonous chemicals offers information about the mechanisms of the disease.

This work is a measurement of cytotoxicity of two chosen substances, phthalic anhydride (PAH) and methotrexate. Cytotoxicity measurements of this study are a part of background research for finding reference chemicals for *in vitro* models of pulmonary fibrosis. The goal of the study is to determine the IC50 and IC20 values for both substances. IC50 value indicates, how much of a specific chemical substance is needed to inhibit a measured biological activity by 50 % (5). IC20 value works similarly, but describes the amount needed for 20 % inhibition of biological activity (6). In this study, the IC values are measured by cell viability. Cells are exposed to different concentrations of PAH and methotrexate and after exposure the number of dead cells is measured with the help of indicators. Sodium dodecyl sulfate (SDS) works as a positive control chemical in this study. Its cytotoxicity effects on cells are known and thus it works as an indicator if the experiment has been conducted correctly.

The *in vitro* model in this study was built with human umbilical vein endothelial cells (HUVEC). These cells are compatible model for lung endothelium and used in endothelium modeling. In addition, HUVEC cells are easily obtainable and simple to subculture and thus were chosen to be the model cells for this experiment. (7) The experiment was conducted three times, and one iteration took two weeks to complete. Cell culture was conducted in the first week and HUVEC cells were sub-cultured twice to ensure sufficient number of cells. During the second week of the experiment, cells were exposed to chemicals of interest and measurements were taken after 24, 48 and 72 hours.

In this thesis the theoretical background is presented in chapter two and includes an explanation about *in vitro* models as well as the mechanisms of both chemicals of interest. Chapter three presents the materials and methods of the experiment and chapter four the results. Chapter five includes analysis and deduction about the results in the form of conclusion.

2. BACKGROUND

This chapter focuses on the theoretical background of the experiment and explains the bio-chemical mechanisms behind the chosen *in vitro* model. The first chapter explains the mechanisms of pulmonary fibrosis as a disease. The following chapters focus on the bio-chemical properties of chosen chemicals and cell culture.

2.1 Pulmonary fibrosis

A healthy alveolar lung tissue consists of multiple layers of tissue that all have their purpose in gas exchange. The innermost tissue layer of alveoli consists of two types of epithelial cells, Type I Alveolar Epithelial Cells (ATI) and Type II Alveolar Epithelial Cells (ATII). Epithelial cells secret pulmonary surfactant to the inner surface of the alveoli. Between the endothelium and vessel endothelial cells, thin extracellular matrix, so called basement membrane, is located. The endothelial cells, that are the focus of this experiment, are located at the wall of the blood vessels that wrap around the alveoli. Pulmonary interstitial tissue is located underneath the blood vessel and mostly consists of fibroblasts and macrophages. (8,9) The structure of the healthy alveolar lung tissue is illustrated in Figure 1.

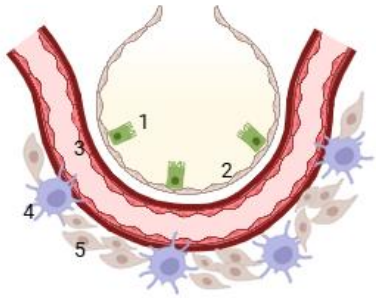

Figure 1. Healthy alveolar lung tissue with cell types marked in the picture: 1. Type II Alveolar Epithelial Cells (ATII) 2. Type I Alveolar Epithelial Cells (ATI) 3. Endothelial cells 4. Macrophages 5. Fibroblast (modified from source (9))

Pulmonary fibrosis is a disease that occurs in lung tissue and includes chronic injury in alveolar epithelium or endothelium. This chronic injury initiates a wound healing process

where excessive amount of fibroblasts and immune cells accumulate to the interstitial lung tissue. (10) This process is a normal reaction for injuries of the tissue, but due to its chronic nature, the scarring of the tissue doesn't stop but continues indefinitely. When the scarring of tissue has advanced enough respiratory failure can occur and patients' quality of life decreases. (11) Even though some treatment methods have been tested for this disease, they have only had minor improving effects for the patient (2). Without proper treatment available, patients with pulmonary fibrosis diagnosis perish eventually. Thus, finding a cure for this disease would be an important step forward.

2.2 Research on pulmonary fibrosis

Diagnosing pulmonary fibrosis correctly on patients has proven to be quite challenging. This is due to the first symptoms of the disease being vague and mild and thus easy to mistake as a cause of another diseases. (12) Late diagnosis complicates the possibility to investigate the effects of the disease in the early stages on real patients. Thus, to understand the early mechanisms of pulmonary fibrosis, disease models are necessary.

Disease models can for example be animals that has been exposed to pulmonary fibrosis inducing substances or they can be cell culture models with human cells. In this study, the approach was chosen to be cell culture model. Several studies have shown that results obtained from animal models could not be replicated in humans. Thus, cell culture models might better represent the progression of the disease in a human body compared to animal models. (13,14)

In addition, animal models have various ethical concerns associated with them. Animal testing often causes pain and distress to animals used as test subjects. Due to ethical concerns of animal testing, the scientific community has agreed upon the principle of the 4 Rs. These letters refer to the words Reduction, Refinement, Replacement and Responsibility. The objective of this principle is to decrease the amount of animal models, ensure reasonable treatment and develop alternative methods for animal testing. (14)

2.3 *In vitro* model

In this study, the cytotoxicity model was chosen to be HUVEC cells. HUVEC cells model the endothelial cells of the blood vessels that wrap around the alveoli. As was established in the previous chapter, endothelial cells have an important role in the structure of lung tissue and gas exchange. According to research conducted with endothelial cells in models of pulmonary fibrosis, they also have a notable effect on the disease formation (15). Endothelial–mesenchymal transition is a mechanism that has been shown to contribute

to the accumulation of fibroblasts in the interstitial lung tissue, which upholds continuous inflammation. (15) In endothelial–mesenchymal transition endothelium loses its cell-cell junctions and doesn't form an organized cell layer. Mesenchymal cells also lose their polarization and apical-basolateral organization of cell organelles. Endothelial–mesenchymal transition is controlled by complicated intercellular signaling pathways and this process can be triggered by extracellular stimuli. (16)

In this study, the used cell culture was simple and only included one cell type. For the chemical exposure, HUVEC cells were attached to 96 micro well plates by ThermoFisher in density of 128 000 cells/ml. For the purpose of investigating the cytotoxicity of potential reference chemical, a simple cell culture is enough. Cytotoxicity of a chemical is in many cases determined with a simple cell culture with one cell type relevant for the investigated drug or chemical (17,18).

2.4 Methotrexate

Methotrexate is a medicine first used as an anticancer drug but nowadays used for rheumatoid arthritis treatment. This drug has an anti-inflammatory effect on cell population and thus is very useful in the treatment of malignant diseases. Methotrexate has biochemical effects on the intercellular pathways and thus causes its anti-inflammatory effect. (19,20)

The IUPAC name of methotrexate is (2S)-2-[[4-[(2,4-diaminopteridin-6-yl)methyl-methyl-amino]benzoyl]amino]pentanedioic acid and it can be classified as a monocarboxylic acid amide and a dicarboxylic acid. Methotrexate is a yellow crystalline structured compound that is odorless. (21)

The mechanism of action of methotrexate is related to dihydrofolate metabolism. An enzyme called dihydrofolate reductase is responsible for regeneration from dihydrofolate to tetrahydrofolate. This regeneration process is crucial in purine and pyrimidine synthesis. Methotrexate works as a competitive substrate and replaces purine or pyrimidine in the process and thus works as an inhibitor. In addition, methotrexate contributes to excess adenosine being released to the blood circulation. Adenosine inhibits superoxide anions, that are produced by neutrophil activation and cause tissue damage. Inhibition happens by affecting specific cell surface receptors. (19,20)

2.5 Phthalic anhydride

Phthalic anhydride (2-Benzofuran-1,3-dione) is an organic compound with white color and powder structure (22). It can be classified as cyclic dicarboxylic anhydride according

to its chemical structure (23). This chemical is widely used in different applications across multiple fields. Material sciences use phthalic anhydride in different polymerization technologies, such as in the production of polyesteramides. (22)

In biological and pharmaceutical field, phthalic anhydride is used as a model of respiratory allergen (24). It is classified as skin and respiratory sensitizer causing irritation to tissues in question (25). Exposure to phthalic anhydride has been reported to increase the concentration of IgE and IL-4 in the serum of test animals. IgE is a type of immunoglobulin that takes part in local defense system of a body. (26,27) IL-4 is an anti-inflammatory cytokine that participates in differentiation of certain leukocytes and activates humoral immunity (28).

2.6 Sodium dodecyl sulfate

Sodium dodecyl sulfate (SDS) is the positive control chemical of this study. This chemical was chosen, because cytotoxicity data of this chemical already exists and it can be used as a reference point for Methotrexate and PAH. SDS is an organic sodium salt with pale yellow liquid or paste structure. This chemical is used in cosmetic products such as toothpaste, hair dyes and cleaning products. (29)

The toxicity of SDS is caused by its capability to disrupt essential proteins in human cells. Human transferrin (HTF) is a glycoprotein that participates to the transportation of iron ions in the bloodstream. This protein acts as a chaperone and binds to the iron thus preventing precipitation and harmful interaction with cellular components. SDS disrupts the structure of this protein and decreases its ability to bind iron ions thus causing a harmful effect. (30)

2.7 Tracer compounds

In order to measure the cytotoxicity and amount of alive cells in the cell culture, tracer compounds are needed. These substances react with an indicator that is dependent on living cells being in the culture and produce a luminescence or fluorescence reaction. Tracer compounds used in this study were Promega's RealTime-Glo™ and CellTox™ Green.

RealTime-Glo™ MT Cell Viability Assay is a method of measuring the cell viability. The method utilizes the ability of living cells to process substances. To perform the measurement, MT cell viability substrate and NanoLuc enzyme will be added to the well plates with cells. All the living cells in the well will process the substrate within their metabolism and create a certain compound. NanoLuc enzyme is capable of binding to that product

compound and produce a luminescent signal. Since dead cells don't have metabolism, they don't produce the compound or the light reaction. The strength of the luminescent signal depends on the concentration of living cells and thus allows the measurement of viability with the help of a reader machine. (31)

CellTox™ Green Cytotoxicity Assay is a toxicity tracer compound by Promega but works differently from RealTime-Glo™. This tracer compound binds to free DNA and causes a luminescent signal when the binding happens. Free DNA in the cell culture indicates decreased cell membrane integrity and dead cells. Unlike RealTime-Glo™, CellTox™ measures the amount of dead cells in the culture. The amount of alive cells can be calculated with the information of dead cell count. (32)

Other possible tracer compounds used in research are for example 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) and alamar Blue (33). CFDA-AM is an esterase compound that has the ability to enter living cells with intact cell membrane. Inside the cell esterase activity causes the CFDA-AM to transform into fluorescent product. (34) This tracer compound works similarly to RealTime-Glo™ used in this study and the strength of the luminescence signal is dependent on living cells with metabolism. Alamar blue works similarly to both CFDA-AM and RealTime-Glo™ by working as a method to measure alive cells with metabolic activity. Alamar blue is processed inside living cells by oxidoreductases and the mitochondrial Electron transport chain. The product of these processes has luminescence capabilities and can be observed from the cell culture medium. (35)

3. MATERIALS AND METHODS

This chapter describes the conduction of the experiment. Experiment consisted of measurements that took two weeks and were repeated three times. Experiment included cell culture, exposure with the chemicals and measurements.

3.1 Cell culture

The experiment began with cell culture to ensure sufficient number of cells. Cell culture phase of the experiment extended the first week. First step to the cell culture was thawing of the cells. Frozen cell ampoule containing $0{,}5 * 10^6$ cells in 1 ml of DMSO (10 % v/v) was acquired from the liquid nitrogen freezer. Cells were thawed in water bath with temperature of +37 °C. The second step in the process was to transfer thawed cells to three Promega's cell culture bottles with pipette (Figure 2). Cells were transferred to 14 ml of HUVEC cell medium by PromoCell. PromoCell does not disclose the composition of their cell culture medium, but it includes at least Fetal Calf Serum, Endothelial Cell Growth Supplement, Epidermal Growth Factor, Basic Fibroblast Growth Factor, Heparin and Hydrocortisone.

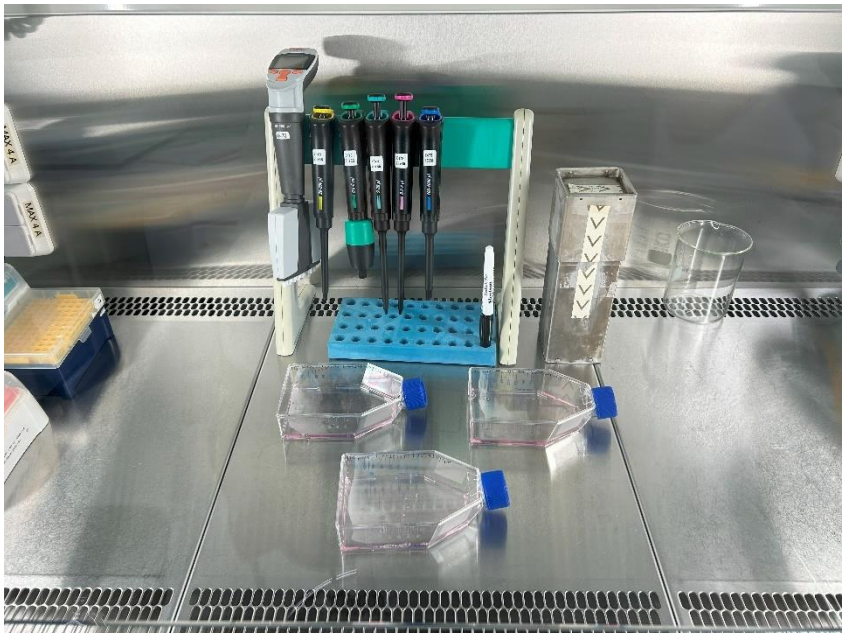

Figure 2. Cell culture bottles in laminar flow hood

After transfer, cell cultures were placed to incubator with temperature of +37 °C and CO_2 level of 5.0%. Cell medium was changed after 24 hours to ensure enough nutrients for the cells.

Cells were allowed to divide and increase in number for approximately 48 hours after they were subcultured. Subculturing is the process of transferring cells to new cultures to ensure enough space and nutrients for cells. The first step was to remove cell media from cultures with a suction pump. Detaching the cells from the culture was done with TrypLE ™ Express enzyme. This enzyme is a protease produced in fungi that detaches cells from surfaces (36). The composition of this TrypLE enzyme solution is not publicly available.

First the surface of the bottle was rinsed with 2 ml of TrypLE ™ Express and then removed with a suction pump. This step was done to ensure that the cell media is completely removed. After rinsing, 3 ml of TrypLE ™ Express was added to the cell culture and was left to take an effect for 8 minutes in an incubator. After processing time, cells were inspected under microscope to ensure detachment and 7 ml of cell culture medium was added on top of TrypLE ™ Express. At this point, the content of three cell culture bottles were combined into one 50 ml test tube that then had cells, medium and TrypLE ™ Express in it. The test tube was placed in a centrifuge for 5 minutes with 130 g. Centrifuged test tube had all the cells at the bottom and cell media and TrypLE ™ Express at the top. The supernatant was sucked away with the suction pump and replaced with 6 ml of fresh cell culture media.

Before transferring the cells to five new bottles, cells were counted with Nexcelom cell counting device Cellometer. Cell counting is conducted by placing cell coloring trypan blue solution and target cells to specific counting slides and inserting that into the device. Counting slide has two chambers that both fit 20 µl of solution that has 10 µl cells and medium and 10 µl of trypan blue. Device gives the number of cells based in both chambers and their mean is the approximate number of cells in the test tube. With the number of cells calculated, 200 000 cells were transferred to each new cell culture bottle, to a total of five bottles, that all had 10 ml of cell culture medium in them.

After 24 hours, cell medium was changed, and subculturing was performed again after 48 hours.

3.2 Exposure chemicals

During the cell culture phase of the experiment, cells were subcultured twice. At the start of the experiment's second week, cultured cells were transferred to three ThermoFisher's Nunc™ MicroWell™ 96-Well Microplates. Transfer procedure was initiated with the same procedure as subculturing. Cells were detached from the culture with 3 ml of TrypLE ™ Express that was allowed to take an effect for 8 minutes in an incubator. After detaching, 7 ml of cell culture medium was added to the culture and detached cells transferred to 50 ml test tube. Test tube with cells in it was centrifuged with the same settings and medium with TrypLE ™ Express was sucked away with suction pump and 6 ml of fresh medium was added.

The target cell density for the well plates was 128 000 cells/ml. To achieve this density, the cell count of the test tube was verified with the Nexcelom cell counting device cellometer. The method of counting was the same as in the subculturing phase. With the cell count of the tube and target cell density, it was possible to calculate the amount of medium to be added to achieve the density of 128 000 cells/ml. The growth medium with cells in it was transferred to the 96-Well Microplates with multi-channel pipette, so that each 100 µl was added to each well, except the outer most wells. The outermost wells were filled with 100 µl fresh cell culture medium. After cell addition, plates were numbered from one to three and placed into incubator for 24 hours.

After 24 hours, cells had attached to the plate and the old medium was removed from the wells. To conduct the viability measurement, a tracer compound was added to the plates. In this study, the used indicators were RealTime-Glo™ MT Cell Viability Assay, NanoLuc substrate and CellTox™ Green Cytotoxicity Assay. 40 µl of both tracer compounds was added to 20 ml of cell culture medium and 50 µl of this solution was added to each well.

Cells were exposed to Methotrexate, PAH and a control chemical SDS. Exposure was conducted with eight different concentrations of each substance. First step of the exposure was to manufacture a dilution series for each chemical. First concentration was made by adding chemical's stock solution to cell culture medium in a test tube. The following step was to transfer certain amount of first solution to the second tube that has fresh medium already in it. By repeating this seven times, the result is dilution series of eight concentrations where every solution is of lower concentration than the previous. The dilution series concentrations can be found in appendix 1.

After manufacturing the dilution series, each concentration was added to one of the columns in the plate filling 8 wells. Each chemical has its own plate. Adding was performed with multi-channel pipette that fills one column at a time and 50 µl was added to each

well. Exposure dilutions were added to the columns 3-10. Vehicle control, which in this case is 50 µl fresh cell culture medium, was added to the columns 1,2,11 and 12. Plate map is depicted in the figure 3.

VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb
VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
VCb	VC1	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	VC2	VCb
VCb	VCb	C_1b	C_2b	C_3b	C_4b	C_5b	C_6b	C_7b	C_8b	VCb	VCb

Figure 3. Plate map: depicts the positioning of cells, chemicals and medium. VCb = fresh cell culture medium $c_x b =$ ***exposure chemical and medium VC1&VC2 = Cells and medium*** $c_x =$ ***Medium, exposure chemicals and cells All wells have tracer compounds***

After adding the exposure chemical and vehicle control, the plates were put to incubator for 24 h before the first measurement.

3.3 Measurement methods

Measurements were conducted with Tecan spark reader machine. The used tracer compounds were RealTime-Glo™ MT Cell Viability Assay and CellTox™ Green Cytotoxicity Assay. Their mode of action was explained in the background part of this thesis.

Tecan spark reader is a machine that can detect luminescence or fluorescence readings from cell cultures. The tracer compounds used in this experiment send luminescence readings based on the amount of alive (RealTime-Glo™) or fluorescence readings based on the amount of dead cells (CellTox™ Green). For the luminescence measurement, all wave lengths of visible light were collected without filtering. In fluorescence measurement wave lengths were filtered and the wave lengths were 485nm in excitation and 560 nm in emission. Measurement happened by inserting the 96- well plates to the Tecan spark reader and starting the measurement program from a computer.

Measurements were performed three times for each plate. The first measurement was after 24 hours, next after 48 and the last was after 72 hours. One experiment round had one plate for each chemical meaning three plates and experiment was performed three times to guarantee repeatability. One example of three 96- well plates is shown in figure 4.

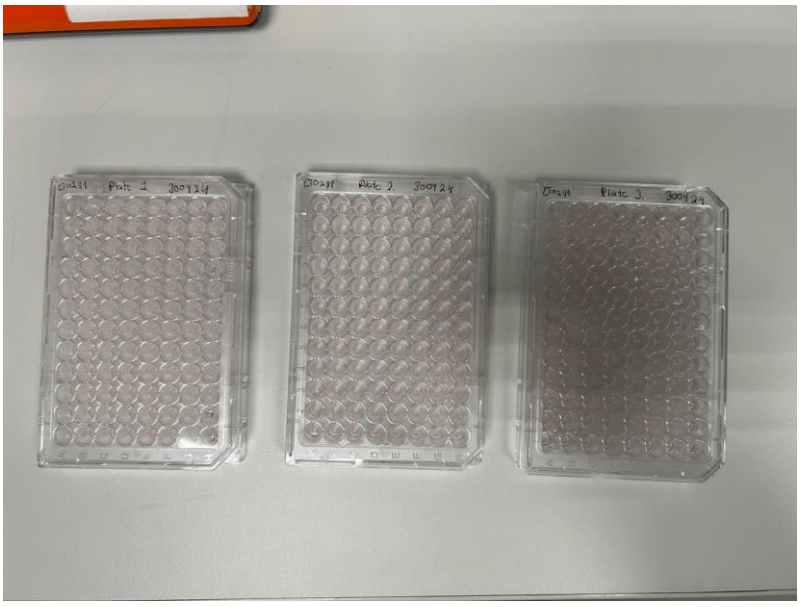

Figure 4. Methotrexate, SDS and phthalic anhydride plates

The results obtained from the Tecan plate reader were Excel files with the meta information about the measurement and then a table in the same format as the plate map. The table contains the luminescence or fluorescence reading of each well in the plate. With the help of different reference wells, it is possible to calculate the viability of cells in the exposed wells. In this chapter, the names used for the wells of the exposure plate are the same as in the figure 3 plate map.

CellTox measures the number of dead cells on the plate. This means that the fluorescence reading obtained from that tracer compound would have to be converted into amount of living cells. Without this conversion, it is not possible to calculate the wanted IC50 and IC20 from the cells. This conversion turned out to be complicated and CellTox results were left out of consideration. The following result calculation was conducted with MTGlo.

Medium, the exposure chemical and CellTox and MTGlo have their own effect on the luminescence or fluorescence reading of a well. Thus, first step in the result calculation is to calculate the mean of the luminescence readings obtained from wells with exposure chemical, medium and tracer compounds for each concentration. For example, for the first concentration it would indicate the mean of $c_1 b$ readings. The next step after that is to subtract this calculated value from the luminescence readings of exposure wells of that concentration. In the case of the first concentration, the mean of $c_1 b$ values is subtracted from each c_1 reading.

VC_1 and VC_2 wells have living cells in a medium but no exposure chemical. Thus, the mean of their reading result works as a reference point. The luminescence reading of each exposure well ($c_1, c_2\ etc.$) is divided by the mean of the VC_1 and VC_2 wells. This calculation result depicts the viability of exposed cells compared to healthy cells in percentages.

The equation to calculate the cell viability for each well is

$$\frac{c_x - mean(c_x b)}{mean(VC_1 + VC_2)} \qquad (1)$$

Where c_x is an exposure well of a concentration, $c_x b$ is a well with that concentration's exposure but no cells and VC_1/VC_2 are wells with cells but no exposure. After calculating the viability of each well, their mean was used as the collective result for that concentration and time point.

4. RESULTS AND DISCUSSION

After processing the data from the Tecan spark reader excel files as was described in previous chapter, the data are used to draw exposure curves. In these curves, y- axis has the viability of exposed cells compared to non-exposed cells (vehicle control). X-axis has the concentration of the exposure chemical on the plate. One figure has three exposure curves that represent the different time point measurements.

As an indicator for the result reliability, standard deviation is calculated from each concentration. Standard deviation depicts how obtained results position around the mean of the results. in other words, small standard deviation indicates that the measurement points are close to their mean. In the exposure curves, standard deviation describes the uncertainty of the result. The actual measured luminescence values and their mean is somewhere in the range of standard deviation.

Results include one exposure curve for each chemical per experiment. Since three experiment rounds was conducted that means 9 exposure curves. In this chapter, only one exposure curve for each chemical is presented and all the exposure curves are presented in Appendix 2.

The best exposure curve of SDS was from week 2 and is presented in the figure 5. All the curves showed consistent results, but this curve has the smallest standard deviations in the results. SDS worked as the positive control chemical of this experiment and its behavior indicates whether the experiment was conducted correctly. The IC50 value of SDS was 19,3749 µg/ml and IC20 value was 18.53691 µg/ml. There is a study conducted with HaCaT Keratinocytes, where 2 different measurement methods were used to find out the IC50. According to this experiment SDS' IC50 value was 42-46 µg/ml (37). This result differs from the result obtained in this experiment, but that is most likely caused by the different cell type used. This keratinocyte experiment was the closest reference point available.

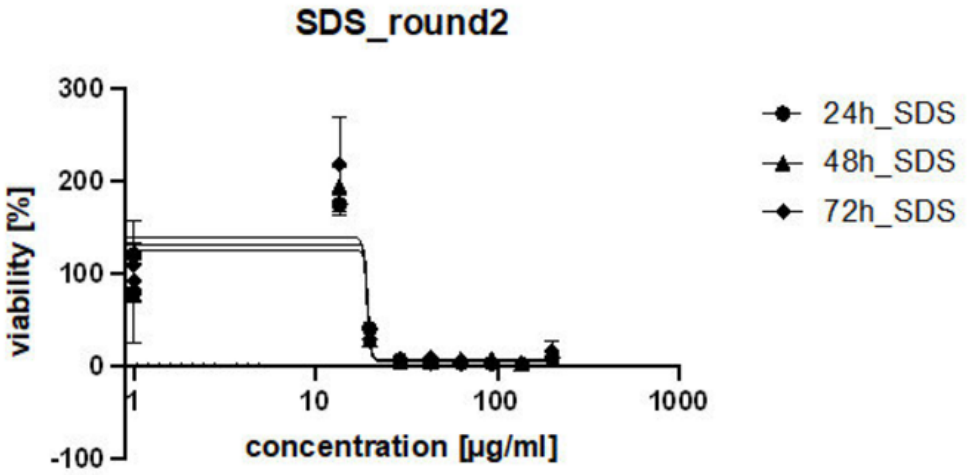

Figure 5. Exposure curve of SDS (week 2). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

The best exposure curve of Methotrexate is presented in figure 6. It was from week 2 and has the most consistent behavior from the curves. The results were similar in all three repeats, which indicates reliable results and repeatability.

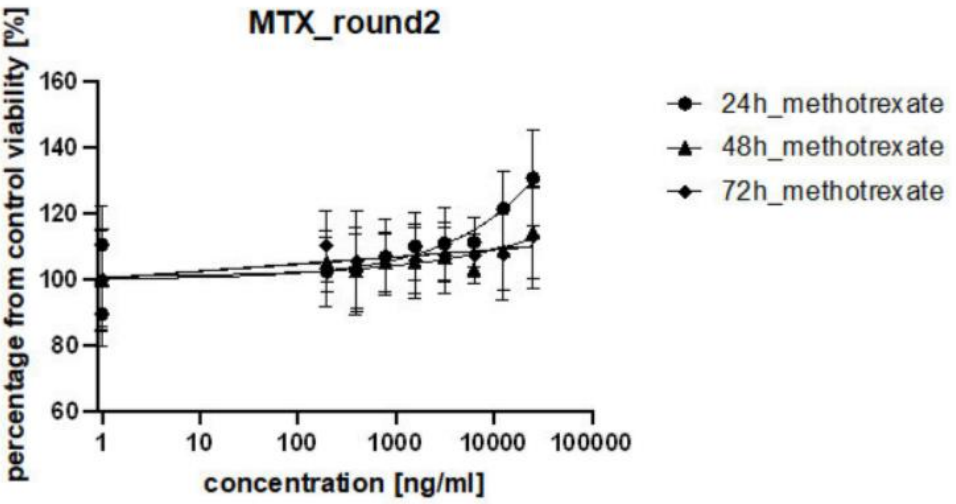

Figure 6. The exposure curve of Methotrexate (week 2). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

The viability of exposed cells did not decrease below the viability of unexposed cells. In addition, the viability increased in higher concentrations. This would indicate that methotrexate is not poisonous enough to kill cells and to work as the reference substance for the in vitro model of pulmonary fibrosis in these concentrations. The increase in viability at the higher concentrations is most likely caused by the cells increasing their metabolism

to get rid of the harmful substance. When cell metabolism works faster, they process the MT cell viability substrate in larger quantities and binding NanoLuc enzyme produces stronger luminescence signal.

The best exposure curve of PAH was from the first week and is presented in Figure 7. The other 2 figures showed similar behavior to methotrexate and the viability of the cells just increased when the concentration of the exposure increased. This behavior is most likely caused by the cells increasing their metabolism, just like in methotrexates case. Due to the viability results in the second and third experiment, additional experiments would have to be conducted to get reliable results about the behavior of this substance. It was also not possible to determine the IC50 and IC20 results to this substance.

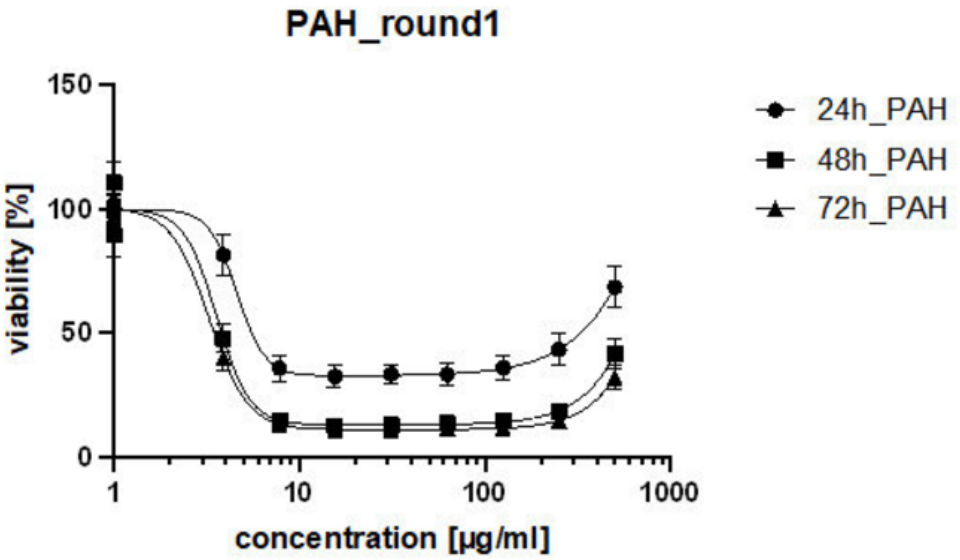

Figure 7. The exposure curve of PAH (week 1). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

In conclusion, the results of methotrexate and PAH were not according to the goal of this experiment. Methotrexate did not decrease the viability of the cells at all and phthalic anhydride had inconsistent results between weeks. The cytotoxicity of methotrexate and PAH has not been studied with HUVEC cells. Hence it was not possible to find reference values from literature. The positive control chemical SDS gave consistent results that are in line with literature. This indicates that the experiment was conducted correctly.

5. CONCLUSION

The aim of this thesis was to contribute to the creation of a complete *in vitro* model for pulmonary fibrosis. This aim is important due to pulmonary fibrosis currently not having a working treatment and the disease leading to the death of the patient. The intended contribution to the *in vitro* model was to find suitable reference chemicals for pulmonary fibrosis causing chemicals. Reference chemicals are needed for comparing the changes any poisonous chemicals cause to cells to specifically pulmonary fibrotic cells. Potential reference chemicals for the model were methotrexate and PAH that were the focus of this experiment.

The positive control chemical of this experiment showed consistent behavior through this whole experiment and it's IC50 values were in similar range to literature values. This would indicate that the experiment was conducted correctly.

According to the results of this experiment, methotrexate was not poisonous enough to reduce the viability of the cells to work as a reference chemical. The viability of methotrexate exposed cells was at the same level as unexposed cells and even exceeded it in the higher concentrations. For future studies, it might be beneficial to use higher concentrations of methotrexate and see if that is able to reduce the viability of the cells.

The results obtained from the other chemical PAH showed inconsistency. The first week's results showed promising behavior by decreasing the cell viability. However, the two other weeks' results showed similar behavior to methotrexate and the cell viability in exposed cells did not decrease. In the future, more experiments would be needed to exactly determine the cytotoxicity of PAH and whether it is suitable as a reference chemical.

Overall, the results of this experiment weren't according to the set objective but some conclusions can be made based on these results. Methotrexate does not appear to be suitable chemical for the in vitro model and PAH's inconsistent behavior also hints towards not being a good choice. In the upcoming experiments, some other chemicals should be considered for the model.

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APPENDIX 1: DILUTION SERIES

Methotrexate

Table 1. Dilution series of methotrexate. The table lists the amount of stock solution added and final concentrations in medium and cell culture.

medium	previous solution	stock [10 mg/ml] µl	medium dilution concentration [µg/ml]	end concentration in cell culture [µg/ml]	Code on 12-well plate
6ml		30	50	25	1
3	3		25	12,5	2
3	3		12,5	6,25	3
3	3		6,25	3,125	4
3	3		3,125	1,5625	5
3	3		1,5625	0,78125	6
3	3		0,78125	0,390625	7
3	3		0,390625	0,195313	8
6ml			0	0	vc

Phthalic anhydride

Table 2. Dilution series of PAH. The table lists the amount of stock solution added and final concentrations in medium and cell culture.

medium	previous solution	stock [200 mg/ml] µl	medium dilution concentration [µg/ml]	end concentration in cell culture [µg/ml]	Code on 12-well plate
6ml		30	1000	500	1
3	3		500	250	2
3	3		250	125	3
3	3		125	62,5	4
3	3		62,5	31,25	5
3	3		31,25	15,6	6
3	3		15,6	7,8	7
3	3		7,8	3,9	8
6ml			0	0	vc

SDS

Table 3. The dilution series of SDS. The table lists the amount of stock solution added and final concentrations in medium and cell culture.

me-dium	previous solution	stock [100 mg/ml] µl	medium dilution concentration [µg/ml]	end concentra-tion in cell cul-ture [µg/ml]	Code on 12-well plate
5ml		20,1	402,0	201,0	1
1,41	3		273,5	136,7	2
1,41	3		186,0	93,0	3
1,41	3		126,6	63,3	4
1,41	3		86,1	43,0	5
1,41	3		58,6	29,3	6
1,41	3		39,8	19,9	7
1,41	3		27,1	13,6	8
6ml			0	0	vc

APPENDIX 2: EXPOSURE CURVES

Methotrexate

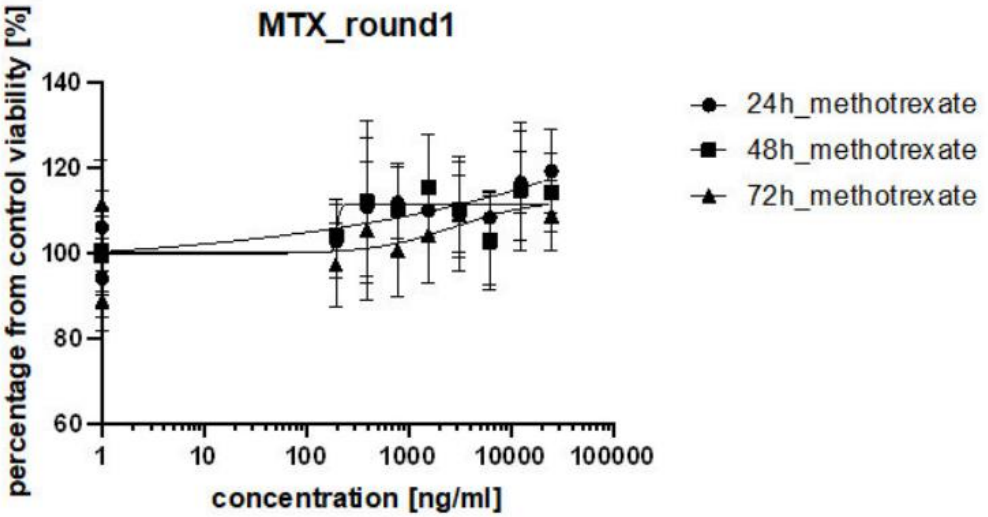

Figure 8. The exposure curve of Methotrexate (week 1). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

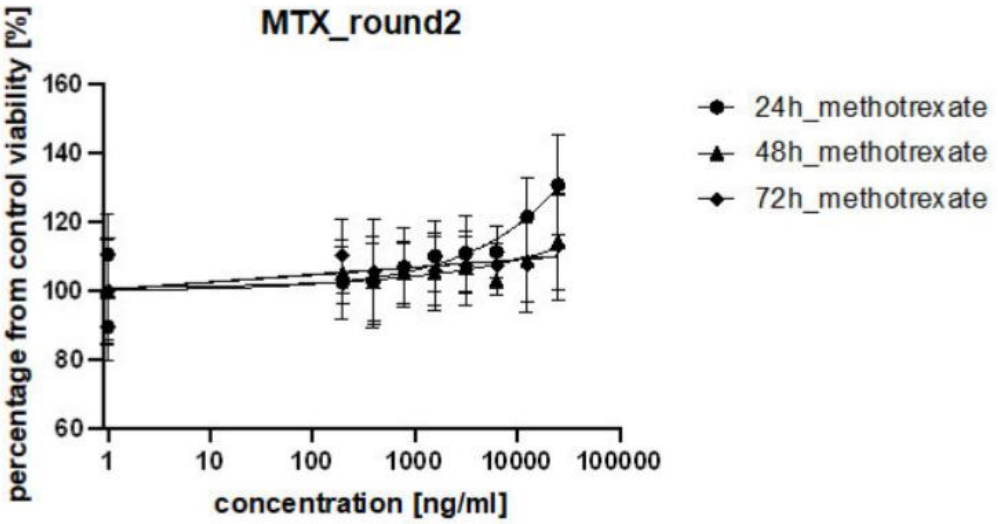

Figure 9. The exposure curve of Methotrexate (week 2). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

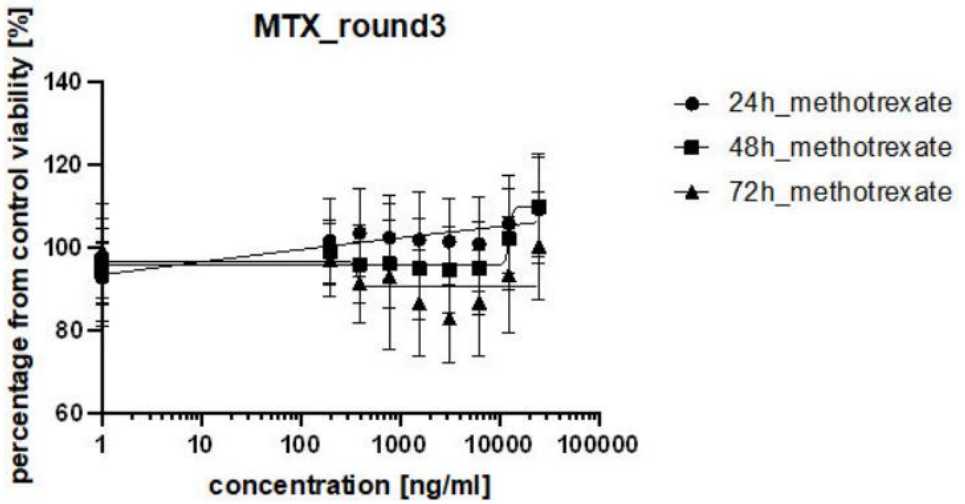

Figure 10. The exposure curve of Methotrexate (week 3). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

Phthalic anhydride

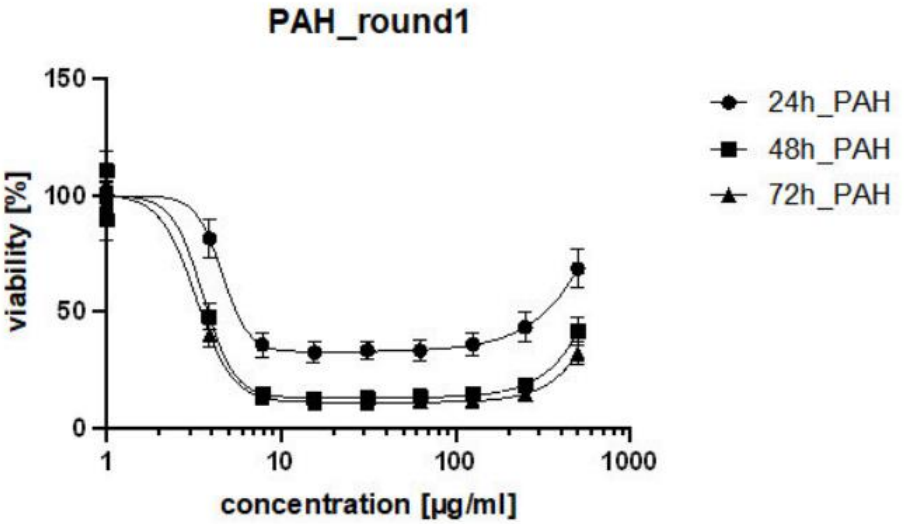

Figure 11. The exposure curve of PAH (week 1). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

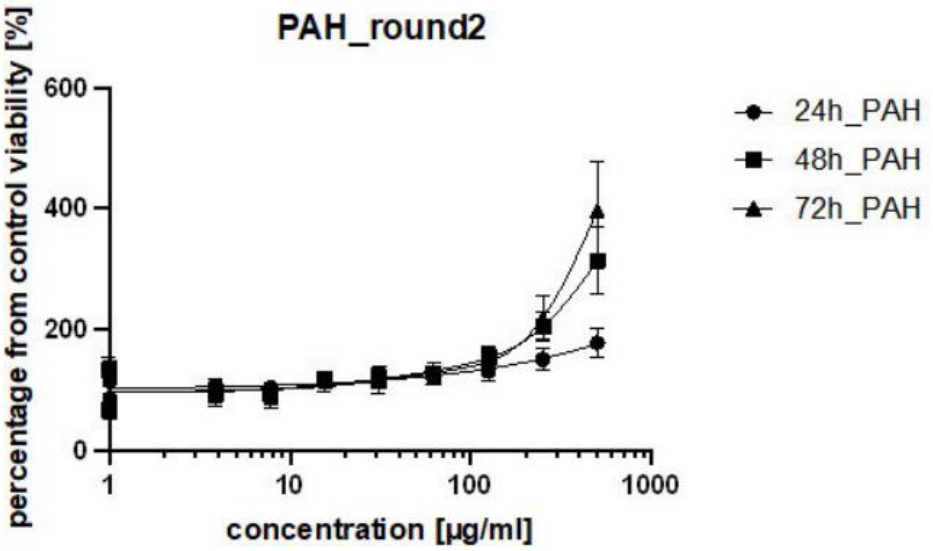

Figure 12. The exposure curve of PAH (week 2). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

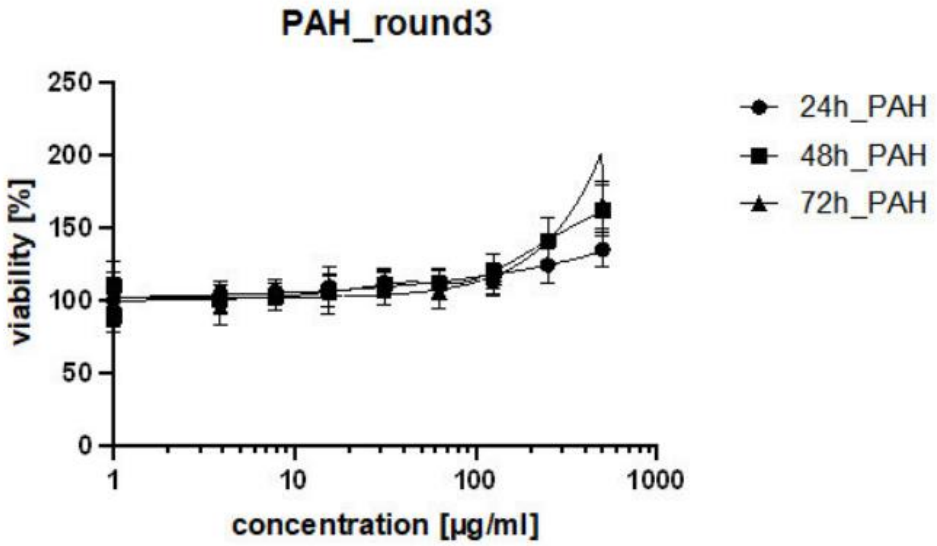

Figure 13. The exposure curve of PAH (week 3). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

Sodium dodecyl sulfate

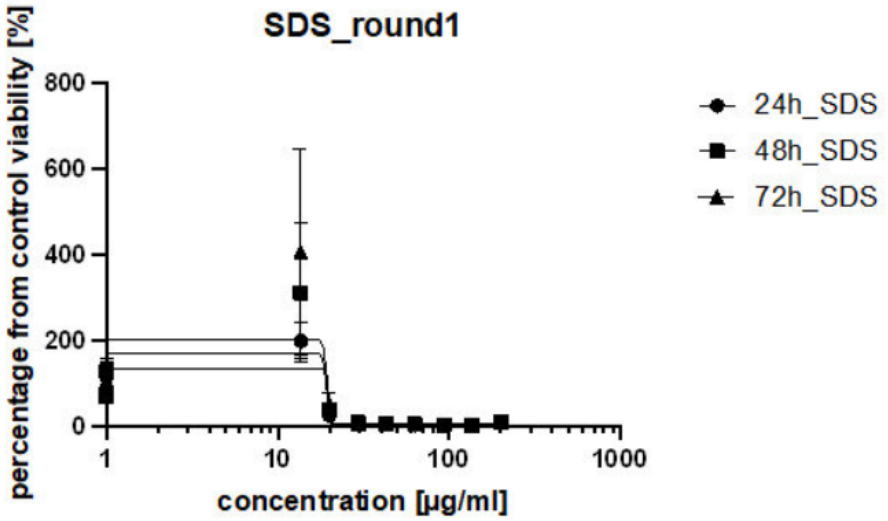

Figure 14. The exposure curve of SDS (week 1). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

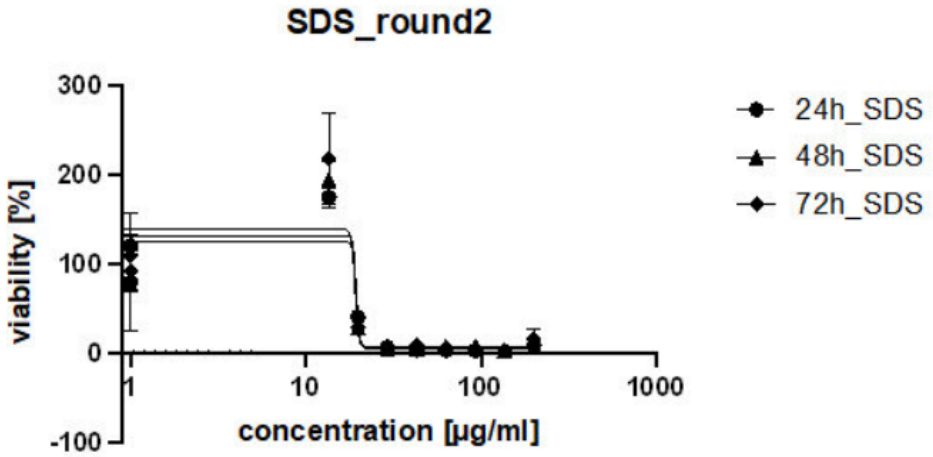

Figure 15. The exposure curve of SDS (week 2). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.

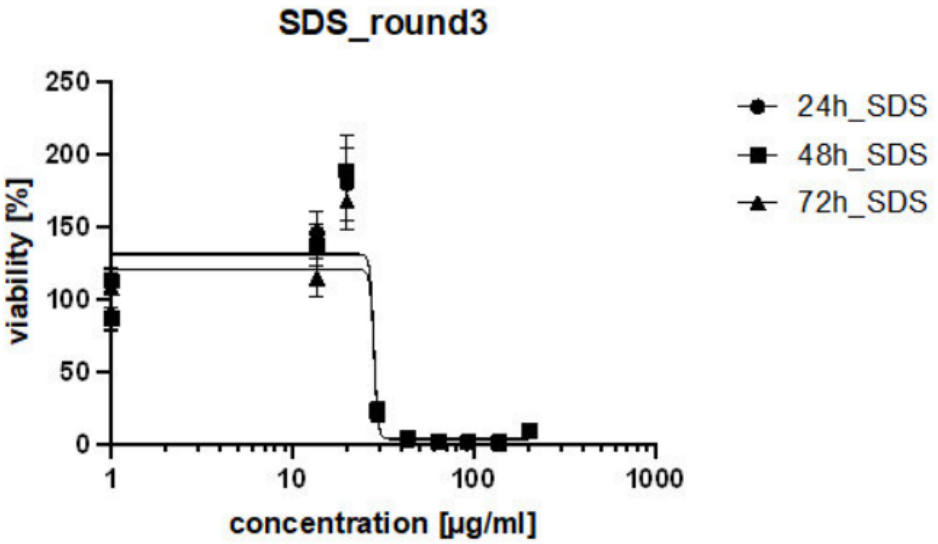

Figure 16. The exposure curve of SDS (week 3). Y-axis has the percentage of alive cells in exposure wells compared to non-exposed cells. X-axis has the concentration of the exposure chemical. The standard deviation of each result is presented in the figure.